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Original Research Article

Cytotoxic, cytostatic, and keratolytic activity of anti-dandruff shampoo formulations

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ABSTRACT

Background: Dandruff is characterized by increased sebum production, yeast colonization, and hyper-keratinization leading to an itchy-flaky scalp. Ancillary non-microbial reasons for flaky scalp include ultraviolet rays and excessive heat from the sun. Thus, apart from traditional anti-fungal activity, anti-dandruff formulations should also possess the keratolytic activity and should be safe to use.
Methods: Selsun, a shampoo formulation with 2.5% selenium sulfide (SS2.5), was tested in-vitro for its cytotoxic and cytostatic activities via cell viability of primary human dermal fibroblasts (HDFs). Its keratolytic activity was determined by assessing anti-proliferative activity on immortalized human keratinocyte (HaCaT) cell line. The shampoo was compared with 3 other shampoo formulations, one containing 2% ketoconazole (K2), second containing 2% ketoconazole with 1% zinc pyrithione (K2ZP1), and third containing 1% zinc pyrithione (ZP1).
Results: Of the tested samples, SS2.5 and K2ZP1 showed cytostatic and keratolytic activity where the IC_{50} values on normal HDFs i.e., 0.521 mg/ml and 0.220 mg/ml, respectively, were higher than those on HaCaT cells (0.162 mg/ml and 0.198 mg/ml, respectively). SS2.5 displayed the highest keratolytic activity at IC_{50} of 0.162 mg/ml, followed by K2ZP1 (0.198 mg/ml) and K2 (0.267 mg/ml). On normal HDFs, SS2.5 showed the highest IC_{50} value (0.521 mg/ml), followed by K2 at 0.264 mg/mL, K2ZP1 at 0.220 mg/ml, and ZP1 at 0.139 mg/ml.
Conclusions: SS2.5 was found to possess the highest cytostatic and keratolytic activities among all the shampoo formulations tested, thereby confirming its safety profile.

Keywords: Selenium sulfide, Ketoconazole, Zinc pyrithione, Immortalized keratinocytes, Human dermal fibroblasts, Cytostatic activity, Keratolytic activity

INTRODUCTION

Dandruff is restricted to the scalp region and affects 50% of the global adult population.[1] Dandruff is responsible for the itchy-dirty condition of the scalp irrespective of gender and ethnicity.[2] The word dandruff and seborrheic dermatitis are often used interchangeably; however, the presence of inflammation and extension of lesions in scaly scalp conditions rules out dandruff as a diagnosis effectively.[3] Dandruff is thus a milder form of seborrheic dermatitis with fungal colonization.[4]

Before treating a condition, it is of utmost importance to diagnose it precisely; thus, it is important to understand the progression and formation of this "itchy-scalp" skin condition. Increased sebum production at the onset of puberty occurs as a result of the influence of androgens.[5] Dandruff is a condition that occurs mostly in the post-pubertal population wherein the activity of sebaceous

glands is maximal owing to the heightened influence of androgens.[5] The microbial etiology of dandruff is associated with the presence of higher numbers of *Malassezia* species that represent 75% of the scalp microbial population along with *Staphylococcus* and *Propionibacterium* spp (now *Cutibacterium*).[6] A recent study on the colonization of the healthy scalp versus normal scalp with microorganisms revealed higher frequencies of *M. restricta* and *Staphylococcu*s spp in dandruff-associate scalp ($p<0.05$) in contrast to a healthy population.[7] Yeast colonization occurs in the stratum corneum, particularly in human keratinocytes and corneocytes.[8] A non-microbial etiology of dandruff can be direct sunlight that results in desquamation of the scalp without primary or original fungal involvement.[9] The susceptibility of the infection by *Malassezia* spp occurs upon disruption of the barrier function of the skin due to the expression of *Malassezia* lipases and the interaction with scalp lipids.[10] Thus, the susceptibility to infection by yeast is higher in the sebum-rich scalp.[11]

