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Research Article

EVALUATION OF ANTIPROLIFERATIVE ACTIVITY OF SIDDHA ANTI-PSORIATIC FORMULATION PANCHAMUGA CHENDHURAM USING CULTURED HUMAN KERATINOCYTE CELL LINES

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ABSTRACT

Objectives: This study was aimed at scientifically evaluating the *in vitro* antipsoriatic activity of Siddha drug Panchamuga Chendhuram (PMC) in human keratinocyte (HacaT) cell lines.

Methods: The Siddha drug PMC tested for antipsoriatic activity on HacaT cell lines was morphologically examined by phase contrast microscopy, and the cell viability was determined by 3- (4, 5 dimethyl thiazole-2 yl) -2.5-diphenyl tetrazolium bromide assay. About 100 µl of different concentrations (2, 6, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml) of the test samples were prepared in the cell culture medium and incubated for 24 h and 48 h to determine the viable cells.

Results: The results revealed that Siddha drug PMC showed hopeful antiproliferative activity. *In vitro* studies showed that after 24 h and 48 h incubation, the inhibitory concentration 50 (IC_{50}) values of PMC (IC_{50} 20 µg/ml) were 72.08±27.56 µg/ml and 43.91±17.71 µg/ml, respectively, as compared with Asiaticoside as a positive control with an IC_{50} value of 20.13 µg/ml.

Conclusion: Thus, this study provides scientific evidence about the efficacy of the Siddha drug PMC against the HacaT cell lines confirming its traditional use in psoriasis treatment and also emphasizes the need for antipsoriatic evaluation in animal models.

Keywords: Siddha drug, Psoriasis, Chendhuram, Human keratinocyte, Antiproliferation.

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INTRODUCTION

Psoriasis is a serious global problem for many decades. Its prevalence rate ranges from 0.44% to 2.8% of *the Indian* population [1]. The name psoriasis was given by the Viennese dermatologist Von Hebra. The name is derived from the Greek word psora which means to itch [2]. Psoriasis is defined on a clinical basis as chronic, relapsing, remitting papulosquamous eruption with typical localization on the extensor surfaces such as elbows and knees involving scalp, genitalia or nails, and other sites [3]. Among the psoriasis patients, the ratio of male to female was 1.1:1. Among the affected patients, 12.5% had a family history of psoriasis [4]. Early onset disease that affects patients at age<40 years accounts for more than 75% of psoriasis cases [5].

Psoriasis is an autoimmune disorder. The immune system is not only the key to what causes psoriatic disease but it may also be the key to treating it, too. In 1979, the researchers discovered an accident that a drug called cyclosporine that suppresses the immune system also clears psoriasis. Since then, many effective treatments directed toward the immune system have been developed for psoriasis and psoriatic arthritis [6]. At present, the treatment of psoriasis composes of a wide range of treatment options, the prescribed synthetic drugs for the treatment of psoriasis are often associated with severe side effects; thus, researchers around the globe are searching for new, effective, and safer drugs from natural resources. Around 60–70% of the world population is using traditional medicines isolated from various medicinal plants. The herbal formulations are used not only that the herbal formulations are economical but also present lesser side effects when administered to control diseases and their secondary complications [7].

It is well known that skin diseases are cured excellently with Siddha medicines. There are lots of Siddha medicines indicated in Siddha textbooks for skin diseases. The medicines are used in two forms one as individual herbs and another as compound formulations. These are some single herbs which have potent efficacy against skin diseases proved by *in vitro* studies, *Aristolochia* bracteolata, *Carum copticum*, *Curcuma aromatica*, *Indigofera aspalathoides*, *Nigella sativa*, *Smilax china*, *Wattakaka volubilis*, *Pongamia pinnata*, *Commiphora myrrha*, *Morinda tinctoria*, *Strychnos nux-vomica*, *Thespesia populnea*, *Cardiospermum halicacabum*, *Clerodendrum inerme*, and *Coccinia grandis*. The popular Siddha compound formulations are parangipattai chooranam (Powder) parangi rasayanam, parangi pattai padhangam, kendhi mezhugu, Mahaveera Mezhugu, nandhi mezhugu, rasagandhi mezhugu, kandhaga parpam (Calx), muthuchippi parpam, sangu parpam, palagarai parpam, irunelli karpam, serangkottai nei, kandhaga sudar thailam, Siddhadhi ennai, and Garudan kilangu ennai. External oil and ointments are vetpalai thailam (oil), karappan thailam, arugan thailam, Punga thailam, Meganadha thailam, sirattai thailam, kundhriga thailam, matthan thailam, Kungiliya vennai, Amirtha vennai, Kizhinjal mezhugu, Vellai mezhugu, and Padai sangaaran [8,9]. Like single herbs the *in vitro* studies of compound Siddha formulations for skin diseases are still lacking; hence, the attempt is made to fill this lacuna. Panchamuga Chendhuram (PMC) is a Siddha Herbomineral formulation used in this study indicated for psoriasis in the classical Siddha textbook Pulippani Vaidhyam – 500 [10]. In this study, cultured cell line (spontaneously transformed and immortalized human keratinocyte [HacaT] cell line) was used which is commonly employed as an *in vitro* test model for the antiproliferative activity of new treatment due to its highly preserved differentiation capacity [11].

