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STUDIES ON AN ANTI-AGING FORMULATION PREPARED USING *ALOE VERA* BLENDED COLLAGEN AND CHITOSAN

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ABSTRACT: Photochemoprotection has nowadays become inevitable to combat aging. Exposure to Ultraviolet radiations accelerates skin aging which eventually culminates in wrinkles, laxity, dyspigmentation, roughness and dryness. Though many active synthetic topicals have been used since years, the present era of treating an aged skin has been diverted towards natural biomaterials as these synthetic topicals pose health and safety risk on human health. In an effort to produce a promising natural anti-aging agent in contemporary cosmetics, this study has been aimed to formulate an anti-aging gel by blending three biopolymers namely Collagen (COL) 3%w/v, Chitosan (CS) 1.5% w/v and *Aloe vera* (AV) gel 0.21% w/v. The AV blended COL-CS gel was characterized by spreadability and moisture uptake test, which indicated good spreadability and increased hydrophyllicity of the gel. Cell culture studies on NIH3T3 mouse fibroblasts cells were carried out using senescence-associated-β-gal [SA-β-gal] as a biomarker. The fibroblasts cells on incubation with AV blended COL-CS gel showed increased proliferation rate and the absence of blue stain indicated that the process of senesce has been reversed. In conclusion, the prepared AV blended COL-CS gel helps in regeneration and rejuvenation of the skin and therefore can be used as a promising anti-aging gel.

INTRODUCTION: In modern days, skin aging has become a major symptom, involving the process of photo induced oxidative reactions and exposure to pollutions resulting from industrialization *etc*. Repetitive exposure to UV radiation, leads to free radical formation which breaks down the lipids to form malondialdehyde [MDA]. This MDA subsequently cross-links with collagen, leading to the loss of skin elasticity and decreased moisture holding capacity which are very much related to the most obvious symptom of skin wrinkling [1].

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Therefore, for quenching the free radicals release and to restore the skin elasticity, we are in need of promising topical treatments.

Skin wrinkling is one of the common symptoms of aging. The identification of new biological mechanisms associated with skin aging and continuous discoveries of new acting forms of compounds which can prevent the appearance of or recover the signs of aging are two fronts, which cooperate for these advances in knowledge [2].

Excessive long-term sun exposure contributes to signs of aging and the damage so produced is most readily recognizable on our external surface of the skin [3]. Stratum corneum, the primary barrier of the skin, is rich in cholesterol free fatty acids and ceramides. Water from this stratum corneum gets evaporated rapidly, thereby resulting in dehydration.

By using moisturizers, this dehydration of the skin can be averted and flexibility can be imparted to the skin [4]. As we know that the dry skin is an early sign of aging, moisturizing the skin is essential to maintain a youthful appearance.

When histology of photoaging were considered, the main features include dermal damage with marked elastotic degenerative change, collagen loss, reduction in number and size of fibroblasts, an increase in proteoglycans and a moderate mononuclear inflammatory cell infilterate [5]. Development of novel strategies and suitable delivery systems that can reduce the occurrence and delay the process of photoaging are highly desirable goals. Photoprotection could be achieved efficiently by the use of sunscreens, moisturizers, keratolytics and antioxidants [6]. The active synthetic molecules as photoprotectives, are being used in a number of cosmetic formulations since years. It has been reported that these active molecules adversely affect the skin via self inducing reactive oxygen species. Therefore the research is now diverted towards natural biomaterial, which in turn helps to overcome such serious side effects of these synthetic molecules [7].

Collagen (COL), one of the most promising natural material, acts as a guide for cell growth and forms the major structural protein of the extracellular matrix [8]. Because of excellent biocompatibility, biodegradability and non-toxic properties [9, 10], COL has been selected as one of the biopolymer in this study. However, increased degradation rate and poor mechanical strength pose limitations for the use of this material. An effective method, which modifies its degradation rate and optimize its mechanical properties is by blending it with another natural polymer [11].

