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IN-VITRO ANTI-INFLAMMATORY ACTIVITY OF SELECTED DIHYDROXY FLAVONE BY TPA INDUCED INFLAMMATION IN POLYMORPHONUCLEAR LEUCOCYTES

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ABSTRACT: The present study involves the investigation of anti-inflammatory activity for 2', 4' dihydroxy flavone by TPA [12- O-Techanoyl 13 – Myristate] induced inflammation in Polymorphonuclear leukocytes [PMNL]. The cell viability was assayed by Trypan blue dye exclusion assay, MTT assay, release of Cathepsin D, Nitrite and TNF- α levels. Diclofenac sodium was used as the standard drug. The selected dihydroxy flavone, 2', 4' dihydroxy flavone showed the significant increase in the number of viable PMNL cells and decreases the release of Cathepsin D, Nitrite and TNF- α . The present work evidenced the *in-vitro* anti-inflammatory effect of 2', 4' dihydroxy flavones.

INTRODUCTION: Flavonoids are the poly phenolic compounds present almost in all parts of the flowering plants. Flavones found to possess wide pharmacological actions, especially as a potent antioxidant, ¹ antinociceptive ², anti-inflammatory ³, antihepatotoxic ⁴, antiallergic ⁵, anti-platelet ⁶, anti-hypertensive ⁷, antiulcerogenic ⁸, anti-microbial, anti-fungal, antiviral ⁹, anti-rhinovirus ¹⁰, antimalarial ¹¹ and anticarcinogenic ¹².

The combination of multiple pharmacological properties in a single nucleus is quite interesting. One of the earliest therapeutic applications of flavonoids is in the treatment of some inflammatory diseases.

In a previous study done by the the authors, it has been proven that 2',3'- dihydroxy flavone, 2', 4' - dihydroxy flavone, 7, 3'- dihydroxy flavone and 5, 3' dihydroxy flavone have proven to have antinociceptive ¹³ and anti-inflammatory action. It has been evidenced that out of four flavones, 2', 4'- dihydroxy flavone was found to have significantly potent anti-inflammatory activity ¹⁴. This *in-vitro* investigation help to understand the mechanism of anti inflammatory action.

Inflammation: is the biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants ¹⁵. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. It is regulated to prevent over activation of the immune system and unwanted immune response. The increase of leukocytes into the site of inflammation is critical to the pathogenesis on inflammatory conditions. The ability of the selected dihydroxy flavone to inhibit tumor necrosis factor [TNF- α] which are pro-inflammatory cytokines, involved in fever,

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inflammation, tissue destruction, determines the mechanism of action for its anti-inflammatory potential. Therefore, inhibition of the cellular reactions in one of these targets are typically used as an *in-vitro* model for evaluation of anti-inflammatory activity.

Polymorphonuclear leukocytes (PMNL) play an important function of resistance against invading microorganisms. Upon stimulation, there is a marked increase in oxygen consumption, which is converted into various reactive oxygen species¹⁶. ROS generated by phagocytes play a crucial role in the inflammatory response, which is required for immune defense and regeneration after injury. Tetradecanoyl Phorbol Acetate (TPA) in PMNL induces superoxide anion generation¹⁷. Thus activated inflammatory leukocytes are considered to play an important role. In acute inflammation, activated polymorphonuclear leukocytes release lysosomal hydrolytic enzymes, lipid mediators, and reactive oxygen species that may damage the surrounding viable tissues^{18, 19}. Thus, this study planned to investigate 2', 4'-dihydroxy flavone capable to modulate ROS metabolism of PMNL.

MATERIALS AND METHODS:

2', 4'-dihydroxy flavone used in the present study was synthesized using standard procedures at Research Organics, Chennai. The authenticities of the compound was done with melting points and UV method. All other chemical and kit were purchased from standard laboratories.

Ethics: The approval for the collection of blood was granted by Institutional Ethics Committee (IEC). IEC reference number: IEC-NI/14/AUG/41/41

Anti inflammatory activity by TPA (12-O-tetradecanoylphorbol – 13 - acetate) induced inflammation model:

Isolation of polymorphonuclear leukocytes^{20, 21}: Polymorphonuclear leukocytes (PMNL) were isolated from healthy individuals, cultured in RPMI - 1650 media and it was maintained in 37° C and 5 % CO₂ incubator. The human blood cells are fractionated to separate polymorphonuclear leukocytes and then they are cultured. The lymphocytes are separated by density gradient

centrifugation method which involves a gradient, ficoll hypaque. The principle underlying the technique involves the differential separation of leukocytes in a solution of density 1.077-1.080, when subjected to centrifugation. The various cells of blood are separated based on their density difference.