METHODS

Preparation of PMC [10]

Raw drugs were purchased from Raw drug shop, R. N. Rajan and co., Parry's, Chennai. Authentication was made by Pharmacognosy, Siddha Central Research Institute, Chennai, and purification was made as per the Siddha classical literature [12].

Ingredients

Purified rasam (Hydragyrum), purified Gandhagam (Sulfur), purified Thalagam (Arsenic trisulfide), purified Lingam (Mercury II sulfide), purified Veeram (Mercuric chloride) each 100 g, and piper betel leaf juice Q.S.

Procedure

All the ingredients were ground well for 1 day and made into pellets (villais). The pellets were allowed to dry, and then it was ignited for small flame (Deepaagni) – 6 h, moderate flame (Kamalaagni) – 6 h, and high flame (Kaadaagni) – 9 h. After self-cooling, the product was again subjected to grinding for 1 day. The final product PMC was weighed and stored in an airtight container.

In vitro antiproliferative activity on HacaT cell lines [13,14]

Cell culture

In vitro antipsoriatic activity was carried out on HacaT cell lines. HacaT cell lines were obtained from NCCS, Pune, India, and the procedure was carried out in Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai. The cells were seeded at a concentration of 1.0 × 105 cells/ml in a 96 well microtiter plate and grown in Dulbecco's modified eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (BioWest). After 24 h, the supernatant was decanted and therefore the monolayer was washed once. Then, 100 µl of a test substance in various concentrations was added to the cells in microtiter plates. Test compounds were prepared in dimethyl sulfoxide (DMSO) and then diluted with DMEM; the final concentration of DMSO was 0.2% in the culture medium. Each sample concentration was tested in triplicates. The plates were then incubated at 370° C for 3 days in 5% CO_2 atmosphere.

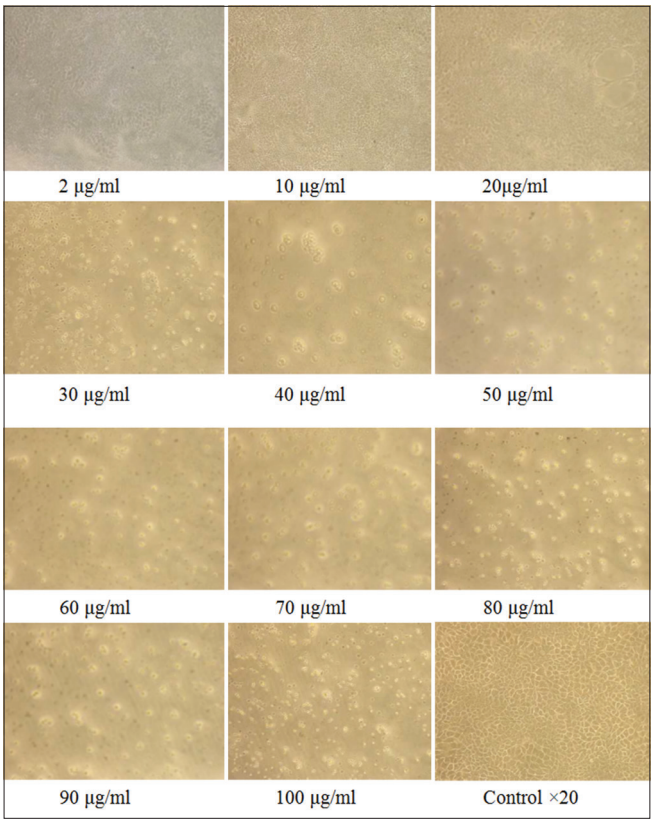

Fig. 1: Panchamuga Chendhuram induced changes in morphology and number of human keratinocyte (HacaT) cells changes shown by phase contrast microscopy. As the concentration increased, the number of HacaT cells gradually decreased, morphological changes occurred

Antiproliferative assay

The antiproliferative activity was assessed by performing the 3- (4, 5 dimethyl thiazole-2 yl) -2.5-diphenyl tetrazolium bromide (MTT) assay. The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective of principle on which the assay is based. The principle involved is the cleavage of tetrazolium salt MTT into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used [15].