Another biopolymer, widely used in a variety of biomedical field is Chitosan (CS) [12]. CS has the ability to induce local cell proliferation and it possess enough stability when integrated with the host tissue [13]. Among the naturally derived polymers, the COL [14 - 15] and CS [13, 16] possess biological and chemical similarities with the natural tissues, because of which they have been given special attention. Also, In a study, it was reported that COL on combining with CS influences the chemical and mechanical properties [17, 18].

Likewise the emollient and anti-aging properties of the *Aloe vera* (AV) gel has made it an important constituent in many cosmetics [19]. Glucomannan, an emollient polysaccharide and a good moisturizer is reported to be present in the gel, which accounts for its use in many cosmetics. In a study, it was proposed that skin hydration was improved by AV gel containing products possibly by means of humectant mechanism. In a study it was reported that glucomannan has the capacity to proliferate fibroblasts cells and increase collagen production and secretion [20].

In addition, the gel is reported to possess an effective anti-inflammatory and anti-bacterial activity [21]. Though fish COL in combination with CS and AV [22] and shark collagen in combination with Aloe [23] have been reported in the preparation of scaffolds for tissue engineering applications, the formulation of bovine COL in combination with CS and AV gel as an anti-aging gel for cosmetic purpose has not been yet reported.

MATERIALS AND METHODS: Chitosan (80% deacetylation) Dulbecco's Modified Eagles's Medium [DMEM], trypsin, EDTA, 5-bromo-4-chloro-3-indolyl-β-D-galactoside[X-Gal], antibiotic and antimycotic solution were obtained from SIGMA. Fetal Bovine Serum [FBS] was purchased from Pan Biotech Company. NIH3T3 [mouse fibroblasts cells were provided by National Centre for Cell Sciences, Pune, India]. Glacial acetic acid and Dimethyl Sulphoxide [DMSO] were procured from SRL chemicals. All other reagents used were of analytical grade.

Collagen Extraction: Collagen was prepared from collagenous tissue of bovine origin by mechanical disintegration followed by swelling in dilute Hydrochloric acid and then treated with pepsin to get collagen solution. The supernatant solution of the enzyme digest was precipitated by using NaCl [30% w/v] followed by washing the precipitate in distilled water. Thus obtained Reconstituted Collagen was re-dissolved and made to swell in dilute acetic acid [0.5 M]. This hydrated collagen mass, after knowing the solid content was used in the preparation of anti-aging formulation.

Seperation of *Aloe vera* Gel: Well matured *Aloe vera* leaves were collected from a single plant and

washed thoroughly with sterile water. The leaf rind was removed and the colourless gel was separated in a sterile condition. The collected inner gel was frozen and lyophilised at -80°C and stored at -20°C until further use.

***Aloe vera* Blended Collagen-Chitosan Gel Preparation:** Solutions of COL (3% w/v) and CS (1.5% w/v) in 0.1 M succinic acid were mixed thoroughly. The collected AV gel (0.21% w/v) was then added to the above prepared solution and blended thoroughly. The AV blended COL-CS gel was then homogenized for good mixing of the three biopolymers and the pH was tested.

Characterization of Anti-aging Gel:

Determination of Spreadability of the Gel: The slide method was used to evaluate the spreadability of the gel in between two slides. About 1 gm of the prepared AV blended COL–CS gel was placed with a pre-weighed plate placed laid over the sandwiched gel. Until the gel stops spreading, additional known weights were added on to the plate. Both the final cumulative weight and the total time taken for the gel to spread were measured. The spreading diameter of the AV blended COL–CS gel was measured based on 2 different parameters. Firstly, the readings were taken with constant cumulative weights but at varying time intervals and secondly at constant time intervals but at varying cumulative weights. The spreaded area of the gel was then traced out using a trace paper. By using a graph paper, the spreaded area of the gel was calculated by counting the number of squares covered by the spreading diameter of the gel and the results were tabulated[24].

Moisture Uptake: A known weight of the lyophilised AV blended COL–CS was soaked separately in distilled water, taken in a measuring cylinder with known initial level of water. The test was carried out at room temperature. The samples were then removed at 5 minutes time interval and the superficial water was gently blotted on a filter paper and weighed. The amount of water uptaken by the lyophilised gel was calculated by measuring the reduced level of water (ml) in the measuring cylinder [25].

