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Evaluation of A Natural Anti-Dandruff Technology in a Shampoo Formulation via *In-vivo* and *In-vitro* Methods

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Abstract

Background: Natural anti dandruff efficacy is an area of interest as there is a consumer need for natural technologies in over-the-counter shampoo formulations.

Aim: This research explored dipotassium glycyrrhizate (DPG) for anti dandruff benefit utilizing in vitro and in vivo methodologies, examining a shampoo formulation with 0.15% DPG.

Patients/Method: The in vitro investigation focused on the evaluation of antimicrobial efficacy of DPG in a shampoo formulation by use of bacteriostatic efficacy testing as well as a short interval kill test against well known dandruff causing microorganism i.e. *Malassezia*. Clinically, 28 female and male subjects possessing visible dandruff were enrolled in a non-comparative study with 1 shampoo formulation containing 0.15% DPG. A trained investigator conducted visual technical assessments evaluating dandruff intensity at baseline and after product use over 2 weeks. A product performance self-assessment questionnaire was also completed for all subjects. The clinical was conducted under the supervision of a dermatologist.

Results: Shampoo formulation with 0.15% DPG showed bacteriostatic efficacy against well known dandruff causing microorganisms i.e. *Malassezia*. The short interval kill test shows that a shampoo formulation with 0.15% DPG is more effective against *Malassezia furfur* (ATCC 14521) compared to the placebo, a non-anti dandruff shampoo. Clinically, a shampoo formulation with 0.15% DPG significantly reduced visible flakes/dandruff after 1, 7 and 14 days of use from baseline and the greatest improvement as compared to baseline was seen at week 2 ($P < 0.001$) with 90% improvement in reduction of visible flakes/dandruff. The self-assessment questionnaire results correlated to the visual technical assessment results.

Conclusion: A shampoo formulation with 0.15% dipotassium glycyrrhizate (DPG) is effective in providing antimicrobial efficacy and clinically, significantly reduced visible flakes/dandruff in the scalp.

Introduction

The scalp consists of a diverse microbiome which contributes to either a healthy or diseased scalp. For instance, the proliferation of certain fungi in the scalp has been linked to seborrheic dermatitis and dandruff. Seborrheic dermatitis generally affects the scalp, face and other areas of the body where high concentrations of sebum are found. In regards to dandruff, this is mainly found in the scalp where it may cause scaling and itchiness with or without inflammation. *Malassezia* spp. is microorganisms well known for being the main drivers for dandruff or seborrheic dermatitis [1]. Most of the species in the genus *Malassezia* are present in human skin where the environment is warm and moist. Based on the genomic sequence analysis of *Malassezia*, this genus is understood to not have the gene necessary for fatty acid synthesis. It contains lipase phospholipase genes instead; this acts on sebum in human skin which in turn releases diglycerides as well as unsaturated and saturated fatty acids on the scalp. Most of these fatty acids are consumed by the fungus for its own growth on the scalp. This becomes a vicious cycle of *Malassezia* growth, release of fatty acids, consumption of those acids, and further growth. In those individuals with sensitive skin where there may be a concern in the skin barrier integrity, the fatty acids can penetrate the stratum corneum and cause further irritation and inflammation, ultimately producing various levels of dandruff and leading to other scalp conditions [11-13].

In today's market, the traditional actives that are found in anti dandruff shampoo products are zinc pyrithione (ZPT) [11-12], climbazole [13] and piroctone olamine. There is extensive clinical research and proven efficacy backing these ingredients [15]. While they are efficacious, all three of these active ingredients in formulations have their own set of challenges in a world that is gearing toward a natural solution. For example, these ingredients can provide an initial relief to a dandruff sufferer by eliminating or reducing *Malasseiza* from the scalp, but this can lead to a further imbalance in the microbiome due to the strength and impact of these active ingredients on the scalp. Beyond the efficacy, anti dandruff formulations tend to not be well-liked by consumers because of a less than ideal effect on hair condition [11-13]. This can impact the continued use of the product by the consumer, which if stopped or switched to non-anti dandruff shampoo, can lead to a relapse of dandruff and inflammation.

