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ANTI-INFLAMMATORY CELL LINE STUDY OF RHUKOT GEL IN POLYMORPHONUCLEAR LEUKOCYTES CELLS

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Abstract: The present study was carried out to investigate anti-inflammatory activity of Rhukot gel in polymorpho nuclear leukocytes cells. Tetradecanoyl Phorbol Acetate (TPA) was used as an inflammatory inducer. The parameters like Cell doubling at various time intervals, IC_{50} (Inhibitory Concentration of 50%) value of TPA, IC_{50} value of Rhukot gel, trypan Blue exclusion assay, MTT assay, nitric oxide synthase activity, cathepsin D assay, nitro blue tetrazolium dye reduction assay and assay of TNF α were carried out in polymorph nuclear leukocytes cells. The cell doubling time of leukocytes was evaluated by counting the number of viable cells with trypan blue dye for every 24 hrs. The maximum doubling time of leukocytes was found to be 48 hrs. About 50 % toxicity of TPA was observed at the concentration of 4 ng and hence this concentration was considered as IC_{50} TPA. IC_{50} value of Rhukot gel was found to be 200 µg. The results showed that increase in concentration of Rhukot gel protects the cells from action of TPA, but increased concentration affect the cell number. Hence, it has dose dependent protective action on cells.

Keywords: Rhukot gel, IC_{50}, TPA and leukocytes cells.

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INTRODUCTION

The most numerous of neutrophils like polymorphonuclear leukocytes (PMNL) in human, play a significant function of resistance against invading microorganisms. On stimulation, the oxygen consumption is increased. It is converted into various reactive oxygen species (ROS). This ROS play an important role in the inflammatory response. It is required for immune protection and restoration after injury [(1)]. Tetradecanoyl Phorbol Acetate (TPA) in PMNL stimulates superoxide anion (O_2-) generation. Thus activated inflammatory leukocytes are regarded to play an essential role. In acute inflammation, activated polymorphonuclear leukocytes liberate lysosomal hydrolytic enzymes, lipid mediators, and reactive oxygen species that may spoil the surrounding feasible tissues [(2)].

The principal underlying the technique involves the human blood cells are fractionated to separate polymorphonuclear leukocytes and culture it. The lymphocytes are separated by density gradient centrifugation method which involves a gradient, ficoll hypaque. The various cells of blood are separated based on their density difference.

MTT assay is a standard colorimetry assay for ensuring cellular proliferation; 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 mitochondria dihydrogenious enzymes are active. A solubilization of solution usually dimethyl sulphoxide is added to dissolve the insoluble formazon product into a purple colored solution. Thus, this study planned to investigate for herbal gel capable to modulate ROS metabolism of PMNL[(3)].

Materials and method

The approval for the collection of blood was granted by Institutional Ethics Committee (IEC). IEC reference number: IEC-NI/14/JAN/38/17.

TPA induced inflammation model

Isolation of polymorphonuclear leukocytes

PMNL were isolated from healthy individuals, cultured in RPMI - 1650 media and it was maintained in 37º C and 5 % CO_2 incubator.

5 ml of peripheral blood was collected and transferred into heparin coated tubes. 5 ml 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 culture flask and 5ml

of RPMI 1650 media supplemented with 10 % v/v fetal bovine serum was added and incubated at 30^{o} C, 5 % carbon dioxide

Experimental design

Group 1: Control (cells without drug)

Group 2: PMNL Cells +DMSO

Group 3: PMNL Cells +TPA

Group 4: PMNL Cells + Rhukot gel

Group 5: PMNL Cells + TPA + Rhukot gel

The following parameters were carried out in PMNL cells:

Cells doubling time assay, IC_{50} of TPA, IC_{50} of Rhukot gel, Trypan blue exclusion assay, Cathepsin D assay, MTT assay, Nitroblue tetrazolium dye reduction assay and Nitric oxide synthase activity and assay of TNF α.

