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IN VITRO EXPRESSION STUDIES OF AQUAPORIN-3 (AQP-3) GENE STIMULATION BY PURE ALOE VERA FACE WASH IN KERATINOCYTES

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ABSTRACT

Aquaporin-3 (AQP-3) is the most abundant and important membrane protein that facilitates water transport in and out of keratinocytes and thus possessing a very crucial role of maintaining stable water levels in our skin. Given the significance of hydration and mosturization in skin health and appearance, cosmetic and heath care companies have a reason to focus upon the activity or presence (expression) of aquaporins, especially AQP-3 stimulatory personal care products. Aloe Vera has been widely used in ayurvedic and herbal cosmetic industries for its skin rejuvenating, soothing and moisturizing (hydrating) activities. The goal of this study was to evaluate Aloe Vera based poly-herbal face wash for its stimulatory effect on AQP-3 genes expression. Results showed that the test substances at concentrations of 350 and 700 µg/ml up-regulated the expression of AQP-3 levels by 0.15 and 0.04 folds, respectively, in comparison to the basal levels of AQP-3 mRNA. This study demonstrates that the test product has the ability to enhance skin hydration by stimulating skin specific AQP-3 gene, thereby providing insight into the molecular mechanisms by which this product could reduce the effects of skin dryness.

Keywords: Gene expression studies, AQP-3 expression, Skin hydration, Pure Aloe vera

INTRODUCTION

Aloe vera plant has been widely known and used since centuries for its health, beauty, medicinal and skin care properties [1]. There are over 300 different species of aloe which grow mainly in the dry regions of Africa, Asia, Europe and America. The botanical name of Aloe vera is Aloe barbadensis miller and it belongs to the Asphodelaceae (Liliaceae) family and it is a shrubby or arborescent, perennial, xerophytic, succulent, pea- green color plant [2, 3]. Aloe vera contains 75 potentially active constituents: vitamins, enzymes, minerals, sugars (polysaccharides), lignins, saponins, salicylic acids and amino acids [1, 4]. It has been widely used in ayurvedic and herbal cosmetic industries for its skin rejuvenating, soothing, moisturizing (hydrating), heeling and antiacne effects and, also for its protective effects against UV-induced skin damage [5,6].

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Also, many scientific studies on aloe vera have demonstrated several of its other health beneficial activities such as - antioxidative, anti-inflammatory (immunomodulatory), antibacterial, antiviral, antifungal, antiulcer, antidiabetic, antihypercholestermic, cardiac stimulant, nutraceutical, and also wound healing activities. Aloe vera could be used in treating various clinical conditions such as, mild to moderate skin burns, UV-induced erythema, genital herpes, seborrheic dermatitis, psoriasis vulgaris, oral lichen planus infections, angina pectoris, ulcerative colitis, alveolar osteitis [7-13]. In general, topical application of aloe vera preparations has been regarded as safe as assessed by the Cosmetic Ingredient Review Expert Panel [14]. However, case reports of the development of hypersensitivity reactions and contact dermatitis in response to topically applied aloe gel preparations have been published [15].

Aquaporins (AQPs) are proteins located in cellular membranes with the important function of forming channels permeable to water and glycerol. Amongst AQPs, one type aquaporin-3 (AQP-3) is the most abundant and important membrane protein that facilitates water transport in and out of keratinocytes in the epidermal layer of the skin and thus possessing a very crucial role of maintaining stable water levels in our skin [16,17].

Given the significance of hydration and mosturization in skin health and appearance, cosmetic and heath care companies have a reason to focus upon the activity or presence (expression) of aquaporins, especially AQP-3 stimulatory personal care products [18,19].

InnoVision's pure Aloe Vera face wash is enriched with the goodness of aloe vera, cucumber and rose, helpful in maintaining the elasticity, provides soothing effect and gives an even tone to the skin. It rebuilds lost moisture from the skin after each wash, eliminating dry and stretched skin and also cleanses and moisturizes the skin making it smooth and supple. It also provides cooling effect on the skin and eliminates skin inflammation. The goal of this study was to evaluate InnoVision's Aloe Vera face wash for its stimulatory effect on AQP-3 (Aquaporin) genes expression in keratinocytes.

Materials and Methods:

Details of the materials and methods that are not specified in the subsequent sections of this study plan are contained in the appropriate Radiant Research standard operating procedures.

Test System:

Test system	HaCat (Human Keratinocyte) cell line has been used as an *in vitro* the test substance, model to evaluate the AQP-3 stimulatory effect of the test substance
Test culture preparation	Cell lines were cultured in DMEM high glucose media supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37^0C until confluent. The cells will be dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures will be grown in 25 cm^2

culture flasks and all experiments will be carried out in 96 microtitre plates.

Test Product: InnoVision's Pure Aloe Vera Face Wash

Preparation of Test Doses

For studies, each weighed test substances were separately dissolved in Media and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Procedure:

Determination of cell viability by MTT Assay

Principle: 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 principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured 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.

