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Extraction and Formulation of hydrolyzed-wool keratin solution for hair growth

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Abstract

Hair growth is controlled by hair Follicles which show patterns of cyclic activity with periods of active growth and hair production (anagen), apoptosis-driven involution (catagen) and relative resting (telogen). These transformations are regulated by different variations like endocrine, vascular, neural, age and nutritional. Keratin biomaterial has been used in regenerative medicine owing to its in-vivo and in-vitro biocompatibility. The present study aimed at extracting keratin from wool by sulfitolysis, filtration by dialysis tube and freeze drying. Qualitative analysis of the keratin extract powder ensured the presence of cysteine, Keratin powder formulated as solution dosage form and pharmaceutically evaluated, Keratin extract was topically applied on the synchronized depilated dorsal skin of telogenic 4 mice and promoted hair growth by changing Hair growth initiation and completion time and quality of hair result, in wool keratin extract solution-treated groups hair growth initiation and completion time reduced and the hair result was hard, long, thick and healthy. Besides, that the keratin extract solution was a safe agent for topical administration, our study demonstrated that keratin extract stimulating hair growth by inducing the growth phase. The topical application of keratin extract may represent a promising biomaterial for the management and applications of hair follicle disorder.

Keywords: Wool keratin, hair follicle, solution dosage form, hair growth promoter.

1. Introduction:

New sources as alternatives to the standard human-made synthetic materials are natural created materials with their amazing properties that which have not been used to their full potential. Nowadays scientists are looking for these new materials, not only for ecological reasons but for their excellent intrinsic characteristics as well [1, 2].

Natural fibers, well known as environmentally friendly materials, have the advantages of low density, low cost, and biodegradability. However, a considerable amount of these fibers has been wasted during industrial processes all over the world [3]. Protein wastes such as by-products from agricultural sources, wool textile industry, and poor-quality raw wool not fit for spinning, and hairs and feathers from butchery constitute an important renewable source of biopolymers [4, 5]. Recycling of these very accessible and cheap protein sources and developing a method for extracting keratin from such wastes have been the objectives of many studies due to their biodegradability and biocompatibility [6-9].

As a result, in recent years, there has been a great interest in developing novel applications of keratinous proteins in various fields, including cosmetics, biodegradable composites, compostable packaging, medical membrane, and agricultural films and coatings. Keratin is the major structural fibrous protein providing outer covering, such as hair, wool, feathers, nails, and horns of mammals, reptiles, and birds [10- 14].

Keratins are regarded as three-dimensional polymers interlinked by intermolecular bonding of disulfide cysteine amino acid and inter- and intermolecular bonding of polar and nonpolar amino acids [15, 16], which are responsible for their high stability and distinctive physical properties [17]. Keratin has been considered in the development of biomaterial in the biomedical and pharmaceutical areas such as wound healing, tissue engineering, and drug delivery systems for its unique capability to assist cell–cell and cell–matrix interactions, cellular infiltration, and proliferation with minor or no immunogenicity [18,19].Wool is constituted largely by a three-dimensional mesh structure of keratin – about 95% keratin proteins – which contain 7–20 % cysteine residue [20,21] and small amounts of lipid (0.1%) and minerals (0.5%).

The main methods of keratin extraction are hydrothermal, acid, alkaline, and enzymatic hydrolysis. During hydrolysis, chemicals break both types of disulfide and peptide bonds in proteins, and as a result the structure of keratin hydrolysate is changed [22]

Wool solubilization occurs by disruption of the complex keratin structure. Keratin obtained from the wool fiber can be classified into four different

molecular weight groups: a low sulfur content (LS) with a MW of 45–60 kDa and a fraction with a high sulfur content with a MW of 11–28 kDa, and fractions with a high glycine or high tyrosine content with a MWof 9–12 kDa [23].The amount of cysteine present in the hair fiber is an indicator of hair health [24].

Andreia Vasconcelos, *et al*. Extracted keratin from wool by sulphitolysis, the extract had high concentration of amino acid cysteine [10]. Wool proteins are resistant to the most of chemical and physical environmental factors. These proteins are insoluble in water and most of weak acids, alkali solutions or organic solvents and resistant to common protein-digesting enzymes such as pepsin or trypsin as well. Keratin has high contents of cysteine, glycine, proline and serine, but it is low in lysine, histidine and methionine, and tryptophan is rarely present [25].