Squalene, a common component of sebum, is found to be peroxidized to form monohydroperoxide squalene (SQOOH) in the dandruff-infected scalp. This SQOOH is responsible for the altered barrier function of the stratum corneum, hyperproliferation of keratinocytes, and classical signs of dandruff such as flakes and itchy scalp.[12] Apart from this, hyperkeratinization is also commonly seen in acne, seborrheic dermatitis, psoriasis, and dandruff. To fight hyperproliferation of keratocytes, certain anti-proliferative agents or keratolytic agents are needed that can reduce hyperkeratinization of the skin and sebaceous glands.[13] Selenium sulfide is one such potent keratolytic agent used in dermatologic shampoos in the treatment of dandruff and seborrheic dermatitis.[14] Reports suggest that selenium sulfide exhibits a cytostatic effect by reducing the turnover of corneocytes and keratinocytes involved in the formation of dandruff.[15] Conditions like dandruff and psoriasis can result in hyperproliferation of keratinocyte cells due to dysregulation in apoptosis.[16] The corneocytes thus clump together and cause flakiness in the scalp skin. Hence, agents that induce apoptosis in keratinocytes can be used for the treatment of dandruff.[17] The anti-proliferative and keratolytic effects of anti-psoriatic agents are often assessed on human immortal keratinocyte (HaCaT) cell line as a model for hyper-keratinization.[18] Thus, anti-dandruff shampoo formulations apart from their antifungal ability should exhibit anti-proliferative ability against keratinocytes or keratolytic potential.[19] However, while exhibiting the keratolytic activity, the growth of the normal cells should not be negatively affected. Cytotoxic agents kill all cells, whereas cytostatic agents inhibit the growth of abnormally differentiated cells without affecting the growth of normal cells. Thus, cytostatic compounds are presumably less toxic and more target-specific in nature.[20]

Hence, in the current study immortalized keratinocyte cells (HaCaT) were used to determine the keratolytic activity of anti-dandruff shampoo formulation containing 2.5% selenium Sulfide. Anti-dandruff shampoos containing ketoconazole and zinc pyrithione are also commonly used. These agents alone and in combination are known to exhibit anti-*Malassezia* activity.[21] In the current study, the keratolytic ability and cytostatic potential of the anti-dandruff shampoo with 2.5% of selenium sulfide (SS2.5) was studied and compared to 3 other anti-dandruff formulations namely, 2% ketoconazole (K2), 2% ketoconazole and 1% zinc pyrithione (K2ZP1), and 1% zinc pyrithione (ZP1), *in vitro*, using human keratinocyte cell line HaCaT and primary human dermal fibroblasts (HDFs), respectively.

METHODS

Materials

Selsun, a shampoo formulation containing 2.5% selenium sulfide (SS2.5) is a product of Abbott Healthcare Pvt Ltd and was obtained from Apollo Pharmacy, Mulund, Mumbai. The other formulations K2, K2ZP1, and ZP1 were also obtained from Apollo Pharmacy, Mulund. Primary HDFs isolated from juvenile foreskin cells (product code: CL011) were procured from Himedia Laboratories, Mumbai, India. The media used was Dulbecco's Modified Essential Media (DMEM), which was procured from Himedia Laboratories, Mumbai, India. Fetal bovine serum (FBS) and 0.25% Trypsin-EDTA (1×) were obtained from Gibco, Thermo Fisher Scientific, India. Antibiotic solution (100×) with 10,000 U penicillin and 10 mg streptomycin per ml in 0.9% normal saline was procured from HiMedia Laboratories, Mumbai, India. The HaCaT cell line was maintained in the laboratory in DMEM media with 10% FBS and 1% antibiotic solution.

Maintenance of the cell lines

The HaCaT cell line was maintained in DMEM media with 10% FBS and 1% antibiotic solution. The media was changed thrice a week. The cells were subcultured in three days with 2 ml of 0.25% Trypsin-EDTA (1×) solution for 2 min in CO_2 incubator at 37ºC and 5% CO_2. The cells were split in a ratio of 1:2. A similar procedure was followed for the primary HDF cells. Only difference was that these cells were grown on pre-coated collagen (HiMedia Laboratories, Mumbai, India) flasks. The cells were used only till four subcultures on arrival.

Cytotoxicity on primary HDFs

The assay was performed in a 96-well tissue culture plate and the methodology mentioned in Utgikar et al was followed.[22] HDFs were trypsinized and seeded in a 96 well plate at a cell density of 0.5×10^4 cells/well. The cells were allowed to attach in pre-collagen coated plates and proliferate for 24 hours in a CO_2 incubator at 37 ºC and 5% CO_2. Next day, the media was removed and cells were treated with shampoo formulations. The four anti-dandruff formulations were diluted in media to obtain concentrations of 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/ml. The cells treated with samples were incubated in a CO_2

incubator at 37ºC and 5% CO_2 for 24 hours. The following day, the cells were stained using sulforhodamine-B assay (Sigma Aldrich), and the cell viability was calculated as described below. The percent viability was calculated using the formula where control represents untreated cells maintained in DMEM media containing 10% FBS and antibiotic solution.

$$\text{Percent viability} = \text{Absorbance of sample} \times 100) / \text{absorbance of control}$$

The percent viability of cell control was considered to be 100%. A graph was plotted for cell viability on the Y-axis and concentrations of the samples on the X-axis. The concentrations of the samples having a cell viability of 50% i.e., the IC_{50} value of the sample were calculated.