Protocol:

- 5ml of peripheral blood was collected and transferred into heparin coated- tubes.
- 5ml of ficoll hypaque was taken in a sterile 15 ml centrifuge tube and-blood was slowly layered onto it.
- The tube was centrifuged at 2000 rpm for 20 min.
- The buffy coat layer was separated which contains polymorphonuclear leukocytes.
- These cells were transferred to 25 cm²-culture flask and 5ml of RPMI 1650 media supplemented with 10 % v/v fetal bovine serum was added and incubated at 30° C, 5 % carbon dioxide.

Experimental design:

Group 1: Control (PMNL cells without drug)

Group 2: PMNL Cells +DMSO

Group 3: PMNL Cells +TPA

Group 4: PMNL Cells + 2', 4'-dihydroxy flavone

Group 5: PMNL Cells + Diclofenac sodium (185 µg)

Group 6: PMNL Cells +TPA + 2', 4'-dihydroxy flavone

Group 7: PMNL Cells +TPA + Diclofenac sodium (185 µg)

The following parameters were carried out in PMNL cells: Cells doubling time assay IC₅₀ of TPA, 2', 4'-dihydroxy flavone, Diclofenac sodium, Trypan blue exclusion assay, Cathepsin D assay, MTT assay, Nitroblue tetrazolium dye reduction assay, Nitric oxide synthase activity assay and Assay of TNF α.

Trypan blue exclusion assay:²²

Trypan blue is a vital stain used to selectively color dead tissue or cells to blue. Principle Cells are very selective in the compounds that were through the cell membrane. In available cell the sodium potassium pump will be active and hence it will not allow the dye to enter the cell. But in dead cell the pump will be inactive and hence the selective permeability of the membrane is lost. Hence the dead cell will take up the dye and will appear blue when viewed under microscope. Since live cells are excluded from staining, this staining method is also described as dye exclusion method.

Method:

- PMNL Cells were seeded in a concentration of 1×10^5 cells /ml in a 96 well plate and it was incubated for 48 hrs in a carbon dioxide incubator at 37⁰C.
- To all wells IC₅₀ concentrations of TPA were added and incubated for an additional 24 hrs in a carbon dioxide incubator at 45⁰C.
- Three wells served as control and the other three wells were served as solvent control, which consists of 0.1 % v/v DMSO. Equal volume of 0.1 % w/v trypan blue were added to the cell and mixed gently and counted using haemocytometer

MTT assay:^{23, 24}

MTT assay is a standard colorimetry assay for ensuring cellular proliferation and it can also be used to determine cytotoxicity of potential medicinal agent and other toxic materials. Yellow MTT (3-(4, 5-dimethylthiazole 2-yl)-2, 5 diphenyl tetrazolium bromide, tetrazole) is reduced to purple formazan in the mitochondria of live cells. This reduction takes place only when Mitochondrial dehydrogenous enzymes are active. Dimethyl sulphoxide is added to dissolve the insoluble formazon product into a purple colored solution.

- PMNL Cells were seeded in a concentration of 1×10^5 cells /ml in a 96 well plate and it was incubated for 48 hrs in a carbon dioxide incubator at 37⁰C.

- To all wells IC₅₀ concentrations of TPA were added and incubated for an additional 24 hrs in a carbon dioxide incubator. IC₅₀ concentrations of sample and standard were added to each well and incubated for an additional 24 hrs in a carbon dioxide incubator at 37⁰C.
- 50 µl of MTT reagent (5mg/ml) were added to each well and the plates were incubated for 24 hrs at 37⁰C. When the purple precipitate was clearly visible under the microscope, 100 µl of DMSO solution were added to all wells.
- The formazan crystals were dissolved completely by to and fro motion and the absorbance of the wells including the blank was measured at 540 nm.

Enzymatic assay of Cathepsin D:²⁵

Isolation of lysosomal preparation is important because cathepsin D an essential marker present in the lysosomes. Lysosomal swelling is the indicator of inflammation. During inflammatory condition lysosomal enzymes are released and resulting in variety of disorder. Many anti inflammatory drugs act by either stabilizing the lysosomal membrane or by inhibiting these enzymes.

- PMNL Cells were seeded in a concentration of 1×10^5 cells /ml in a 96 well plate and it was incubated for 48 hrs in a CO₂ incubator at 37⁰C.
- To all wells IC₅₀ concentrations of TPA were added and incubated for an additional 24 hrs in a carbon dioxide incubator. IC₅₀ concentrations of sample and standard were added to each well and incubated for an additional 24 hrs in a carbon dioxide incubator at 37⁰C.
- Then the well contents were transferred into respective eppendorf tubes and then centrifuged at 10,000 rpm for 5 min. 1 ml of PBS were added to the pellet and again centrifuged at 10, 000 rpm for 5 minutes. To the pellet 500 µl of 0.1N HCl were added and sonicated for 10 min.