Cells were fixed by adding 25 µl of ice-cold 50% trichloroacetic acid on top of the growth medium, and the plates were incubated at 40°C for 1 h after that plates were washed to get rid of traces of medium, drug, and serum. MTT reagent (10 µl; 0.4% in 1% acetic acid) (Sigma) was added to each well and left in contact with the cells for 30 min after which they were washed with 1% acetic acid, rinsing 4 times until only dye adhering to the cells was left. The plates were then dried and 100 µl of 10 mm Tris buffer (Sigma) added to every well to solubilize the dye. The plates were shaken gently for 5 min and absorbance was read at 550 nm using a microplate reader. The readings were taken at two different incubation time (24 h and 48 h). In this study, no positive control was used, but as the data available previously the results were compared to Asiaticoside. Asiaticoside is a major chemical constituent present in the plant *Centella asiatica*. The percentage inhibition was calculated from this data using the following formula [16]:

$$\% \text{ viability} = \frac{\text{Mean OD of untreated cells (control)} - \text{Mean OD of treated cells}}{\text{Mean OD of untreated cells (control)}} \times 100$$

Table 1: IC_{50} of PMC in HacaT cell lines at 24 h and 48 h

Concentration (µg)	% of cell inhibition in 24 h	% of cell inhibition in 48 h
2	19	27
6	27	25
10	35	30
20	92	23
30	93	28
40	87	38
50	89	48
60	84	67
70	85	69
80	86	68
90	85	52
100	83	52

HacaT: Human Keratinocyte cell lines, IC_{50}: Inhibitory concentration, PMC: Panchamuga Chendhuram

Table 2: IC_{50} of PMC in HacaT cell lines at 24 h and 48 h

Cell line	PMC	
	24 h IC_{50} (20 µg)	48 h IC_{50} (60 µg)
HacaT	72.08±27.56*	43.91±17.71

Effect of PMC on HacaT at 24 h shows significant inhibitory concentration, IC_{50} (24 h) = 72.08±27.56* ($p<0.05$). PMC: Panchamuga Chendhuram, HacaT: Human Keratinocyte cell lines, IC_{50}: Inhibitory concentration

Table 3: MTT assay 24 h PMC

Concentration (µg)	1	2	3	4	5	6	7	8	9	10	11	12
A	3.096	3.598	3.013	0.22	0.234	0.931	0.387	0.6	0.56	0.79	0.661	0.628
B	3.047	2.407	2.397	0.226	0.144	0.461	0.302	0.649	0.432	0.487	0.541	0.78
C	2.828	2.736	2.435	0.293	0.159	1.005	0.307	0.582	0.554	0.522	0.392	0.562
D												
E	2.334	3.133	2.985	2.587	3.766	3.066	3.042	3.007	2.847	2.706	2.886	2.775

MTT: 3- (4, 5 dimethyl thiazole-2 yl) -2.5-diphenyl tetrazolium bromide, PMC: Panchamuga Chendhuram

Table 4: MTT assay 48 h PMC

Concentration (µg)	1	2	3	4	5	6	7	8	9	10	11	12
A	2.777	2.906	2.97	2.969	2.789	2.101	1.748	1.378	1.314	1.207	1.862	1.788
B	2.349	2.965	2.689	2.966	3.324	2.545	2.014	0.853	0.955	1.438	1.412	2.041
C	2.832	2.886	3.159	3.184	3.521	2.368	2.932	1.255	1.199	1.188	2.057	1.84
D												
E	2.117	2.844	3.881	3.761	2.962	Overflow	Overflow	Overflow	Overflow	3.36	2.885	2.469

A1- A12=2 µg, 6 µg, 10 µg, 20 µg, 30 µg, 40 µg, 50 µg, 60 µg, 70 µg, 80 µg, 90 µg, 100 µg, B1-B12=Duplicate, C1-C12=Triplicate, E1-E12=Cell control. MTT: 3- (4, 5 dimethyl thiazole-2 yl) -2.5-diphenyl tetrazolium bromide, PMC: Panchamuga Chendhuram

Statistical analysis

The data were given as mean ± standard deviations (SD). The statistical differences were evaluated using one-way ANOVA followed by the least significant difference as *post hoc* analysis (SPSS Version 19.0; SPSS Inc., Chicago, IL, USA). p=0.05 was considered statistically significant.

RESULTS

The inhibitory concentration 50 (IC_{50}) was determined as the dose that would be required to kill 50% of the cells with the respective preparation and duration. The proportion of dead cells and type of cell death after treatment was better illustrated from the morphological observations (Fig. 1). The drug PMC at 24 h incubation possess significant antiproliferative activity (IC_{50} 20 µg/ml) the mean and SD was 72.08±27.56 µg/ml, ($p<0.05$) (Tables 1 and 2), whereas in 48 h incubation the activity decreases (IC_{50} 20 µg/ml) the mean and SD was 43.91±17.71 µg/ml.