Cell doubling time assay

1x10^{5} cells/ml were seeded in 96 well plates and cell counting was performed using trypan blue, loaded in haemocytometer. Cell counting was performed in triplicates for every 24 hrs.

IC_{50} analysis of TPA

PMNL Cells were incubated for 48 hrs in a carbon dioxide incubator at 37^{o} C. Different concentrations (2, 4, 6, 8 & 10 ng) of TPA were added to each well. Then the cells were incubated for an additional 24 hrs and the cells were counted in a haemocytometer using trypan blue.

IC50 analysis of Rhukot gel

PMNL Cells were incubated for 48 hrs in a carbon dioxide incubator at 37^{o} C. To all the wells IC_{50} concentrations of TPA were added and incubated for an additional 24 hrs in a carbon dioxide incubator. Various concentrations (50, 100, 150, 200 & 250 µg) of sample were added to each well and incubated for an additional 24 hrs in a carbon dioxide incubator. After incubation the live cells were counted in a haemocytometer using trypan blue staining.

Trypan blue exclusion assay

IC_{50} concentrations of Rhukot gel was added to each well and incubated for an additional 24 hrs in a carbon dioxide incubator at 37° C. Equal volume of 0.1 % w/v trypan blue were added to the cell and mixed gently and counted using haemocytometer.

MTT assay

IC_{50} concentrations of TPA were added and incubated for 24 hrs in a carbon dioxide incubator. IC_{50} concentrations of Rhukot gel was added to each well and incubated for an additional 24 hrs in a carbon dioxide incubator at 37° C. 50 µl of MTT reagent (5 mg / ml) were added to each well and the plateswere incubated for 24 hrs at 37° C. 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

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 45° C 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 37° C. 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.

Nitric acid Synthase activity

To all the wells IC_{50} concentrations of TPA were added and incubated for 24 hrs in a carbon dioxide incubator. IC_{50} concentrations of sample and standard were added to each well and incubated for an additional 24 hrs in a carbon dioxide incubator at 37° C. After incubation, the supernatants (0.1 ml) were added to solution of 0.1 ml Griess reagent (1 % w/v sulfanilamide and 0.1 % w/v naphthyl ethylene diamine dihydrochloride in 5 % w/v H_3PO_4) to form a purple azo dye. Using $NaNO_2$ as standard, nitrite production was measured by UV-Visible spectrophotometer at 540 nm.

Nitroblue tetrazolium dye (NBT) reduction assay

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 37° C for 30 minutes and then incubated at 25° C for 20 minutes.

TNF α assay

A capture antibody highly specific for human TNF α has been coated to the wells of the microtitre strip plate provided during manufacture. TNF α samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti- TNF α secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The absorbance of the color complex is then measured and the generated.

RESULTS AND DISCUSSION

Cell doubling time assay

The cell doubling time of Leukocytes was evaluated by counting the number of viable cells with trypan blue dye for every 24 hours. It was found that the doubling time of leukocytes was maximum at 48 hours.

Table-1: Cell doubling at various time intervals

Day	Hours	Cells/well
0	0	80,000
1	24	1,25,000
2	**48**	**1,99,000**
3	72	1,93,200
4	96	1,04,000
5	120	96,000

IC_{50} of TPA

Minimum inhibitory concentration at which 50 % of the population is affected is known as

MIC 50 and it was determined for TPA. The IC_{50} value of TPA was found to be **4ng.**

Table-2: IC_{50} value of TPA

TPA concentration (ng)	No.of Cells/ml
Control	44,500
DMSO	43,750
2	35,600
4	**34,300**
6	24,160
8	20,440

10	19,480

IC_{50} of Rhukot gel

The inhibitory concentration of Rhukot gel was determined. The IC_{50} value of Rhukot gel wasfound to be 200 ng.

Table-3: IC_{50}value of Rhukot gel

Sample Concentration (µg/ml)	No. of Cells /ml (Rhukot gel)
50	33000
100	30000
150	26000
200	**25000**
300	16000

Trypan Blue Exclusion Assay

Trypan blue stains only the dead cells and the live cells remain transparent. The results indicated that there was a significant increase in the cell number in almost all the groups which indicate that the sample have potential anti-inflammatory effects against TPA induced inflammation in PMNL.