Clara Barba et al. applied Keratin peptides and proteins to chemically treated hair showed to restore the fiber surface hydrophobicity with an increase on the value of its contact angle. As well as an improvement in the moisture content of virgin and pretreated hairs the result mean Keratin peptides and proteins demonstrated to restore the internal strong bonds of chemically damaged fibers, inducing an increase of the fiber elasticity thus, improving the mechanical properties of the fibers.[26]. Also the mechanical and thermal properties of relaxed hair were improved after the administration of keratin peptide to relaxed hair, this the result shown by M. M. Fernandes *et al*. [27] Cystine has an important role in determining the physicochemical properties of wool keratin. Wool keratin with 4–8 wt% sulfur is known as hard keratin whereas keratin found in the epidermal tissue of skin has 2% sulfur and 50–75% moisture and is considered as soft keratin. [28].

The Hair follicle (HF) is one of the most complex mini-organs of the human body with the capacity to reconstitute itself [29]. During postnatal life, HFs show patterns of cyclic activity with periods of active growth and hair production (anagen), apoptosis-driven involution (catagen) and relative resting (telogen) [30].

Hence Arecent trial of a keratin-based biocomposite hydrogel reported that the hydrogel promoted wound healing in the skin as well as the maturation of hair follicles and epithelial connective tissue [31].

Md Rashedunnabi Akanda, Hak-Yong Kim *etal*. Extracted keratin and applied on the synchronized depilated dorsal skin of telogenic C57BL/6 mice. The results show that the keratin extract stimulating hair follicle growth by inducing the growth phase; anagen in telogenic C57BL/6 mice and thus the topical application of keratin extract may represent a promising biomaterial for the

management and applications of hair follicle disorder [32].

Physiologically hair follicle growth process includes a number of growth factors, cytokines, hormones, and development-associated biomaterials that are interrelated with the regulation of hair follicle formation, development, and differentiation are governed through transcription factor-mediated signaling pathways [33] Mice hair follicles are similar to that of human beings with respect to essential features of organization and function. Several authors established follicular similarities on mice and human beings. Apart from cell type similarities, both follicles experience repetitive cyclic hair growth [10, 11, 34, and 35].

Solution as dosage forms are very interested among the other pharmaceutical dosage forms. [36]. Generally, the topical solutions employ an aqueous vehicle, whereas the topical tinctures characteristically employ an alcoholic vehicle. As required, cosolvents or adjuncts to enhance stability or the solubility of the solute are employed.

Most topical solutions and tinctures are prepared by simple dissolving. However, certain solutions are prepared by chemical reaction. Mostly they are self-preserved and just need to be packaged in containers that make them convenient to use. Keratin amino acid is soluble in water that formulated as solution dosage form, better patient compliance, easy to be administrated and rapid absorption dosage form[26,27,32].Considering the favorable biological properties of keratin that affect tissue regeneration, thus the aim of this study; to extract and Formulae hydrolyzed-wool keratin solution as a hair growth promoting agent.

2 Materials and instrument:

2.1 Materials:

Sheep wool was purchased from Slaughterhouse/Omdurman/Sudan, Petroleum ether, Sodium dodecyl sulfate, Sodium metabisulfite were purchased from SD Fine Chem Limited/India and urea were purchased from Dop organic Kimya/Turkey.

2.2 Methods:

2.2.1 Extraction of Keratin:

The purchased wool sheep was washed with tap water and then air-dried under the shadow. The dried wool was extracted using Soxhlet apparatus with petroleum ether for 12h. The lipid free-wool (10g) was immersed in 100ml of mixture solution containing 8M urea, 0.2M sodium dodecyl sulfate and 0.5M sodium metabisulphate. Then the mixture was heated to 100 °C for 30 min. After that the solution was diluted with 900 ml of distilled water and filtered by filter paper. The filtrate liquid was dialyzed against 10L distilled water using cellulose tubing (molecular-weight cutoff of 12–14kDa) for 3 days (10). The outer

solution was changed every day, the samples were collected from the outer solution every 6hours for three days and analyzed using U.V – Vis spectroscopy at the absorbance range (185-320) nm. After that the collected solution was frozen in the freeze dryer.