Cell Culture Studies: NIH3T3 mouse fibroblasts were cultured in DMEM containing 10% FBS, 100units/ ml Penicillin and Streptomycin and 1 mg/ ml Amphotericin at 37°C under humidified atmosphere supplemented with 5% CO_2. The culture medium was changed once in every 2 days. For *in vitro* biocompatibility evaluation, both the control and the test were conducted in 24 well plates separately and the results were recorded. The test plate consists of the evenly spread AV blended COL–CS gel and subsequently the well plate containing the culture medium was treated as control. Prior to seeding with cells, both the control and the test were sterilized under UV light for 2 hrs. Afterwards the specimens were washed thrice in sterile PBS for 30 minutes each and then washed twice with cell culture medium. NIH3T3 mouse fibroblasts (2600/µl) were then seeded to both the well plates and incubated. The senescence associated β- galactosidase (SA- β gal) activity of the senescent cells were assessed.

Determination of Morphology of Senescent Cells: Before seeding the cells to both the well plates, the morphology of senescent cells were observed underphase contrast on Leica microscope [Leica trinocular phase contrast microscope, Germany] at 100X magnification and the cells were photographed.

Detection of SA-β-galactosidase activity: Both the control and the test were seeded with fibroblast cells. Senescence in NIH3T3 mouse fibroblasts cells was induced by subculturing the cells from the culture of 95 - 100% confluency. Seeding of fibroblasts was continued after each subcultivation, until the onset of senescence. The cells at a density of 2600 cells per µl were seeded and were maintained for 3 days as a low density culture. Thereafter as described by Dimri *et al.*, the cells were stained for β-galactosidase activity and was measured by the cytochemical assay [26].

The adhered cells were washed twice in phosphate -buffered saline (PBS), then fixed with 3% formaldehyde in PBS for 3 min, followed by washing in PBS for 2 times and then stained with fresh β- galactosidase staining solution for 18h at 37 °C. The solution was composed of 20 mg of 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) in 1 ml DMF to prepare 20X stock solution, 400mM citric acid/ sodium phosphate, 500mM potassium ferrocyanide, 500mM potassium ferricyanide, 1.5M

NaCl, 20mM $MgCl_2$, pH 6.0. After staining, the fibroblasts cells at sub confluence [maximal 75% confluence] were viewed by phase contrast microscope [Leica trinocular phase contrast microscope, Germany], at 100X magnification and were photographed.

RESULTS:

Characterisation of the Prepared Gel:

i. Determination of Morphology of Senescent Cells: The pH of the prepared gel was measured using a pH paper and was found to be about 4.5 as the gel is in succinic acid medium.

ii. Spreadability of the Gel:

The spreadability values for the gel obtained at

- Varied time intervals without changing the cumulative weights and
- Varied cumulative weights but fixed time intervals were plotted and illustrated in **Table 1(a)** and **1(b)** respectively.

TABLE 1(a): SPREADABILITY OF THE PREPARED GEL AT VARIED TIME INTERVALS

S. no.	Time (min)	No. of squares covered by the gel (sq.mm)	Cumulative weight placed (gm)
1.	2	540	101.53
2.	4	590	101.53
3.	6	600	101.53

The spreadability of the gel at different time intervals were evaluated successfully and the number of squares covered by the gel at 2 min was found to be 541 mm^2, at 4 min was found to be 590 mm^2 and at 6 min was found to be 600 mm^2 when subjected to a cumulative weight of 101.53 gm respectively.