Keratolytic activity on HaCaT cells

The assay was performed as per the protocol mentioned in Robles-Escajeda et al with slight modification.[23] HaCaT cells were trypsinized and seeded in a 96-well plate at a cell density of 2×10^4 cells/well. The cells were allowed to attach and proliferate for 24 hours in a CO_2 incubator at 37ºC and 5% CO_2. After 24 hours of incubation, the media was removed and cells were treated with different concentrations of the antidandruff formulations after the cytotoxicity of the samples was noted. The cytostatic activity of the formulation is its ability to affect the proliferation of abnormal cells versus normal cells; hence the IC50 value of keratinocyte cell line is expected to be lower in comparison to HDF. All the formulations were diluted to concentrations of 0.0625, 0.125, 0.25, and 0.5 mg/ml. The culture plates were incubated in a CO_2 incubator at 37ºC and 5% CO_2 for 24 hours. The following day, the cells were stained using sulforhodamine-B assay and the cell viability was calculated.

Sulforhodamine B staining

After the cytotoxicity and keratolytic assay were set up, the plates were processed for measurement of cell viabilities with the help of sulforhodamine-B.[24] The method is used to determine the protein content of the cells which screens the cell viability or cell cytotoxicity in adherent cells in a 96-well plate. For sulforhodamine-B staining, the plates, after incubation with target compounds, were fixed with 40 µl of cold 10% trichloroacetic acid in water for 1 hour at 4 ºC. After incubation, the plates were washed four times with distilled water and then dried with paper towels to remove any traces of water. The plates were then stained with 40 µL of 0.057 (w/v) sulforhodamine-B solution and incubated at room temperature for 30 mins. Excess dye was removed. The unbound dye was then washed with 1% glacial acetic acid four times, and the plates were dried with paper towels. The dye bound to cell protein was then extracted using 10 mM tris-base (pH 10.5) and after shaking for 1 min, absorbance was measured in a microplate reader (Thermo Lab Systems, MRX revealer software) at 510 nm. The percent viability was calculated using the formula: percent viability=(absorbance of sample×100)/absorbance of control, where control represents untreated cells maintained in DMEM media containing 10% FBS and 1% antibiotic solution. The percent viability of cell control was considered to be 100%.

Statistical analysis

All the measurements in the triplicate data set were processed as mean±standard deviation using Microsoft excel. A graph was plotted for concentrations of the shampoo formulations on the X-axis and the percent viability of the samples on the Y-axis for both the cell lines in the Microsoft excel software and non-linear regression was used to calculate IC_{50} values.

RESULTS

Cytotoxicity of antidandruff formulations on primary HDFs

Dose-dependent percent viability of the four antidandruff shampoos, namely, SS2.5, K2, K2ZP1, and ZP1 is shown in Table 1. From the data obtained, it was observed that SS2.5 exhibited the least cytotoxic potential with the highest IC_{50} value of 0.521 mg/ml (Figure 1). K2 showed an IC50 value of 0.264 mg/ml, K2ZP1 with an IC_{50} value of 0.220 mg/ml and finally ZP1 with the least IC_{50} value of 0.139 mg/ml. Because the highest IC_{50} value among all shampoos belonged to SS2.5 at 0.521 mg/ml, keratolytic activity for all the shampoos was tested at concentrations ≤0.5 mg/ml.

Keratolytic activity of antidandruff formulations on HaCaT cells

The dose-dependent keratolytic activity of the four anti-dandruff shampoos is shown in Table 2. All shampoos possessed keratolytic potential at concentrations at or below 0.355 mg/ml (Figure 2). The samples SS2.5showed the least IC_{50} values at 0.162 mg/ml. K2P1 Showed IC_{50} values at 0.198 mg/ml. Further K2 and ZP1 with higher IC_{50} values at 0.267 mg/ml and 0.355 mg/ml, respectively, thus 3 shampoos showed lower keratolytic activity as compared with SS2.5. SS.2.5 had the highest keratolytic amongst the four anti dandruff shampoo.

Table 1: Percent cell viability by concentration of the shampoo formulations on primary HDFs.