With that in mind, the aim of this research was to evaluate an alternative active ingredient that falls in the realm of natural - a gentle ingredient that would not be harsh on the scalp stratum corneum while also providing relief from dandruff. DPG comes from licorice, a plant originating in Asia, North Africa and Europe, belonging to the Glycyrrhiza genus that comprises multiple species. Constituents of *Glycyrrhiza glabra* are well known ingredients in a variety of medicinal preparations, either in the ayurvedic medicine or traditional remedies where multiple benefits are associated with the full plant consumption, its roots or extracts derived from a specific part of the plant. The benefits reach as far as antioxidant, anti asthmatic, anti diabetic, and

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even skin whitening activity has been attributed. Among the multiple phytochemicals present in *Glycyrrhiza glabra* root, the DPG is known for its anti-inflammatory and antimicrobial properties [8-10]. The active constituents of *Glycyrrhiza glabra*, found in DPG, have been documented as effective against fungi and bacteria [14]. This leads us to focus on DPG in a shampoo formulation. This research helps to continue the conversation on the scalp and dandruff, including exploration of the use of a shampoo formulation with DPG to reduce dandruff.

Materials & Methods

In Vitro Methods

Bacteriostatic Efficacy [3]: *Malassezia* furfur ATCC 44344 (test microorganism) was prepared in suspension. A stock of microorganism culture was taken from the freezer and a small amount of the frozen material was scraped from the surface with an inoculating loop and inoculated onto a Lecithin Tween-80 Nutrient Agar (LNA) plate. The plates were incubated at 36°C for 5-7 days. A single colony was transferred from the plate with an inoculating loop to spread on a freshly prepared LNA plate. After incubation (36°C for 5-7 days), the microorganism was washed with 0.85% saline solution. The level of initial microorganism suspension was about 1.0×10^5 CFU/ml. This was vigorously vortexes.

5.0 ml diluted shampoo sample (1:10 dilution with standard sterile hard water) was pipette into a tube, then kept at 20°C for 5 min. 0.1 ml of microorganism suspension was added to the tube containing 5.0 ml of diluted shampoo. The shampoo was vigorously mixed with the microorganism suspension thoroughly. At certain time points (30 s, 1 min, 1.5 min, 2 min, 5 min, 10 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h), 0.5 ml of the mixture of microorganism suspension and shampoo was transferred to a tube containing 4.5 ml of Phosphate Buffered Saline (PBS). This was vigorously vortexed. After 10 mins, serial 10-fold dilutions with 0.85% saline were made and thoroughly mixed by vortexing; an aliquot of 1 ml was pipette into a plate. 15-20 ml melted LNA agar (maintained at 44-48°C) was poured into the plate. The plate was rotated several times to disperse the product dilution. The plates were inverted and incubated at 36°C for 5-7 days after the agar was solidified. The colonies were counted to calculate the survival microorganism and bacteriostatic rate [3].

For the positive control, PBS was used instead of shampoo and the above steps were repeated.

Acceptance criteria

Bacteriostatic rate (%) = (I-II)/I*100.

I-Control sample colonies

II-Tested sample colonies

Bacteriostatic rate ≥50%~90%, indicates test sample has antibacterial effect

Bacteriostatic rate ≥90%, indicates test sample has strong antibacterial effect

Samples	Name	Remark
#1	Non-anti dandruff shampoo	Negative Control
#2	DPG prototype	Test sample
#3	ZPT shampoo	Positive Control

Short Interval Killing Test (SIKT) [4-7]: 4.5 g of each test sample was aseptically weighed into sterile homogeneous bags. 4.5 mL of sterile DI water was aseptically pipette into a test tube to serve as a test control.