TABLE 1(b): SPREADABILITY OF THE PREPARED GEL ON VARIED CUMULATIVE WEIGHTS

S.no	Time (min)	No. of squares covered by the gel (sq.mm)	Cumulative weight placed (gm)
1.	2	517	101.53
2.	2	598	182.69
3.	2	626	256.38

The spreadability of the gel resulting on subjecting to different cumulative weights were evaluated successfully and the number of squares covered by the gel when subjected to a cumulative weight of 101.53 gm was found to be 517 mm^2 and at 182.69 gm was found to be 598 mm^2 and at 256.38 gm was found to be 626 mm^2 when observed at a time interval of 2 min respectively.

iii. Moisture Uptake: The volume of water absorbed by the lyophilised AV blended COL-CS gel at different time intervals were recorded (**Table 2**)

TABLE 2: MOISTURE UPTAKE

S.no	Time interval (min)	Initial volume (ml)	Final volume (ml)	Volume of water absorbed by the lyophillised gel (ml)
1	5	10	9.81	0.19
2	10	10	9.70	0.3
3	15	10	9.60	0.40
4	20	10	9.59	0.41
5	25	10	9.59	0.41
6	30	10	9.59	0.41

The volume of water absorbed by the gel at 5 min was found to be 0.19ml, at 10 min was found to be 0.3ml, at 15 min was found to be 0.40 ml, at 20 min was found to be 0.41 ml at 25 min was found to be 0.41 ml and at 30 min was found to 0.41 ml.

Cell Culture Studies:

Determination of Morphology of Senescent Cells: The senescent cells showed an increased volume and exhibited flattened morphology, when observed under microscope of 100X (**Fig**. **1a** and **b**) magnification.

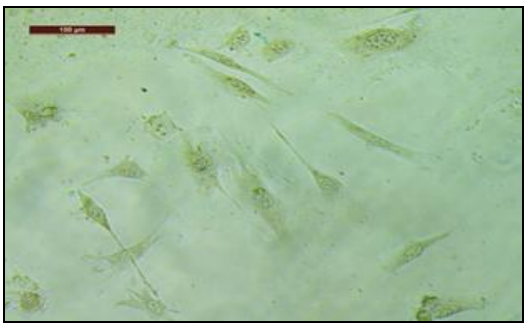


FIG. 1(a)

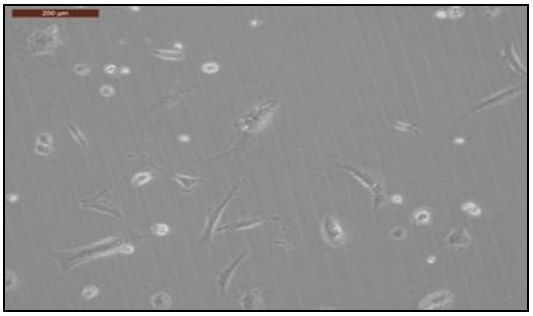


FIG. 1 (b): NIH3T3 FIBROBLASTS CELLS EXHIBITING SENESCENT STAGE AT 100X MAGNIFICATION

SA-β-galactosidase (SA- β-gal): The senescent cells produced blue stains on incubation with X-gal, taken as control (**Fig. 2**).The fibroblasts cells of the control group were compared with the senescent cells incubated with test. It was observed that the cells incubated with AV blended COL-CS gel (test) showed increased proliferation rate with the absence of blue stain, indicating that the formulation has inhibited the β- galactosidase activity (**Fig. 3 a** and **b**). Therefore the prepared gel has been confirmed to possess anti-aging property.

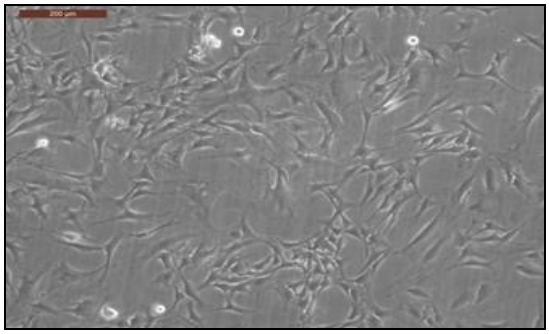
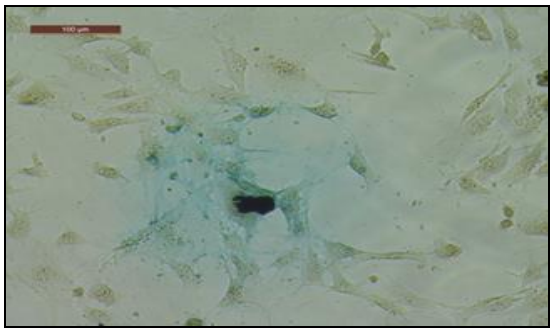
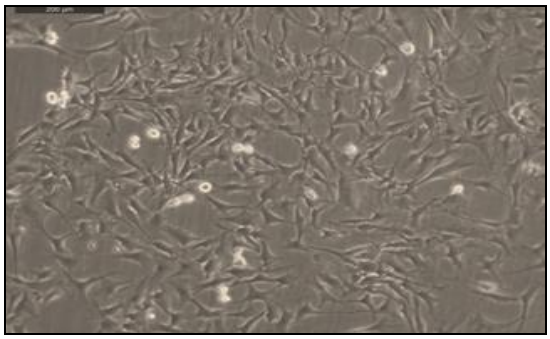