Concentration (mg/ml)	Percent cell viability							
	SS2.5	SD (SS2.5)	K2	SD (K2)	K2ZP1	SD (K2ZP1)	ZP1	SD (ZP1)
0.0625	92.009	0.0025	76.956	0.019	79.909	0.006	75.720	0.003

Continued.

Concentration (mg/ml)	Percent cell viability							
	SS2.5	SD (SS2.5)	K2	SD (K2)	K2ZP1	SD (K2ZP1)	ZP1	SD (ZP1)
0.125	94.276	0.0139	77.745	0.007	68.673	0.005	50.861	0.005
0.25	80.597	0.0164	64.604	0.014	49.931	0.007	27.451	0.006
0.5	50.001	0.0435	20.370	0.019	17.755	0.001	19.352	0.004
1	26.284	0.0044	17.277	0.003	17.723	0.001	-	-

HDFs: human dermal fibroblasts, SD: standard deviation.

Table 2: Keratolytic activity of the shampoo formulations on HaCaT cells.

Concentration (mg/ml)	Percent cell viability							
	SS2.5	SD SS2.5	K2	SD K2	K2ZP1	SD K2ZP1	ZP1	SD ZP1
0.0625	66.957	0.002	64.058	0.003	70.556	0.001	73.333	0.003
0.125	52.893	0.003	54.821	0.002	61.299	0.003	64.124	0.001
0.25	42.448	0.003	51.563	0.001	43.889	0.002	50.400	0.003
0.5	32.011	0.000	44.444	0.001	33.069	0.002	48.148	0.005

HaCaT- immortal human keratinocyte cell line, SD, standard deviation.

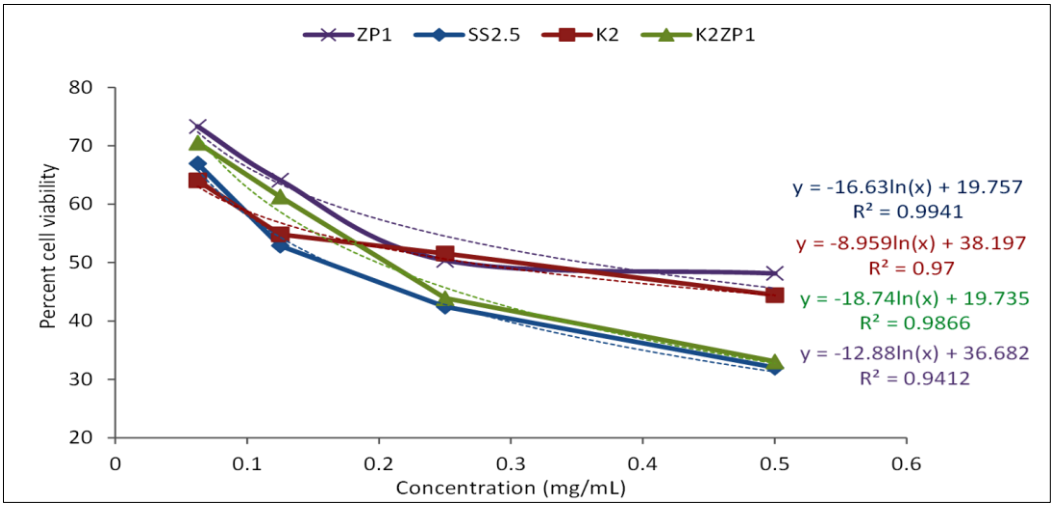

Figure 1: Dose-response curves for cytotoxic activity of various antidandruff formulations on primary HDFs.
IC_{50} values on HDF cells of SS2.5, K2, K2ZP1, and ZP1 were 0.521, 0.264, 0.220, and 0.139 mg/ml, respectively.