Table - 4: Effect of Trypan Blue on PMNL cell

Group	Control	DMSO	Cells + TPA	Cells + Rhukot gel	Cells + TPA+ Rhukot gel
Cells/ml	75000±5000	72400± 5000	34500±2887	63200±6534	57200±5437

MTT Assay

The significant decrease in cell viability was found in group 3 which may be due to inflammatory action of TPA. All the groups showed increased cell viability as compare to TPA induced inflamed PMNL cells. The results have revealed that the sample showed very less toxic effect.

Table- 5: Effect of MTT on PMNL cell

Group	Cell viability (%)
Control	100± 5.243
DMSO	97.76 ± 4.564
Cells + TPA	41.20±5.083
Cells + TPA+ Rhukot gel	87.78± 9. 342

NOS Activity

A significant difference in the release of nitrite was absorbed in the above groups. Also the release of nitrite was nearing to control which indicates that the sample have potential anti-inflammatory activity against TPA induced inflammation in PMNL.

Table- 6: Effect of Nitric Oxide on PMNL cell

S.No.	Group	µM of nitrite release
1	Control	18.45±1.786
2	DMSO	18.87± 1.765
3	Cells + TPA	58.13±2.341
4	Cells + TPA + Rhukot gel	20.89± 1.089

NBT assay

The NBT dye reduction test was used to determine the superoxide radical production by measuring formazan deposit in PMNL's cells. The formazan crystals formed more in Group 3 due to the action of inducer; less formazan was seen in drug treated groups.

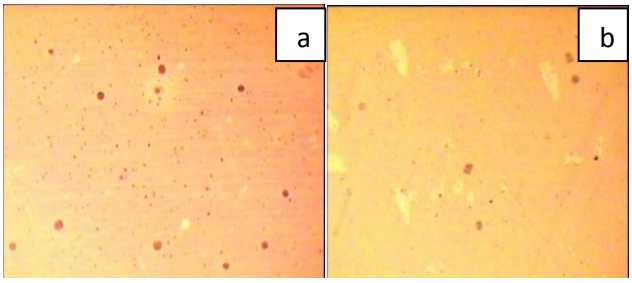

Figure - 1: (a) NBT+TPA+Control (b) NBT+ TPA+Sample

Cathepsin D

Cathepsin D is a lysosomalanzyme released during inflammatory condition. Group 1 and 2 showed the minimal amount of cathepsin D which indicates no lysosomal swelling of the cells in the group 1.The maximum amount of the cathepsin D released showed by group 3 this is because of the action of TPA which is responsible for including inflammation. Very less amount of cathepsin D was found in drug treated group.

Table- 7: Effect of Cathepsin D on PMNL cell

Group	Units/ml
Control	0.06045 ± 0.0675
DMSO	0.0809± 0.0489
Cells + TPA	2.815 ±0.5019
Cells + TPA + Rhukot gel	0.0983 ± 0.0314

TNF α Assay

Anti-inflammatory activity of the herbal gel and standard drug were compared with TPA induced group. There is a highly significant values were observed in groups 2 and 3 when compared with group 2 and also these values are near normal when compare to control. Increased level was showed in group 3 which is due to inflammatory action of TPA. Slightly increase in levels of TNF α was observed in the standard and sample treated groups which indicate that these drugs have potent anti-inflammatory activity against TPA induced inflammation in PMNL.

Table- 8: Effect of TNF α on PMNL cell

Group	Concentration (pg/ml)
Control	169.3±19.45
DMSO	175.7 ±12.54
Cells + TPA	1987±20.98
Cells + TPA + Rhukot gel	465.8±36.48

CONCLUSION

All the studies performed provide a strong evidence for the use of the Rhukot gel as an anti-inflammatory agent.

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