- The solution was again centrifuged at 10,000 rpm for 10 minutes. Supernatant was discarded and to the pellet added 1 ml of RPMI 1650 media and stored in ice cold condition throughout the experiment.
- 1 ml of buffered substrate (2.5 % Hb) was added to all the test and blank tubes. Then 300 µl of enzyme supernatant was added to only test sample tubes and not to the blank. Then the tubes were incubated to 45⁰C for 2 hrs.
- 1 ml of 10 % TCA was added to only blank tubes and all the tubes were incubated for exactly 30 min at 37⁰C.
- Then 1 ml of 10 % TCA was added to only test sample tubes and 300 µl enzyme supernatant was added to only blank tubes. The tubes were centrifuged at 1500 rpm, 5 minutes.
- 1 ml of supernatant was taken and 2 ml of 0.8 N NaOH were added to it and 600 µl of Folin's Ciocalteu Phenol reagent were added.
- Then the tubes were incubated in dark for 10 minutes at room temperature and absorbance was measured at 620nm.

Nitric acid Synthase activity: ²⁶

NADPH and L-arginine are required for the continual operation of NOS and production of nitric oxide (NO). In aqueous solution, NO rapidly degrades to nitrate and nitrite. Spectrophotometric quantification of nitrite using Griess reagent thus provides an accurate determination of total NOS activity

- PMNL Cells were seeded in a concentration of 1 x 10⁵ cells /ml in a 96 well plate and it was incubated for 48 hrs in a carbon dioxide incubator at 37⁰C. To all wells IC₅₀ concentrations of TPA were added and incubated for an additional 24 hrs in a carbon dioxide incubator. IC₅₀ concentrations of sample and standard were added to each well and incubated for

an additional 24 hrs in a carbon dioxide incubator at 37⁰C.

- After incubation, the supernatants (0.1 ml) were added to solution of 0.1ml Griess reagent (1 % w/v sulfanilamide and 0.1 % w/v naphthyl ethylene diamine dihydrochloride in 5 % w/v H₃PO₄) to form a purple azo dye.
- Using NaNO₂ as standard, nitrite production was measured by UV-visible spectrophotometer at 540 nm.

Nitro blue tetrazolium dye (NBT) reduction assay:

This assay focuses on the ability to produce oxygen radicals (O₂ - and OH) and measures the reduction of yellow soluble NBT to blue insoluble formazan by the O₂ - (superoxide) generated in stimulated leukocytes by phorbol myristate acetate.

- PMNL Cells were seeded in a concentration of 1 x 10⁵ cells /ml in a 96 well plate and it was incubated for 48 hrs in a carbon dioxide incubator at 37⁰C.
- To all wells IC₅₀ concentrations of TPA were added and incubated for an additional 24 hrs in a carbon dioxide incubator.
- IC₅₀ concentrations of sample and standard were added to each well and incubated for an additional 24 hrs in a carbon dioxide incubator at 37⁰C.
- 100 µl of NBT solution in 1 mg/ml of PBS was added to the above 100 µl of the pretreated cells and incubated at 37⁰C for 30 minutes and then incubated at 25⁰C for 20 minutes
- Finally, smears were prepared and stained for differential counting of formazan deposits in polymorphonuclear Leukocytes (PMNL) (102). to the above 100 µl of the pretreated cells and incubated at 37⁰C for 30 minutes and then incubated at 25⁰C for 20 minutes Finally, smears were prepared and stained for differential counting of

formazan deposits in polymorphonuclear Leukocytes (PMNL)

TNF α assay:

- PMNL Cells were seeded in 96 well plates at a concentration of 1×10^5 cells/ml and incubator for 48 hrs in a carbon dioxide incubator at 37°C .
- To all wells IC_{50} concentrations of TPA were added and incubated for an additional 24 hrs in a carbon dioxide incubator.
- IC_{50} concentration of the test sample, standard, TPA treated groups, control and DMSO were added to appropriate wells and incubated for 24 hours in a carbon dioxide incubator at 37°C . Three wells served as control and the other three wells served as solvent control, which consist of 0.1% V/V DMSO.
- Added $50 \mu\text{l}$ of prepared biotinylated anti-TNF α to all wells. The plate was covered and incubated at room temperature (18 to 37°C) for 3 hrs.