2.2.2. Qualitative analysis of cysteine in the keratin extract:

2.2.2.1. Solubility test:

0.1gm of the keratin extract powder was put in a test tube**,** and then 2 ml of water was added.

2.2.2.2. Ninhydrine test:

One gram of freeze-dried keratin powder was taken and 2ml of 0.2% ninhydrine solution in acetone was added, then it was boiled using water bath for 2min and allowed to cool [37]

2.2.2.3 Lead - Sulfide test:

Two gram of keratin powder was boiled after the addition of 2mlof 40% NaOH for 2min then cooled. After that few drops of the sodium plumbate were added to the solution [38].

2.2.3 Formulation of keratin extract into solution dosage form:

The solution was prepared by dissolving 0.1g of keratin extract powder in 100ml distilled water.

2.2.4 Evaluation of solution dosage form:

2.2.4.1. Visual inspection:

The final product solution was carefully examined for its appearance.

2.2.4.2 pH measurement:

The pH of the keratin solution was measured using pH meter**.**

2.2.4.3 Physical stability:

All the visual tests and pH test were measured again after three months, six months and year**.**

2.2.5. Viscosity test of keratin solution:

The viscosity test was done by Ostwald viscometer, the sample was prepared by dissolving 1g of keratin powder in100 ml of distilled water.

The density of the sample was calculated by the following equation:

Density of the sample=weight of the solution/volume of the solution

The average of time of flow of sample was measured, the instrument must first be calibrated with distilled water (with known viscosity and density) [39].

2.2.6. Drug content test of keratin solution:

Keratin was scanned in the range of 190-250nm to fix the maximum wavelength and UV spectrum was obtained. Then a calibration curve was obtained as follow, accurately weighed 0.1gm of standard keratin powder was dissolved in 100 ml of distilled water, from this stock solution 10 ml was withdrawn and transferred into 100 ml volumetric flask. Volume was made with distilled water in order to get standard stock solution 0.01g/100ml. From this standard stock solution, a series of dilution (0.005, 0.01, .02, .04, .08 g/100ml) were prepared using distilled water. The

absorbance of these solutions was measured spectrophotometrically against blank of distilled water at 216 nm for cysteine [40]. Finally, the content of Keratin, as cysteine, in the solution dosage form was calculated.

2.2.7. In vivo hair growth of keratin solution in mice:

Experimental mice and management:

Eight mice were kept in standard mouse cages with a supply of food and water. Temperature (room temperature), humidity (60%), and photoperiod cycle (12 h light and 12 h dark) were maintained over the experimental period. Mice were adapted to the laboratory conditions for 1 week before starting the experiment.

Briefly, eight mice were randomly divided into two groups: a control group and experimental group in order to study the hair follicle promoting activity of keratin solution. The hair of dorsal skin of mice was synchronously removed with razor. The treatment procedure was started the day after hair depilation. Control mice were treated with distilled water, whereas experimental mice were treated with keratin solution (100 mg/ml). Treatments were applied to the dorsal skin of mice twice per day, in the morning and evening. Visible hair growth was recorded by take pictures at 0, 7, 14, and 21 days. Qualitative hair growth was evaluated by visual observation of three parameters; (a)hair growth initiation time, that is minimum time to initiate perceptible hair growth, (b)hair growth completion time, that is, minimum time taken to cover the denuded skin region with new hair completely, and (c) quality of hair growth result.

2.2.8. Skin irritation study:

Albino rats were selected to detect the irritation of keratin extract solution by watching the change in the skin like erythematic and burning. The keratin extract solution was applied on the shaved albino rats for 72h.