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^{o} C for 3 days in 5% CO^{2} atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^{o} C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilise the formed formazan. The absorbance

was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

Gene expression

Culture treatment

The HaCat cells were cultured in 60 mm petridish and maintained in DMEM medium for 24 hrs. The DMEM medium was supplemented with FBS and amphotericin. Twenty four hours after plating, HaCat cells were treated with test substances of nontoxic concentration i.e. AFW – 07/15 (700 µg/ml) and AFW – 07/15 (350 µg/ml), cell control (Culture media) and positive control all-trans retinoic acid (ATRA) incubated for 24 hr. After incubation, the supernatant solution from the cultures was discarded and cultures were processed for total RNA extraction.

***RT-PCR* Procedure**

The mRNA expression levels of AQP-3 carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, after 24 hour incubation period, Total cellular RNA was isolated from the untreated (control) and treated cells using Tri-Reagent according to manufacturer's protocol. cDNA was synthesized from total isolated RNA by reverse transcriptase kit according to manufactures instructions (Thermo scientific). Then 20µl of the reaction mixture was subjected to PCR for amplification of AQP 3 cDNAs using specifically designed primers procured from Eurofins India, as an internal control, the house keeping gene GAPDH was co-amplified with each reaction. PCR was carried out in MJ Mini Thermocycler (Bio Rad, U.S.A) and PCR conditions for genes were initial denaturation at 95 °C for 5 min followed by 35 cycles consisting of denaturation at 95 °C for 1 minute, annealing of primers (refer table for temperature) for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 10 minutes.

DETAILS OF THE PRIMERS:

Primer type	**Oligonucleotide bases**	**Annealing temperature**	**Product size (base pairs)**
AQP 3	Forw 5' GCTGTCACTCTGGCATCCTG'3' Rev 5'GCGTCTGTGCCAGGGTGTAG'3'	61	150
GAPDH	Forw 5'ACC ACA GTC CAT GCC ATC AC 3' Rev 5'CAC CAC CCT GTT GCT GTA GCC 3'	60	500

Results

Results suggested that the test substance showed regulatory effects on the levels of AQP-3 gene expression in keratinocytes. The standard retinoic acid increased the level of AQP-3 mRNA by 0.39 fold when compared to that of control. Whereas, the test substances at concentrations of 350 and 700 µg/ml up-regulated the expression of AQP-3 levels by 0.15 and 0.04 folds, respectively, in comparison to the basal levels of AQP-3 mRNA seen in untreated control HaCat cells (Figures 1, 2 and 3). Furthermore, cytotoxicity associated results indicat-

ed that the test substance in the concentration range tested for AQP-3 stimulation had insignificant toxicity on keratinocytes and was found to be safe for usage (Table 1).

Table 1: Cytotoxic properties of test substances against HaCat cell line

Sl. No				Name of Test sample	Test Conc. (%)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	RR 2638	1000 500 250 125 62.5	82.25±1.3 23.99±1.1 1.12±0.8 0.86±0.3 0.83±0.6	723.23±7.6			

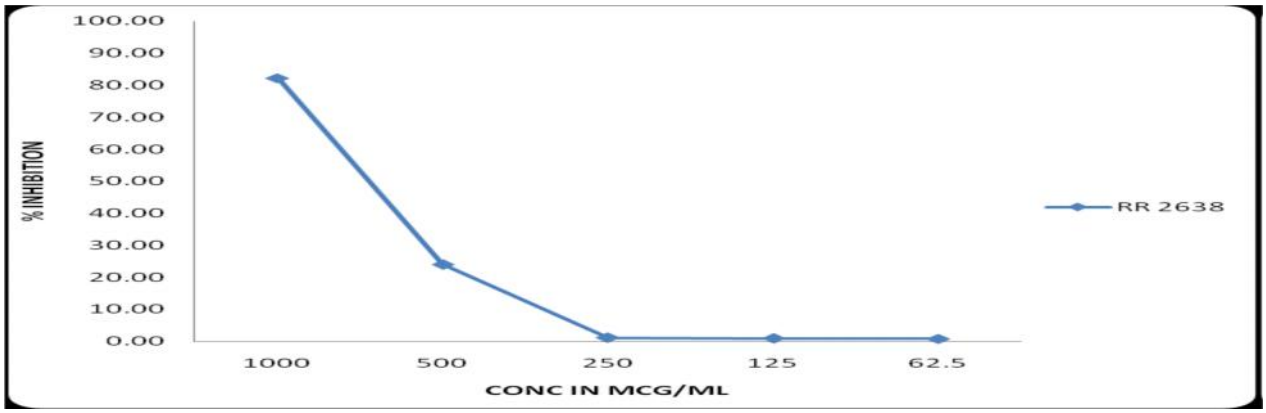

Fig. 1: Cytotoxic effect of the test substance on HaCat Cell line.