Each sample was inoculated and controlled with 0.5 mL of prepared *Malassezia furfur* ATCC 14521 (approximately 10^6 CFU/L). It was mixed properly with a wooden applicator. Timer started immediately after inoculation. At the end of the selected time point (1 min, 2 min, 5 min), 0.5 ml of inoculated sample was added to 4.5ml of Dey/Engley (D/E) broth and vortexes until properly homogenized. This tube was denoted as 10^{-1} dilution and made dilutions up to 10^{-5} (performed dilution up till 10^{-7} for Negative control) in D/E broth and plate 1ml of each dilution in petri plate in duplicates, add 15-20 mL of melted Dixon Agar.

Plates were incubated at 30°C for at least 2-5 days. After incubation, plates were read and results recorded at 48hrs (2 days) and then at day 5 [4-7].

The higher kill rate and larger Log reduction indicate the better performance.

Samples	Name	Remark
#1	DPG prototype	Test sample
#2	ZPT shampoo	Positive Control

In vivo Method

30 female and male subjects with visible dandruff 18-56 years of age were enrolled in a clinical study. Following completion of an Independent Ethics Committee (IEC) approved informed consent (CONEP) and after meeting all inclusion criteria and none of the exclusion criteria, subjects with a minimum sum score of 10 points on the whole head through an dandruff intensity assessment done by a trained technician were enrolled in the study. Subjects were not allowed to use other anti dandruff products throughout the course of the study aside from the study product. The study was conducted under the supervision of a dermatologist.

All subjects who participated in the clinical research signed an Independent Ethics Committee (IEC) of Investiga - Instituto de Pesquisa, registered by the National Research Ethics Commission (CONEP) approved consent, and Good Clinical Practice guidelines were followed. The study was conducted in compliance with the Declaration of Helsinki principles.

A daily application of the test product (shampoo formulation with 0.15% DPG) was completed by a trained technician five (5) times a week on the weekdays for 2 consecutive weeks for each subject. Subjects were provided with the test product (shampoo formulation with 0.15% DPG) and instructed to use on the weekends at home following use-instructions learned at the facility. Subjects were allowed to continue the use of their own standard conditioner for the duration of the study.

The design was a non-comparative study, where the test product was tested on the subject's scalp/hair. On the initial visit (D0), after the visual technical assessment, an image was captured of the scalp before product use. Then, a controlled application of the test product was performed for each subject following a standardized method at the facility by a trained technician. After 24 hours (Day 1), week 1 (Day 7) and week 2 (Day 14), with continued daily application of the test product, new visual technical assessments and images of the scalp

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were captured via a Nikon camera with a Canfield Epiflash system (Canfield Scientific, Parsippany, New Jersey, USA). The images were captured under standard lighting from the top view of the scalp which was divided into four (4) quadrants i.e. five images were captured for each subject, one for each quadrant and one top view image during each evaluation. At the completion of the study, a self-assessment via questionnaire was completed by each subject.

Washings followed normal use instructions by applying 5-10 ml of test product depending on hair length, washing/massaging the hair with the test product for 60 seconds and rinsing for 60 seconds followed by application of the subject's standard conditioner.

Thirty (30) subjects were enrolled in the study and 28 twenty-eight subjects completed the study. One subject voluntarily withdrew and was discontinued from the study. Separately, there was one adverse event reported in which the subject experienced pruritus and scaling due to an individual sensitivity; the subject was voluntarily withdrawn from the study.

Statistical significance was defined as P-value less than or equal to 0.05.

The results of the self-assessment questionnaire at the end of the study were analyzed with a Binomial Test for within treatment: comparisons between frequencies of top box evaluations (2, 1) and bottom box (-1, -2) evaluations were performed. A significance level of 0.05 (5%) was chosen for all statistical analysis. Statement 3 of self-assessment correlates directly to the visual technical assessments i.e. improvement in dandruff.