- The plate was covered and incubated at room temperature for 15 min. Added $100 \mu\text{l}$ of H_2SO_4 : Stop Reagent into all wells and the absorbance was measured on spectrophotometer using 650 nm.

RESULTS AND DISCUSSION: The IC_{50} value of the standard Diclofenac Sodium and 2', 4' dihydroxy flavone was found to be between 150ng & 200ng (**Fig.1**). The trypan blue assay showed significant increase in cell number in cell treated with 2', 4' dihydroxy flavone when compared to TPA alone treated cells. A significant decrease in the release of cathepsin D was observed in 2', 4' dihydroxy flavone when compared to TPA alone treated cells. The cells viability showed maximum in diclofenac and 2', 4' dihydroxy flavone in treated groups. The release of nitrite was reduced significantly in 2', 4' dihydroxy flavone and Diclofenac Sodium treated groups. TNF α was markedly reduced in 2', 4' dihydroxy flavone and Diclofenac Sodium treated groups. In cells treated with drug alone showed no significant change in the values of the parameters analysed in the study when compared to control.

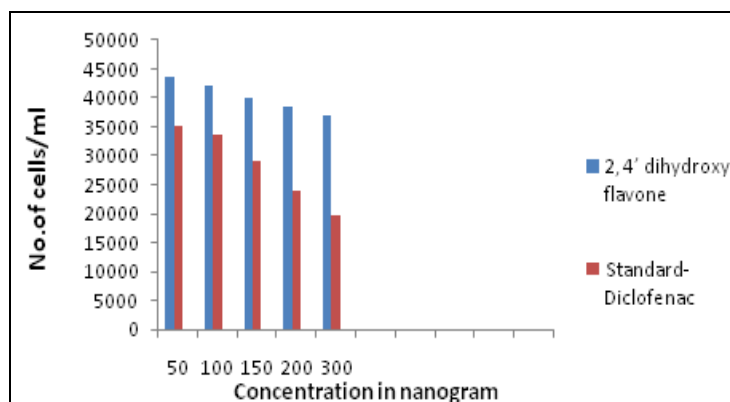


FIG.1: IC_{50} ANALYSIS OF DICLOFENAC SODIUM AND 2', 4' DIHYDROXY FLAVONE

TABLE 1: SHOWS THE EFFECT OF STANDARD DICLOFENAC AND 2', 4' DIHYDROXY FLAVONE ON TPA INDUCED INFLAMMATION ON PMNL CELLS

Group	Trypan blue exclusion assay	Cathepsin D assay	MTT assay	Nitric oxide synthase activity	TNF α (pg/ml)
Control	75000 \pm 4.99	0.07 \pm 0.01	100	16.26 \pm 3.44	170.33 \pm 10.69
PMNL Cells +DMSO	72400 \pm 2.36	0.08 \pm 0.01	97.25 \pm 2.71	16.92 \pm 4.13	172.23 \pm 15.58
PMNL Cells + TPA	34500 \pm 2.77	2.71 \pm 0.38	52.58 \pm 6.30	49.45 \pm 0.36	1665.66 \pm 66.71
PMNL Cells + dihydroxy flavone	65300 \pm 4.28	0.15 \pm 0.25	94.05 \pm 2.40	20.85 \pm 4.20	-
PMNL Cells +	69600 \pm 0.26	0.09 \pm 0.01	97.58 \pm 6.30	17.15 \pm 3.22	-

Diclofenac					
PMNL Cells +TPA+ DHF	63700±6.89	0.23±0.40	89.84±6.55	22.44±8.01	430±4.56
PMNL Cells +TPA+ Diclofenac	68300±4.10	0.12±0.01	95.57±5.26	21.39±2.72	394±0.45

Each value represents mean± SD of three observations.

The Trypan blue exclusion assay showed a significant increase in cell number in cells treated with 2', 4' -dihydroxy flavone and Diclofenac when compared to TPA alone treated cells. Cathepsin D is a lysosomal enzyme released during an inflammatory condition and it is released more in case of inflammation. A significant decrease in the release of Cathepsin D was observed in 2', 4' -dihydroxy flavone and Diclofenac treated groups compared to TPA induced group which indicates less lysosomal swelling of the cells (decreased inflammation). MTT, cell viability assay showed a maximum in Diclofenac and 2, 4' -dihydroxy flavones treated groups. TNF- α levels (Table-1) were markedly reduced in 2', 4' -dihydroxy flavone and Diclofenac treated groups.

CONCLUSION: The anti-inflammatory activity of 2', 4' dihydroxy flavone shows their protective role in inflammation through cytokine mediated pathway.

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