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Exploring *in vitro* anti-inflammatory activity by COX inhibition of flavone isolated from the *Calophyllum inophyllum* Linn leaves in lipo-polysaccharide stimulated RAW264.7 macrophage cell line

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Abstract

Cell lines are being used in vaccine production, testing drug metabolism and cytotoxicity, antibody production, study of gene function, generation of artificial tissues and synthesis of biological compounds, thus, the field of scientific research is being revolutionized by cell lines. Inflammation is an inherent natural process of the immune system. When inflammation persists for a long period, various chronic diseases are triggered. Recent studies have reported the identification of novel natural compounds with anti-inflammatory properties and these compounds offer the potential to decrease excessive inflammation associated with various diseases. Activated macrophages produce different cytokines which, play an important role in the induction of acute and chronic inflammatory diseases. Lipopolysaccharides regulate inflammatory mediators. Thus, suppression of inflammatory mediator synthesis is one of the useful therapeutic strategies in the treatment of inflammatory diseases. COX-2 is not expressed under normal conditions in most cells, but inflammation mediators induce COX2 expression in a number of cellular systems. Pharmacological inhibition of COX by nonsteroidal anti-inflammatory drugs (NSAID) can provide relief from the symptoms of inflammation and pain. Shih-Chang Tsai *et al* explored the anti-inflammatory activity of acetone extract of *Calophyllum inophyllum* leaves and reported the plant can be used in prevention of inflammatory diseases, and its mechanism may be partially associated with blocking COX-2 and iNOS of RAW 264.7 cells. The present work aimed to demonstrate that the anti-inflammatory activity by COX inhibition in lipo-polysaccharide stimulated RAW264.7 macrophage cell line is due to the flavone isolated from the leaves of *Calophyllum inophyllum*. According to the current investigation, isolated flavones from *Calophyllum inophyllum* appear to have anti-inflammatory property. When compared to the IC_{50} value of the standard, diclofenac, the isolated flavone displayed a good IC_{50} value, which indicates that the ethanolic leaf extract of *Calophyllum inophyllum* has the potential to be developed as a non-steroidal anti-inflammatory drug (NSAID).

Keywords: Cell Lines; Inflammation; COX; RAW264.7; *Calophyllum inophyllum*; Flavone

1 Introduction

Several advantages, which include cost effectiveness, easiness to use, unlimited supply of material and bypassing of ethical concerns associated with the use of animal and human tissue, offers immortal cell lines to be used in place of primary cells in research. Cell lines provide a consistent sample and reproducible results, since it provides a pure population of cells, which is valuable. The field of scientific research is being revolutionized by cell lines and they are being used in vaccine production, testing drug metabolism and cytotoxicity, antibody production, study of gene function,

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generation of artificial tissues (e.g., artificial skin) and synthesis of biological compounds e.g., therapeutic proteins [1]. Inflammation is an inherent natural process of the immune system. When inflammation persists for a long period, various chronic diseases such as autoimmune disorders, arthritis, cardiovascular diseases, diabetes, Parkinson's, and cancer are triggered. Novel natural compounds with anti-inflammatory properties have been identified recently and studied, which offer the potential to decrease excessive inflammation associated with various diseases.

Different cytokines such as interleukin-1β (IL-1ß), IL-6, reactive oxygen species (ROS), reactive nitrogen species (RNS), inducible nitric oxide synthase (iNOS), prostaglandin (PGE) and tumor necrosis factor-α (TNF-α), produced by activated macrophages plays an important role in the induction of acute and chronic inflammatory diseases [2]. The mRNA expression of inflammatory cytokines and mediators has been found to increase by exposure to bacterial lipopolysaccharides (LPS), a well-known component of the cell wall of gram-negative bacteria [3]. Lipopolysaccharides regulate inflammatory mediators such as TNF-α, IL-6, and NO [4]. Thus, suppression of inflammatory mediator synthesis is one of the useful therapeutic strategies in the treatment of inflammatory diseases.

Cyclooxygenase (COX), also known as Prostaglandin G/H synthase, prostaglandin-endoperoxide synthase (PTGS, EC 1.14.99.1), is an enzyme that is responsible for oxidation of arachidonic acid to important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane. In the biosynthetic pathway to prostanoids from arachidonic acid, COX is the central enzyme. COX-1 (expressed form) and COX-2 (inducible form) are known to exist in isoenzymes. COX-1 is constitutively expressed in many tissues and is the predominant form in gastric mucosa and in kidney. COX-2 is not expressed under normal conditions in most cells, but inflammation mediators such as growth factors, cytokines and endotoxin induce COX2 expression in a number of cellular systems. Pharmacological inhibition of COX by nonsteroidal anti-inflammatory drugs (NSAID) can provide relief from the symptoms of inflammation and pain.