Results and Conclusion

In vitro results

In Table 1, the bacteriostatic efficacy rates of the test product and controls (negative control: non-anti dandruff shampoo, positive control: anti dandruff shampoo with ZPT) prior to the 1 hr time point were low (< 24%). After 1 hr, the bacteriostatic efficacy rates of the test product and positive control showed high antibacterial efficacy, >76%, as compared to the negative control, <1%. This indicates that the test product is parity in efficacy to the positive control.

In Figure 1, the SIKT results show that the log reduction of test product versus water is 3.2, correlating to a 99.9% bacteria kill rate. The log reduction of placebo versus water is 2.4, correlating to a 99% bacteria kill rate. The test product is significantly better (1 log reduction) in achieving antibacterial efficacy when compared to the placebo.

See Figure 4 for representative images of the culture plates for these evaluations. The results show a reduction of *Malassezia* after exposure to the test product.

In vivo results

The results show (Table 2) that shampoo with DPG was significantly effective in reducing dandruff/visible flakes at all time-points ($p<0.05$).

Tables 3 and 4 are scales used to score the degree of dandruff of the scalp in each quadrant for each subject and the severity of dandruff in each quadrant for each subject. Scores from both scales were used to calculate the overall intensity of dandruff in the scalp for each subject [2].

To calculate the intensity of dandruff for each subject, scales were used for scoring and a calculation after dividing the scalp into four (4) quadrants as seen in Figure 2.

Calculations:

- Intensity of dandruff of the right side (Q2 and Q3) = (upper right score of Degree of Dandruff X upper right score of Severity of Dandruff) + (Lower right score of Degree of Dandruff X lower right score of Severity of Dandruff)
- Intensity of dandruff of the left side (Q1 and Q4) = (upper left score of Degree of Dandruff X upper left score of Severity of Dandruff) + (lower left score of Degree of Dandruff X lower left score of Severity of Dandruff)
- Total intensity of dandruff per subject = Intensity of dandruff of the right side (Q2 and Q3) + Intensity of dandruff of the left side (Q1 and Q4)

The questionnaire evaluations were summarized by counts and percentages of scores based on questions asked in Tables 5-7. Further, the categories of disagree (-1, -2), agree (2, 1), and neutral (0) questionnaire answers were given accordingly.

Visual images were captured by a Nikon camera with the Canfield Epiflash system of subjects 029, 056, and 033 from the study (Figure 3 and 4). Results show a significant reduction in visible flakes.

Table 1: Summary of bacteriostatic efficacy rates.

Samples	30s	1min	1.5min	2min	5min	10min	1h	2h	3h	4h	5h	6h
Negative Control (Non-anti dandruff shampoo)	-4%	11%	5%	15%	9%	2%	-2%	-7%	0%	17%	50%	74%
Test Product (DPG prototype)	5%	17%	0%	0%	13%	-2%	91%	98%	98%	98%	99%	99%
Positive Control (ZPT shampoo)	18%	11%	14%	20%	26%	23%	76%	86%	93%	97%	99%	99%

Table 2: Summary of dandruff intensity scores, N=28.

	Mean +/- STD						
	Baseline D0	Day 1	Day 7	Day14	Δ (Day 1 – Baseline)	Δ (Day 7 – Baseline)	Δ (Day 14 – Baseline)
Shampoo with 0.15% DPG	25.6 ± 2.1	19.4 ± 2.2	13.5 ± 2.0	10.0 ± 2.1	-6.2 ± 1.4	-12.1 ± 2.0	-15.6 ± 2.1
95% CI	21.4; 29.8	14.8; 24	9.3; 24	5.6; 14.4	-9.1; -3.3	-16.3; -7.9	-19.9; -11.3
Δ % Mean Improvement in relation to D0					24.2	47.3	60.9
% Subjects with Reduction					75.0	89.3	92.9
P-value					<0.001*	<0.001*	<0.001*
*Significant at a 5% level (Student T-test)							