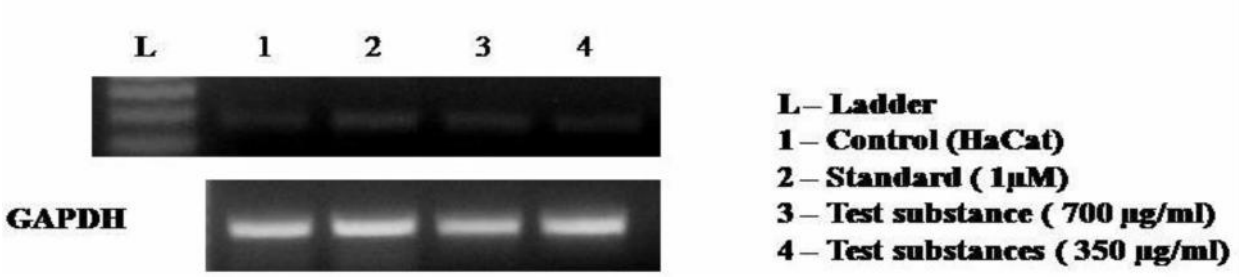

Fig. 2: **RT-PCR profile of AQP 3 gene amplified from test substance treated HaCat cells**

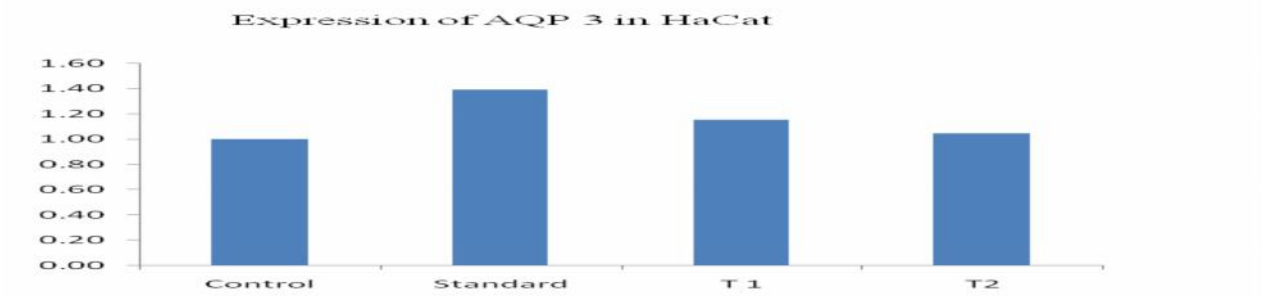

Fig. 3: Densitometric analysis of gene transcripts. The relative level of AQP-3 gene expression is normalized to GAPDH. Values shown depict arbitrary units.

Discussion

Skin is a very crucial and primary most defence organ of our body, covering the internal organs and protecting them from external invasions by mechanical, thermal, chemical, microbial and UV-radiation agents etc [20]. Skin also helps in trafficking of water and heat in and out of the body. The epidermis which is the outer most layer of the skin, consisting of keratinocytes mainly, is responsible for the physiological homeostatic functioning and requires balanced hydration (water levels) and maintains the outward appearance of the skin. Imbalanced, i.e., Over/under hydration results in alterations in the physical and functional properties of the skin, leading to disruption of skin tissues/layers by, repair mechanisms that mount inflammation in the affected skin sites, and by the colonization of foreign microorganisms, bacteria, fungi and others, eventually resulting in a dry and diseased skin [21].

Thus, it is well known that maintaining an optimum level of skin hydration is fundamental in any skin care regimen. It is also clinically very well known that skin aquaporins (AQPs), especially aquaporin-3 (AQP-3) triggered mechanisms are widely involved in maintaining balanced skin epidermal hydration and play an important modulatory role that could implicate in the overall wellness/appearance of the skin [22-24]. The results of this study demonstrate that the product tested has the ability to enhance skin hydration by stimulating skin specific AQP-3 gene, thereby providing insight into the molecular mechanisms by which the test product could reduce the effects of skin dryness. The active ingredients present in the composition of the test substance such as, vitamins, minerals, sugar alcohols (polysaccharides) and other active constituents might be responsible for having a direct effect on AQP-3 gene expression. There are many instances where such classes of compounds — caffeine, sugars, and vitamins — have worked as AQP-3 stimulators, either, by acting directly on AQP-3 or by indirect fashion triggering osmotic stress with the capacity to upregulate AQP-3 gene expression [25,26]. Thus, the scientific data presented in this study should make the tested product, Innovision's pure aloe vera face wash, due to its aquaporin harnessing features, a prime candidate in the product line of skin care regimen, especially suited for dry and sensitive skin.

Conclusion:

Innovision's pure aloe vera face wash has a positive effect on AQP-3 gene expression. The product supposedly utilizes the mechanism of AQP-3 stimulation to create an effective product with hydrating and moisturizing effect by increasing AQP-3 levels in skin.

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