FIG. 2: SA-β- GAL POSITIVE NIH3T3 MOUSE FIBRO BLASTS CELLS (CONTROL)



(a)



(b)

FIG 3: SENESCENT CELLS IN THE TEST (AV BLENDED COL-CS GEL)

DISCUSSION: The physiological skin surface pH is just below 5. As age increases, the skin surface pH increases up to 6. An increased pH correlates with reduced barrier, integrity/cohesion. In a study it was postulated that long-term acidic skin care (pH 3.5 to pH 4.0) normalizes the increased skin pH of the elderly, improves the integrity of the epidermal barrier and therefore reduces skin problems in these individuals [27]. The pH of our prepared AV blended COL-CS gel was found to be 4.5, which indicates that our prepared gel is perfect to be used as a long term skin care product. It is evident from the spreadability property that the gel can be applied easily on the skin surface for anti-aging treatment.

The results also suggested that by applying force, the gel can be easily spread over the skin. Also, in a study, it was postulated that the inclusion of amino acids in collagen- chitosan scaffolds, resulted in increased hydrophillicity of the scaffolds [28]. In the prepared gel, the addition of aloe gel containing amino acids [29] into the COL-CS composite may be responsible for the increased hydrophillicity of the gel. Therefore our prepared AV blended COL-CS gel has been reported to possess increased hydrophillicity.

In this study, the enlarged and flat cell morphology of the aged fibroblasts cells and their SA - β- gal activity were used as key phenotypes of senescence. The cytochemical detection of senescence associated β- galactosidase was carried out using NIH3T3 mouse fibroblasts, as this assay is particularly simple and allows quick histochemical detection in tissue samples [26]. The lysosomal-β-gal expressed by most cells is optimally active at pH 4 [30]. As this SA-β-gal, detectable at pH 6, is expressed by senescent but not by presenescent, quiescent and by terminally differentiated cells [26], we have used it as a potential biomarker for aging in this study. The senescent cells on incubation with X-gal, a chromogenic substrate, resulted in blue stains, thereby confirming the senescent stage of the cells. Studies have also confirmed that preparation of scaffolds by the inclusion of AV to the COL-CS biocomposite have possessed, all requisite physical and biological properties to recruit, attach and proliferate the fibroblasts [31]. The fibroblasts cells on incubation with the prepared AV blended COL-CS gel has resulted in increased proliferation rate with the absence of blue stain, which in turn indicates, that the process of senesce has been reversed.

Aloe is said to moisturize the skin because of its water holding capacity [32]. Also studies have shown that aloe, by stimulating the fibroblasts production, makes the skin more elastic and less wrinkled [33]. Hence for rapid hydration and regeneration of skin, aloe is intended to be used in this formulation to make the skin smooth and elastic. Though many studies have shown the incompatibility of skin fibroblasts with chitosan, it has been reported that the addition of collagen facilitates the attachment and proliferation of skin fibroblasts [34-37].

CONCLUSION: The prepared AV blended COL-CS gel has been reported to possess good spreadability, increased hydrophyllicity, increased proliferation rate of the cells and in addition has reported to reverse the process of senescence. From the above studies, it can be concluded that the prepared AV blended COL-CS gel helps in regeneration and rejuvenation of the skin and therefore can be used as an anti-aging gel.

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CONFLICT OF INTEREST: The authors have declared that there is no conflict of interest.

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