Balsam, an oleoresin from the bark of *Calophyllum inophyllum* is used as cicatrisan [5]. It is reported that, traditionally the decoction of the leaves is used to relieve eye irritation and conjunctivitis [6, 7]. Kashman *et al* reported that the presence of calanolides in *Calophyllum lanigerum*is responsible for the anti-HIV activity [8]. From the leaves of *Calophyllum lanigerum* and *Calophyllum inophyllum* isoprenyl coumarins was isolated and are reported to be the active substances in inhibiting the HIV-1 reversed transcriptase activity. Shih-Chang Tsai *et al* explored the anti-inflammatory activity of acetone extract of *Calophyllum inophyllum* leaves and reported the plant can be used in prevention of inflammatory diseases, and its mechanism may be partially associated with blocking COX-2 and iNOS of RAW 264.7 cells [9]. The present work aimed to demonstrate the anti-inflammatory activity of flavone isolated from the leaves of *Calophyllum inophyllum* by COX inhibition in lipopoyssacharide stimulated RAW264.7 macrophage cell line.

2 Material and methods

2.1 *In-vitro* anti-inflammatory activity in cell lines

2.1.1 Principle

RAW 264.7 cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and were grown in Dulbecco's modified Eagles medium, DMEM (Sigmaaldrich, USA). DMEM is a complete media with nutrients, proteins, amino acids, buffering system and vitamins, which provides the conditions and physicochemical properties of environment for the growth and maintenance of cell cultures.

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/mL), Streptomycin (100µg/mL) and Amphotericin B (2.5µg/mL). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany) until the cells were confluent. Serum is a complex mixture of proteins, source of minerals, lipids, hormones, growth and adhesion factors. Fetal bovine serum (FBS) and newborn calf serum (NCS) are most common. The amino acids like cysteine, L-glutamine and tyrosine are essential for growth and cell proliferation. The major ions-Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, PO_4^{3-}, SO_4^{2-}, HCO_3^--affect osmolarity of culture media. Antibiotic solutions like pencillin and streptomycin and antimycotic agents like kanamycin or amphotericin B are applied in cell cultures to reduce the frequency of contamination. The buffering system is essential to maintain proper pH. For establishing physiological pH for cells CO_2 is dissolved in the culture medium. Carbon dioxide establishes equilibrium with bicarbonate ions. The bicarbonate buffers not only show low toxicity, but also help in glucose metabolism. Generally, most of cell lines are maintained at 37°C [10, 11].

2.2 Pro-inflammatory activation of RAW264.7 cells

The RAW 264.7cells were grown to 60% confluency followed by activation with 1 µL lipopolysaccharide (LPS: 1µg/mL). Stimulation with lipopolysaccharide (LPS) is to trigger the inflammatory response. LPS stimulated RAW cells were exposed with different concentration (25, 50, 100 µg/mL) of sample solution. Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the sample was added and incubated for 24 hours. After incubation the anti-inflammatory assay for quantification of COX concentration was performed using the cell lysate.

2.3 COX Inhibition assay

The COX activity was assayed by the method of M. C Gierse Walker and J. K. Gierse 2010 [12] 1mL of reaction mixture containing Tris-HCl buffer (pH 8), glutathione 5 mM/L and hemoglobin 20µg/L was used for incubating 100µL cell lysate for 1 minute at 25°C. Arachidonic acid 200 mM/L was used for initiating the reaction. After 20 minutes of incubation at 37°C, 200µL of 10% trichloroacetic acid in 1 N hydrochloric acid was added for terminating the reaction. The supernatant taken after centrifugation was treated with 1% thiobarbiturate. COX activity was determined by reading absorbance at 632 nm. Diclofenac was used as standard and the reaction mixture without any tested compound served as control. COX activity inhibition was expressed in percentage using the following relation.

$$\% \textbf{ of inhibition} = \frac{\textbf{Absorbance of control} - \textbf{Absorbance of test}}{\textbf{Absorbance of control}} \times \mathbf{100}$$