3. Results and discussions:

3.1. Keratin extraction:

The method of extraction of keratin from10gm of wool sheep gave 3g of keratin powder. The yield percentage of the method of extraction= (weight of extract after lyophilization*100)/ (weight of wool sheep) $\frac{3}{10} * 100$ = 30% of keratin. The yield percentage of the keratin was 30%. Lipid and impurities were firstly removed from cleaned wool sheep to facilitated hydrolysis of protein to amino acid. We used continuous heat extraction method because it is simple, small amount of solvent used, high efficiency and complete extraction. The solvent used was petroleum ether which was cheap and nonpolar enough to remove all lipids. The filtration process time which was done is complete enough that by observation no turbidity in the solution and by analytical method the U.V.

analysis results in Fig. (1) Confirm this observation.

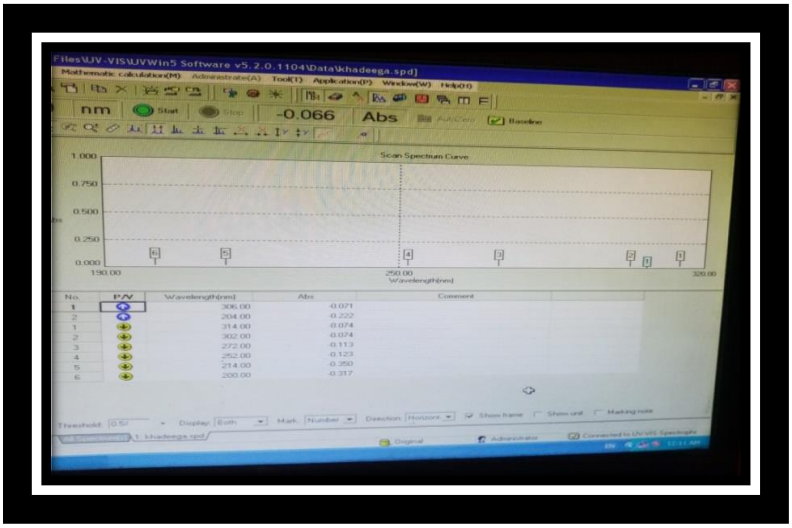
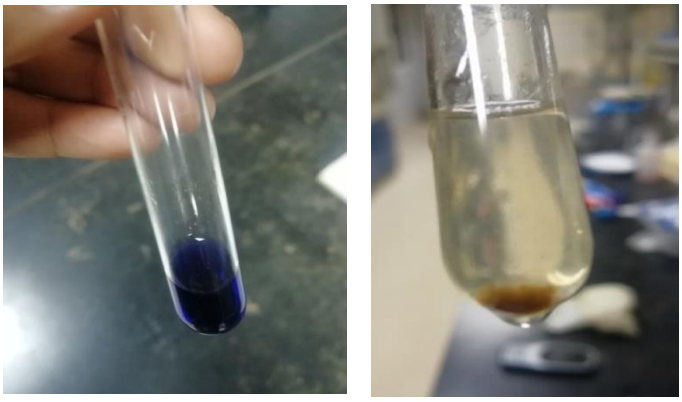


Figure (1): UV analysis result

No valuable amino acid in UV analysis at the absorbance range (185-320) nm, after 3 days of filtration method

3.2 Qualitative analysis of cysteine in the keratin extract:

The blue color was formed after ninhydrine test which indicated the presence of the amino acid cysteine in the extract. Also the brown color and precipitate were formed after lead-sulfide test that could confirm the presence of cysteine Fig.(2) These results suggest that the keratin powder extract contains amino acid mainly cysteine and cysteine.



(a) (b)

Figure (2): qualitative determination of the cysteine and cysteine presented in Keratin extract, (a) Blue color in ninhydrine test, (b) The brown color and precipitate in lead-sulfide test

3.3. Viscosity of keratin solution:

The density of keratin solution=weight of solution (weight of solution with beaker-weight of beaker)/volume of solution the density of keratin solution = (70.542-20.952)/50 = 49.708/50 = 0.992 g/cm3

Viscosity of keratin solution=

$$\eta k = \frac{\rho k t k * \eta k}{\rho w * t w}$$

where; ηw; absolute viscosity of water, tw; time of flow of water, ρw; density of water, ηk; absolute viscosity of keratin solution, tk; time of flow of keratin solution, ρk; density of keratin solution

Tk=(tk1+tk2+tk3)/3=(29+28+27)/3=28 s

Tw=(tw1+tw2+tw3)/3=(27+28+28)/3=27.6 s

Pw=1g/cm3

$\eta w = 0.891$mPa.s

Keratin solution is Newtonian fluid. Low concentration of protein or polymer solution might display a constant viscosity regardless of shear rate with large anisotropic molecules. Although used 1gm to measure viscosity, but still Newtonian, that mean 0.1%w/v is Newtonian.