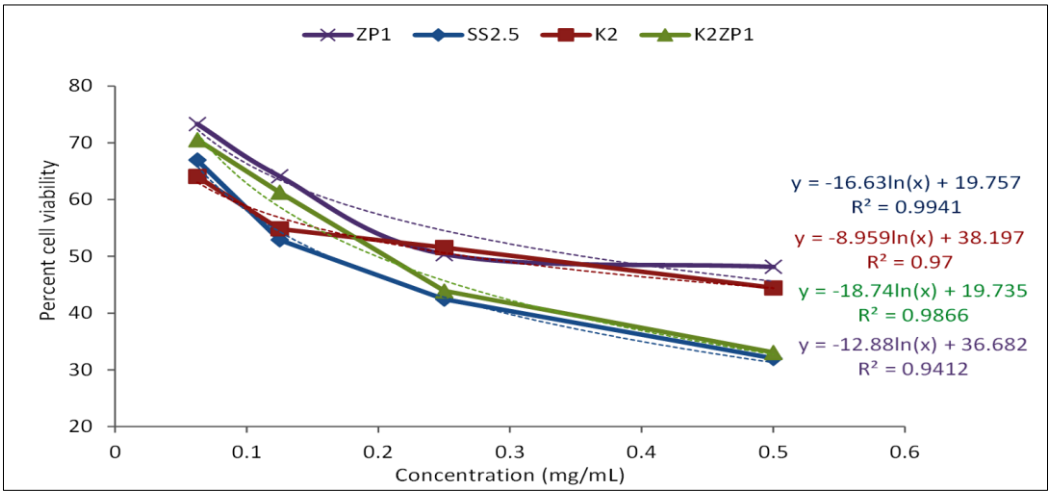

Figure 2: Dose-response curves for the keratolytic activity of various antidandruff formulations on HaCaT cells.
IC_{50} values on HaCaT cells of SS2.5, K2, K2ZP1, and ZP1 were 0.162, 0.627, 0.198, and 0.355 mg/ml, respectively.

DISCUSSION

In the current study, four anti-dandruff shampoo formulations were tested for their cytostatic and keratolytic activities. All the samples showed keratolytic activity in HaCaT cell line at concentrations equal to or less than 0.355 mg/ml. It was observed that formulations containing only K2 and P1 had lower keratolytic activity with an IC_{50} value of 0.267 and 0.355 mg/ml whereas K2ZP1 exhibited an IC_{50} value of 0.198 mg/ml. However, SS2.5 alone showed the highest keratolytic potential with IC_{50} of 0.162 mg/ml as compared with K2ZP1.

The cytotoxic potential tested on primary cultures of normal human dermal fibroblasts showed that formulation containing both 2% ketoconazole and 1% zinc pyrithione has an IC_{50} value of 0.220 mg/ml. This was higher than its IC_{50} value on HaCaT cells at 0.198 mg/ml. However, SS2.5 with 2.5% selenium sulfide showed a higher IC_{50} value at 0.521 mg/ml, indicating high cytostatic and low cytotoxic potential. To conclude, SS2.5 showed better keratolytic activity than K2ZP1 (2% ketoconazole and 1% zinc pyrithione) and was also found to be safer on HDF cells.

The shampoo formulations containing K2 and ZP1 alone had IC_{50} values on HDFs lower than those on HaCaT cells, indicating that these formulations showed toxicity on normal HDF cells. On the other hand, the formulations SS2.5 and K2ZP1 showed a cytostatic effect *in-vitro* as the IC_{50} value on the primary culture of normal human dermal fibroblast was higher than their keratolytic effect on the immortalized human keratinocyte cell line. Of them, 2.5% selenium sulfide shampoo formulation showed the highest efficiency and presumably lower toxicity *in-vitro* followed by formulation with 2% ketoconazole with 1% zinc pyrithione. These cytostatic compounds were less toxic but target-specific in nature.[20] It is also reported in the literature that selenium sulfide has anti-dandruff activity due to its anti-*Malassezia* activity, its cytostatic activity on normal epidermal cells, effectiveness in reducing the turnover of cells, and anti-seborrheic activity.[17]

CONCLUSION

Selsun, a shampoo formulation with 2.5% selenium sulfide, was tested for its non-microbial related anti-dandruff activity by targeting its ability to treat hyperproliferation of keratinocytes. It was compared against three other combinations of keratolytic agents such as formulations with 2% ketoconazole, 1% zinc pyrithione, and 2% ketoconazole with 1% zinc pyrithione. It was found that the efficacy of SS2.5 as a keratolytic agent was as high as the formulation containing both ketoconazole at 2% with 1% zinc pyrithione. However, SS2.5 showed a higher IC_{50} value on HDF cells in comparison to 2% ketoconazole with 1% zinc pyrithione, which explains its high selectivity towards hyperproliferative keratinocytes in comparison to the normal cells. This explains that SS2.5 showed a higher cytostatic and therapeutic value in comparison to all the other samples tested. SS2.5 was found to possess the highest cytostatic and keratolytic activities among all the shampoo formulations tested, thereby confirming its safety profile. Clinical trials should be conducted to corroborate the present *in vitro* data. It is important to have a streamlined drug trial conducted to formulate efficacious anti-dandruff shampoos with keratolytic ability and low cytotoxic potential.

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