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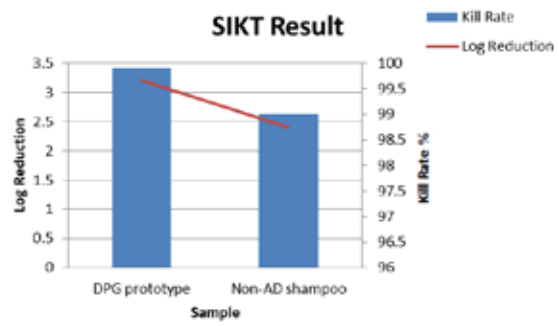

Figure 1: Short Interval Kill Test results of test product vs placebo.

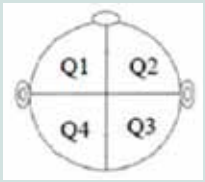

Figure 2: Division of the scalp for visual technical assessment.

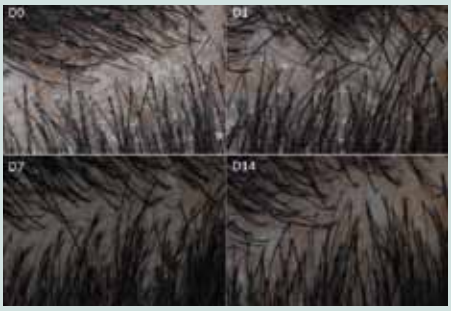

Figure 3: Before and after treatment images. Subject 029, D0=baseline, D1=24hr, D7= wk 1, D14= wk 2.

Subject 056, D0=baseline, D1=24hr, D7= wk 1, D14= wk 2; Subject 033, D0=baseline, D1=24hr, D7= wk 1, D14= wk 2;

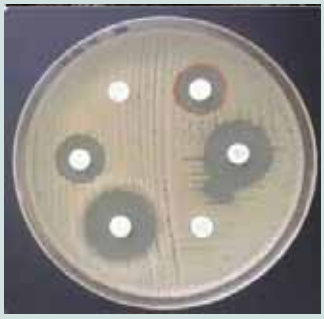

Figure 4: Representative images of the in vitro studies, Bacteriostatic Efficacy

SIKT, *The above images were not captured during the study; they are representative of the types of in vitro evaluations conducted during this phase.

Table 3: Scale: Degree of dandruff on scalp.

Affected Area	Score
Below 10%	0
10-30%	1
30-50%	2
50-70%	3
Above 70%	4

Table 4: Scale- Severity of dandruff on scalp.

Severity	Score
Small flakes resembling a white powder	1
Intermediate	2
Big flakes stuck to the scalp, Resembling an irregular surface	3
Intermediate	4
Flakes apparently frozen together, in the shape of yellowish plaques attached to the scalp, a few with evidence of exudation or erythema	5

Conclusion

In vitro testing confirmed the antimicrobial efficacy of dipotassium glycyrrhizate at 0.15% in a shampoo formulation through bacteriostatic efficacy testing and short interval kill test. The results for the short term interval kill test showed that the test product performed better in eliminating *Malassezia* when compared to the negative control (placebo).

Clinically, the shampoo formulation with 0.15% DPG significantly reduced visible flakes/dandruff after Day 1, 7 and 14 of use from

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Table 5: Summary of self-assessment questionnaire on Day 14, N=28.