3 Result and Discussion

Inflammation is a pathophysiological reaction that induces injury to living tissue to cause a localized build-up of plasmatic fluid and blood cells [13]. Inflammation is managed by a group of drugs known as NSAIDs, or non-steroidal anti-inflammatory medicines [14]. On continuous usage current anti-inflammatory medications have many side effects. Antioxidant-rich plants have been proposed as possible sources of chemicals with anti-inflammatory properties with the least side effects [15]. An important role in inflammatory processes is played by macrophages and they produce inflammatory mediators, such as nitric oxide (NO) and prostaglandin E2 (PGE2), which are generated by activated inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. When activated by appropriate stimuli, macrophages also produce different cytokines, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α). Thus, activated macrophages has a pivotal role in inflammatory diseases *via,* excess production of inflammatory mediators, such as NO and PGE2, as well as pro-inflammatory cytokines, to promote the inflammatory response [16, 17, 18]. The pro-inflammatory transcription factor NF-κB controls the up-regulation of iNOS and COX-2 during inflammation. Flavonoids that inhibit the induction of this factor are regarded as interesting tools for inflammation control [19, 20].

The standard used in this evaluation of the cyclooxygenase (COX) inhibitory activity was diclofenac, an anti-inflammatory medication with very high COX inhibition. The percentage of inhibition and IC_{50} values of the standard and the isolated flavone is shown in Table 1 and its graphical representation is shown in Figures 1 and 2.

Table 1 Percentage of inhibition and IC50 values of the standard and the isolated flavone

Conc. of extracts (µg/mL)	Percentage inhibition	
	Diclofenac (Standard)	Isolated flavone
25	43.35±0.0004	48.32±0.0006
50	65.21±0.0012	61.58±0.0006
100	84.91±0.0002	71.36±0.00011
IC_{50}	31.07µg/mL	22.58 µg/mL

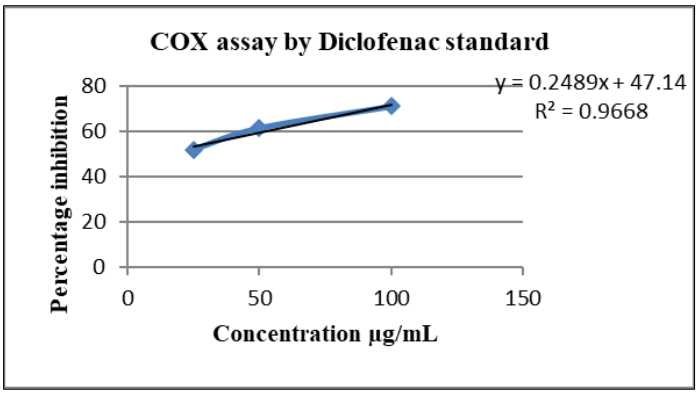

Figure 1 Graphical representation of percentage inhibition of COX enzyme activity by Diclofenac standard

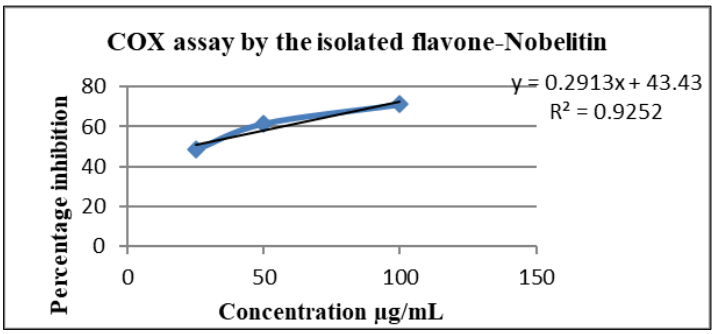

Figure 2 Graphical representation of percentage inhibition of COX enzyme activity by the isolated flavones

In the COX analysis, the Diclofenac exhibited 84.91 percentage of inhibition at a concentration of 100 µg/mL with an IC_{50} value of 31.07. Isolated flavone exhibited 71.36 percentage of inhibition at a concentration of 100 µg/mL and the IC_{50} value was obtained as 22.58µg/mL.

According to the investigations, isolated flavone from *Calophyllum inophyllum* appears to have anti-inflammatory properties. When compared to the IC_{50} value of the standard, the isolated flavone displayed a good IC_{50} value, which indicates that the flavone from ethanolic leaf extract of *Calophyllum inophyllum* has the potential to be developed as a non-steroidal anti-inflammatory drug (NSAID).

4 Conclusion

For the assessment of the anti-inflammatory potential the *in vitro* cyclooxygenase assay is commonly used. To estimate the predicted levels of COX inhibition of the compounds *in vitro* assay is used because it is a quick and simple method as compared to clinical research. In the current work, COX enzyme inhibition assay is performed for the isolated flavone from *Calophyllum inophyllum* leaves. It can be concluded that the isolated flavone appears to have anti-inflammatory properties, so it can serve as a lead and can further be modified to obtain various derivatives with more potent anti-inflammatory activity with improved safety profiles.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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