DISCUSSION

Skin is the largest exposed organ of the body and is easily targeted for allergic and immunological reactions. Skin ailments, specifically dermatitis, urticaria, angioedema, psoriasis, etc., are immune-mediated disorders that are chronic, inflammatory, and proliferative in nature [17]. Psoriasis has become an important area of scientific study due to its severe effect on the quality of life, cost of treatment and toxicity, and the side effects of available medication [18,19]. Inhibition of hyperproliferation of epidermal keratinocytes is one of the key mechanisms by which most of the available antipsoriatic drugs act. HacaT cells are human spontaneous immortal keratinocyte cells and are often used as an effective model instead of primary-cultured keratinocytes and the data obtained from this model have shown a good correlation with *in vivo* skin irritation [11].

The drug PMC at 24 h incubation possesses significant antiproliferative activity, whereas in 48 h incubation the activity decreases (Tables 3 and 4). The effect of PMC at 48 h treatment shows only a few dead cells. In a study conducted by Khazaei *et al.*, the cytotoxic activity of methanolic extract of bulb of *Allium atroviolaceum* (BAA) in *MCF7*, *MDA-MB-231*, *HeLa* and *HepG2* cell lines, the results showed the BAA extract on *MDA-MB-231* treated cells entered early apoptosis stage in IC_{25}, IC_{50}, and IC_{75} concentrations after 24 and 48 h. However, at 72 h, the proportion of early apoptotic cells decreased compared to 24 and 48 h, this suggested that the cell death might occur due to nutrient depletion in the growth media or contact inhibition [20]. In the same way, PMC at 24 h showed an increase in anti-proliferative activity, but after 48 h exposure, the activity decreases (Fig. 2) this may be due to the same

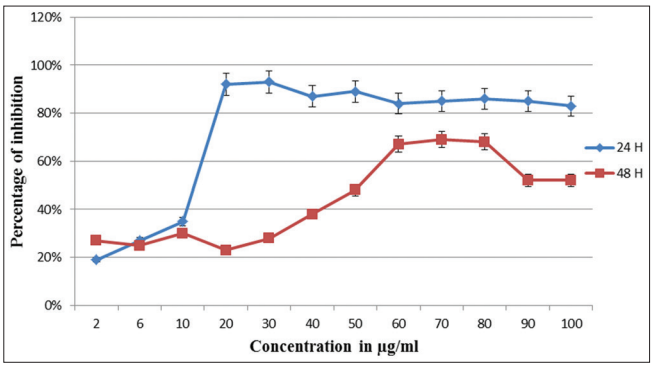

Fig. 2: *In vitro* antiproliferative activity of Panchamuga Chendhuram (PMC) in human keratinocyte (HacaT) cell lines. 3- (4, 5 dimethyl thiazole-2 yl) -2.5-diphenyl tetrazolium bromide assay indicated that PMC inhibited the viability of HacaT cells in a dose-dependent manner. The drug PMC at 24 h incubation possess significant antiproliferative activity (inhibitory concentration 50 [IC_{50}] 20 µg/ml) the mean and standard deviations (SD) was 72.08±27.56 µg/ml, whereas in 48 h incubation the activity decreases (IC_{50} 20 µg/ml) the mean SD was 43.91±17.71 µg/ml

reason dealt in the above study. This trial drug PMC was also tested in human breast cancer (MCF-7) cell lines in that study the IC_{50} values are constantly increasing in 24 and 48 h, this is contrary to the present study [21]. In the future, this study has to be carried out in animal models for a better understanding of the mechanism action of PMC. The PMC is shown IC_{50} 20 µg/ml in 24 h with good antiproliferative activity when compared with Asiaticoside as a positive control with an IC_{50} value of 20.13µg/ml.

A successful antipsoriatic drug that targets the epidermis is defined as a compound that ideally shows low toxicity and restores skin homeostasis by suppressing keratinocyte hyperproliferation, abnormal differentiation, or both [22]. The effect of PMC was observed in HacaT cell lines, and it is evident that the Siddha drug PMC has the ability to induce cell death. The probable mechanism in causing the cell death is by interacting with the cell membrane proteins and making the cell leak its cellular constituents and finally leading death, or maybe it is able to interact with the DNA or cell signaling pathways and manipulating the cellular pathways leading or triggering the cell death pathways [23]. To know the exact mechanism of Siddha drug

PMC, the evaluation of antiproliferative activity in an animal model is a must.

CONCLUSION

This study reveals the promising skin keratinocytes antiproliferative activity of Siddha drug PMC. Hence, the hypothesis that PMC can potentially be used in antipsoriatic therapy is shown. Further works on Siddha drug PMC need to be carried out in the treatment of psoriasis.

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AUTHORS' CONTRIBUTIONS

The authors have contributed equally to this work.

CONFLICTS OF INTEREST

No conflicts of interest.

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