	Count and Frequency of Answers for Self-Assessment Questionnaire, Day 14										Total	
	Strongly Disagree (-2)		Somewhat Disagree (-1)		Neutral (0)		Somewhat Agree (1)		Strongly Agree (2)			
	n	%	n	%	n	%	n	%	n	%	n	%
Q1	1	3.57	1	3.57	2	7.14	7	25.00	17	60.71	28	100
Q2	0	0.00	1	3.57	2	7.14	6	21.43	19	67.86	28	100
Q3	1	3.57	2	7.14	0	0.00	7	25.00	18	64.29	28	100
Q4	0	0.00	3	10.71	0	0.00	8	28.57	17	60.71	28	100
Q5	0	0.00	1	3.57	2	7.14	9	32.14	16	57.14	28	100
Q6	3	10.71	2	7.14	1	3.57	6	21.43	16	57.14	28	100
Q7	0	0.00	2	7.14	1	3.57	7	25.00	18	64.29	28	100
Q8	1	3.57	0	0.00	4	14.29	5	17.86	18	64.29	28	100

Table 6: Binomial test of disagree and agree on Day 14, N=28.

	Binomial Test of Disagree and Agree, Day 14						
	Total	Disagree (-2, -1)		Agree (2, 1)			
	n	n	%	n	%	P-Value	Result
Q1	26	2	7.14	24	85.71	<0.001	*
Q2	26	1	3.57	25	89.29	<0.001	*
Q3	28	3	10.71	25	89.29	<0.001	*
Q4	28	3	10.71	25	89.29	<0.001	*
Q5	26	1	3.57	25	89.29	<0.001	*
Q6	27	5	17.85	22	78.57	0.004	*
Q7	27	2	7.14	25	89.29	<0.001	*
Q8	24	1	3.57	23	82.15	0.001	*
*Significant at a 5% level, n.s.: not significant (P>0.05)							

Table 7: Self-assessment questionnaire questions.

Q1	The test product helps reduce the itchiness of my scalp.
Q2	The test product is mild.
Q3	The test product helps provide improvement in dandruff.
Q4	The test product helps my scalp feel healthy.
Q5	The test product helps my scalp feel refreshed.
Q6	The test product leaves my hair looking and feeling good.
Q7	The test product helps improve my scalp's health.
Q8	Overall, I liked the test product.

baseline and the greatest improvement as compared to baseline was seen at week 2 ($P < .001$) with 90% improvement in reduction of visible flakes/dandruff. The self-assessment questionnaire results correlated to the visual technical assessment results.

This study explored the use of DPG as a natural solution to dandruff and over all, helped to gently prevent the scalp from dandruff proliferation.

Based on preliminary research, literature and the series of tests as captured in this paper, we hypothesize that there are four (4) key factors that provide anti dandruff benefit from a DPG shampoo formulation. First, it's documented in various publications as mentioned previously, that DPG provides anti inflammatory and antimicrobial benefits [14]. Secondly, the complete formula is at a pH of 4.0 which provides a mild acidic environment in order to inhibit bacterial and fungal growth. In addition, the formula contains a high level of surfactants which helps to enhance overall cleansing as well as antibacterial and antifungal benefits. Lastly, the formula contains menthol which provides a soothing feel as to prevent itching of the scalp. The synergy of the above factors delivers significant anti dandruff benefits.

A limitation in this research is that the clinical study did not contain a comparative control to help distinguish any benefit over traditional anti dandruff shampoos. In addition, the evaluations were restricted to only dandruff and flaking. To continue understanding the impact of natural ingredients such as DPG on the scalp in a clinical setting, future research should include a control test product as well as evaluations such as anti inflammatory biomarkers and microbiome shifts from baseline to after product use. This research can be used as the foundation work for future research in the area of scalp health as related to the scalp's microbiome via metagenomics and sequencing [17].

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Conflict of Interest

This research was funded by Colgate-Palmolive and all of the authors are employed by Colgate-Palmolive.

Author Contribution

All authors participated in the conduct of the research and writing of the manuscript.

Ethical Approval

All subjects who participated in the clinical research signed an Independent Ethics Committee (IEC) of Investiga - Instituto de Pesquisa, registered by the National Research Ethics Commission (CONEP) approved consent, and Good Clinical Practice guidelines were followed.

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