Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
https://www.phytojournal.com
JPP 2023; 12(2): 08-10
Received: 05-10-2022
Accepted: 13-12-2022

Rajesh S Mony
Head QA/QC, Vaidyaratnam Oushadhasala, Vaidyaratnam Road. Thaikkattussery P O, Ollur, Thrissur, Kerala, India

Corresponding Author:
Rajesh S Mony
Head QA/QC, Vaidyaratnam Oushadhasala, Vaidyaratnam Road. Thaikkattussery P O, Ollur, Thrissur, Kerala, India

Studies on anti-inflammatory activity of herbal extract mixture using inflammation induced human keratinocytes (HaCaT) cells

Rajesh S Mony

DOI: https://doi.org/10.22271/phyto.2023.v12.i2a.14619

Abstract
Inflammation is a general name for reactions occurring in several types of tissue injuries, infections, or immunologic stimulation as a defense against foreign or altered Endogenous substances. This comprises of a series of changes of the terminal tissues, which tend to eliminate the injurious agents and to repair the damaged tissue.
Chemokines are a family of small proteins secreted by various cell types that play a role in the immune cell infiltration into inflammatory or infectious sites [1, 2]. Skin inflammation is closely associated with the production of Th2 chemokines [3]. These chemokines released from keratinocytes bind to and attract CCR4-positive Th2 cells into inflammatory tissues. It was reported that GJBRH inhibited the production of inflammatory cytokines by dermal endothelial cells. This study aimed to investigate whether GJBRH has inhibitory effects on TNF-α and IFN-γ-induced Th2 chemokine production and to identify the underlying molecular mechanisms in HaCaT human keratinocyte cell line.
The present study was planned to evaluate the Herbal Extract sample for anti inflammatory activity using human skin keratinocytes

Keywords: *Boswellia serrata, Terminalia bellerica*, LPS induced inflammatory hacat cells

Introduction
Background
Inflammatory process is the collective response of cytokines and immune cells to injury and infection Cytokines and immune cells create a micro environment for either pro or anti tumor progression (Hanahan *et al*., 2011) [7]. Chronic inflammation without resolution can lead to the development of cancer (Kuper *et al*., 2000) [8]. Cyclooxygenases (COXs) COX1 and COX2 isoforms catalyze critical step in the oxidation of arachidonic acid (AA) to the prostanoids.

Objective
To determine the Antiinflammatory activity of extract sample, by inhibiting the multiplication of LPS induced inflammatory HaCaT cells in *in vitro*.

Principle
The Caspase-3 apoptotic assay is helps to detect the activity of caspase-3 in cell lysates. It contains substrate called (N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin or Ac-DEVD-AMC) in caspase-3.The principle behind of this assay explaines type of caspase enzymes involved apoptosis process in humans. Particluarly caspase 3 involved in apoptosis while reoccurring of skin cells. During the assay, in the stage of delayed process, apoptosis cannot be taken place so if drug has efficacy to induce apoptosis caspase 3 will be activated and it will motivate the inflamed cells to be die. At the same time, drug might reduce the inflammation and make the inflamed cells to reoccur back to normal, these two ways can be done by drug depends on stages of psoriasis, it will be targeted apoptosis, or reviving back (doesn't mean reduce the inflammation).so in apoptosis caspase 3 will be bind to substrate called Ac DEVD AMC, form enzyme substrate complex. So increasing the drug concentration either cell will be reviving or cells will be died. According to that drug efficacy is interpreted.

Materials and Methods
Extract Test Sample
Equal Mixture of Hydroalcoholic Extract (90:10) of *Boswellia serrata* and *Terminalia bellerica*
Human keratinocyte cell line (HaCaT) from NCCS, Pune, Maharastra, India

Lipo polysaccharide (LPS) from sigma aldrich
Caspase 3 substrate
Dithiothreitol (DTT)
Hydroxyethy l piperazineethane sulfonic acid (HEPES)
Triton X
Ethylene DiamineTetraAcetic acid (EDTA)
Dulbecco's modified Eagle's medium (DMEM)
Fetal Bovine Serum (FBS)
Phosphate buffer solution (PBS)
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
Penicillin, Streptomycin, isopropanol
Cell culture facility control DMSO were also maintained (n=3).

Medium preparation

Dulbecco's modified Eagle's medium (DMEM) was purchased from PAN Biotech supplemented with 10% FBS (Invitrogen Life Technologies) 1% penicillin and streptomycin it was stored at 4 °C.

The cells were observed under an inverted microscope for appearance of detached cells. About 1ml of thawed DMEM media was added to the trypsinized cells and gently pippetted to avoid clumping of cells. About 1ml of the final volume of cells was pippetted out and reseeded in a fresh UV sterilized culture flask. The flask was labeled appropriately. To both the flasks, 3-4ml of DMEM media was added and kept inside the incubator with culture conditions (37 °C/5% CO_2).

Caspase 3 Apoptotic Assay Procedure

HaCaT Cells were seeded on 24 well tissue culture plate with 90% cell density and incubated overnight. Inflammation was induced in HaCaT cells using LPS (1µg/1ml) and kept overnight incubation. Then medium was discarded and replaced with fresh medium with different (Extract sample) drug concentrations (from 0.2 to 10 mg). Maintained Methotrexate (10 mg/ml) as positive control and incubated for 48 hrs under CO_2 incubator. After incubation medium was discarded, cells washed with ice cold PBS (1ml) then added 40 µl of lysis buffer to each well and kept in 4 °C for 30 min.

Then scrap the cells by using cell scrapper and transfer into centrifuge tube with PBS. centrifuge at 15000 rpm of at 4 °C for 20 min, collected supernatant and transfer 35 µl to 96 well plate. Then added 55 µl of assay buffer and 10 µl of 2mM caspase substrate (0.2 mM).x. Then covered the plate with foil kept overnight incubation at 37 °C under CO_2.then read at 405 nm.

Statistical analysis

The CASPASE 3 assay was analyzed by one-way ANOVA followed by Turkey's test. (Using Origin pro.8 software)

Results

The results of anti inflammatory activity for Extract Sample were shown in Table1. The dose dependent inhibition was observed in the product. Statistical analysis were observed as significant for the present study. The anti inflammatory activity was observed in sample A. It showing anti psoriatic activity when compared to positive control (methotrexate).it showing inhibitory concentration 4.9mg/ml (IC_{50}) and it might be conclude that there was potential ANTI INFLAMMATORY effect for the given sample without cytotoxicity (needs to be proved).

Discussion

Keratinocytes-The body's initial line of defense is often the skin, which is composed of the epidermis and the dermis. Keratinocytes are the principal cell type comprising the epidermis, and constitute 90% of total epidermal cells. The HaCaT human keratinocyte cell line is a spontaneously immortalized human epithelial cell. The drug was activated in two ways simultaneously, this will be depends on stages of inflammatory cells. Either drug can induce apoptosis and reduce the inflammation through plant marker compounds. It is having antioxidant and anti inflammatory so that directly the drug can act against inflammation and reduce it. While inducing apoptosis the inflammatory cells will die which could not revive again and also might be because of that final stage of disease. Hence in this scenario caspase 3 will be activated abundantly and also depends on programmed cell death.

Table 1: *In vitro* Apoptotic activity (caspase3) of human inflammatory skin cell line (HaCaT) cells exposed to Sample A

S. No.	Concentration mg/ml	OD value ± SD	Caspase 3 activity%
1	Solvent blank	0.149 ± 0.0040	100
3	0.2	0.094 ± 0.0172	61
4	0.4	0.088 ± 0.014	58
5	0.6	0.086 ± 0.003	57
6	0.8	0.081 ± 0.0062	54
7	1	0.0793 ± 0.014	53
8	2	0.079 ± 0.003	52
9	3	0.079 ± 0.008	52
10	4	0.077 ± 0.006	51
11	5	0.077 ± 0.006	50
12	10	0.065 ± 0.002	43
13	10 (positive control)	0.074 ± 0.001	47

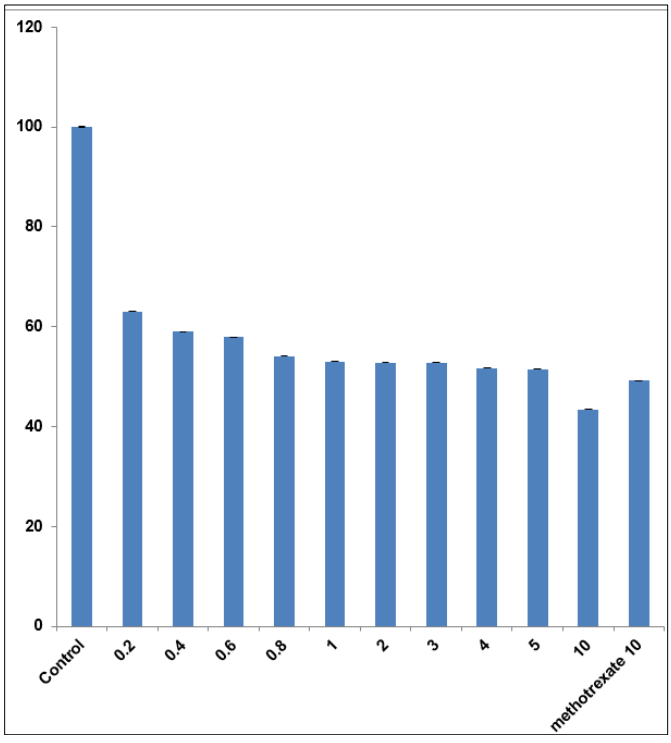

Fig 1: Effect of sample on inflammatory HaCaT Cells

IC_{50}= 4.0mg/ml

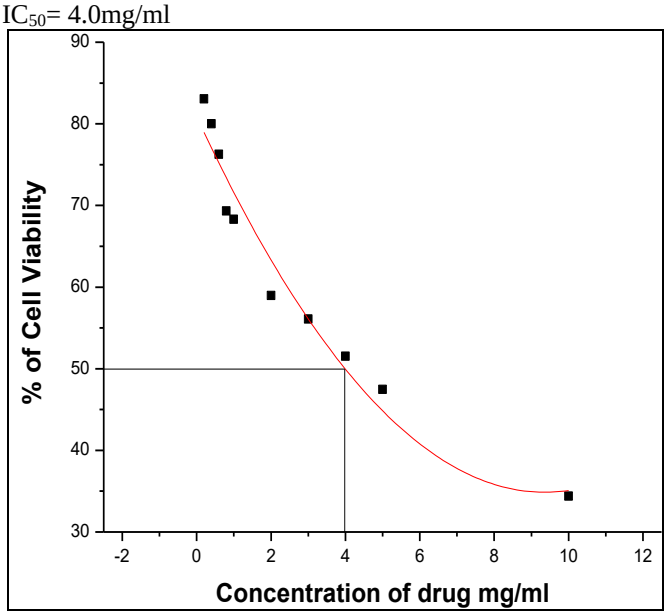

Fig 2: Caspase 3 apoptotic activity of Extract sample

Conclusion

The study supports the ANTI inflammatory activity of Extract sample.

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