3.4. Keratin extract as solution dosage form:

There are different dosage forms of Keratin extract available in the market such as shampoo, hair cream and spray. In this study keratin was formulated as solution dosage form (0.1%w/v), (there was no cross-contamination with distilled water [32] which may be rapidly absorbed, easily administrated and has good patient compliance [32, 41, 26].

3.4.1. Evaluation of keratin solution dosage form:

3.4.1.1. General appearance and pH of keratin solution:

Table (1): Physical stability of keratin solution for one year:

Time Properties	**Zero**	**3 months**	**6 months**	**12 months**
Appearance	Elegant	Elegant	Elegant	Elegant
Color	whitish	whitish	whitish	whitish
Odor	Palatable	Palatable	Palatable	Palatable
Solubility	Soluble	Soluble	Soluble	Soluble
Ph	6.8	6.8	6.8	6.8

As shown in table (1), Keratin solution showed a good organoleptic characteristic and a suitable pH for hair environment.

3.5. Drug content test of keratin solution:

As shown in table figure (1), the concentrations of keratin solution showed a linearity with the absorption in U.V-visible spectrophotometrically analysis with $R^2 = 0.999$

Table (2): Different concentrations and U.V absorption of keratin solution:

Concentration of keratin solution	**Absorbance**
0.005	0.061
0.01	0.130
0.02	0.325
0.04	0.65
0.08	1.300
Unknown concentration	0.556

From the equation; Y=16.45X-0.014

Concentration of unknown keratin solution = 0.556+0.014= 0.57/16.45 = 0.0346g /100ml.

That meaning 0.1g keratin powder contain 0.0346g of cystine. From UV-visible spectrophotometrically 1g keratin powder contain 346mg cysteine

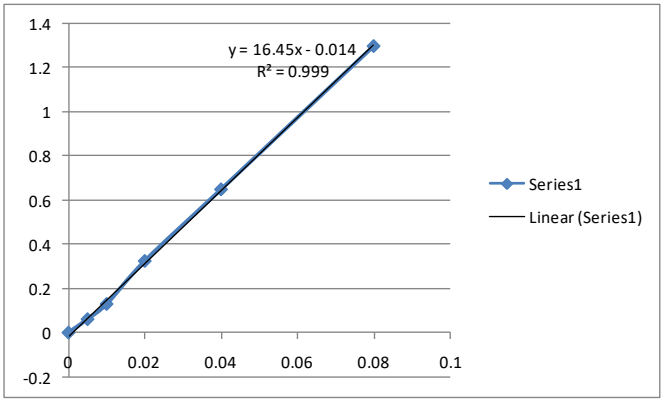


Figure (2) calibration curve of keratin solution

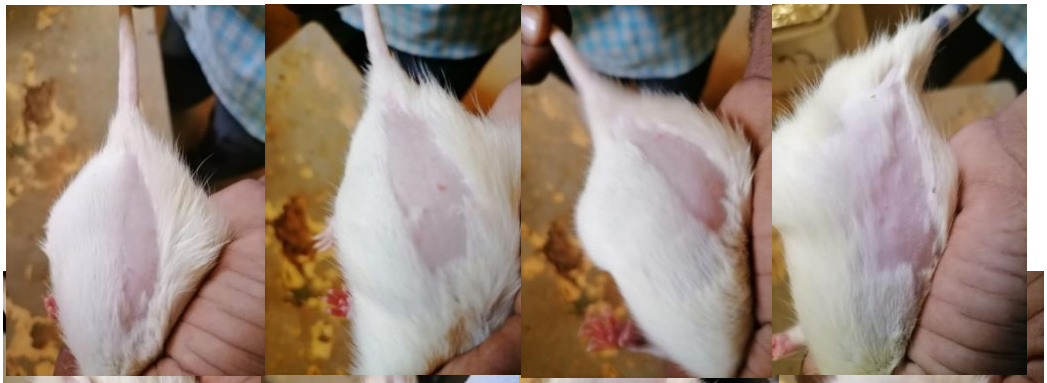
3.6. In vivo hair growth of keratin solution in mice:

CD0

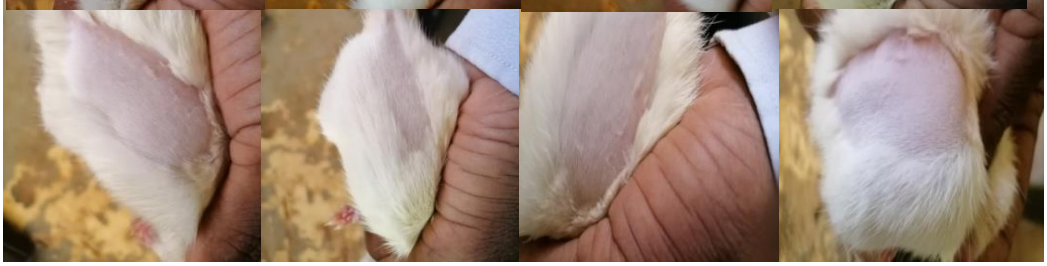
SD0

CD7

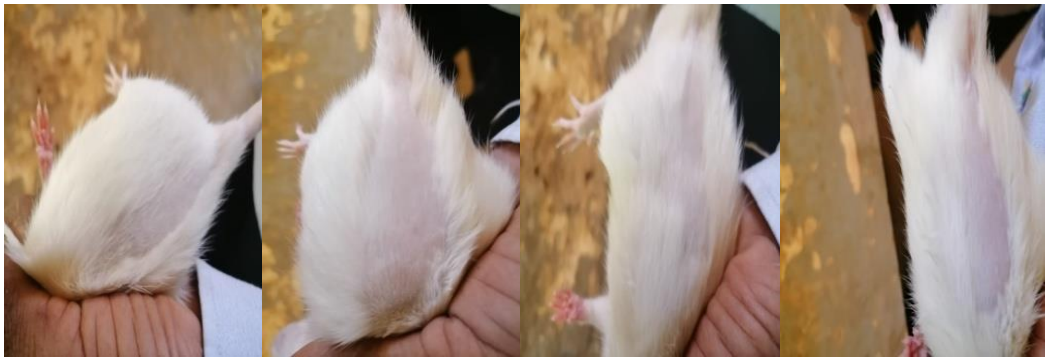
SD7



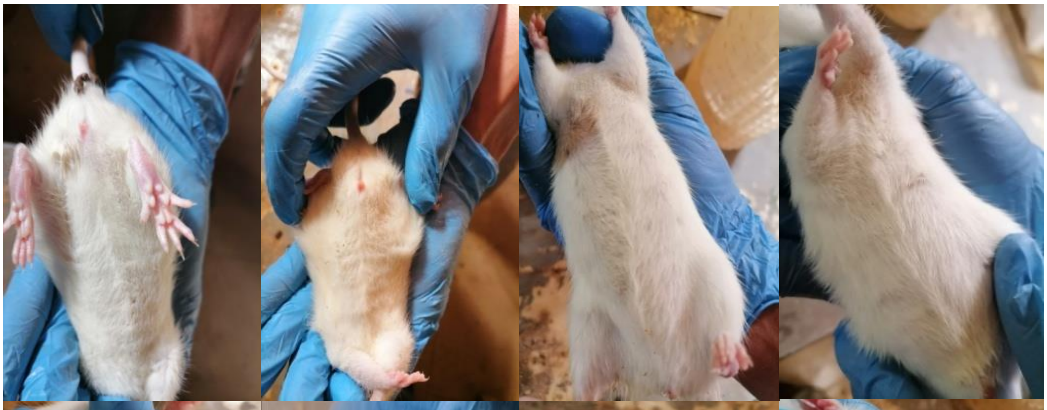
CD14



SD14



CD21



SD21

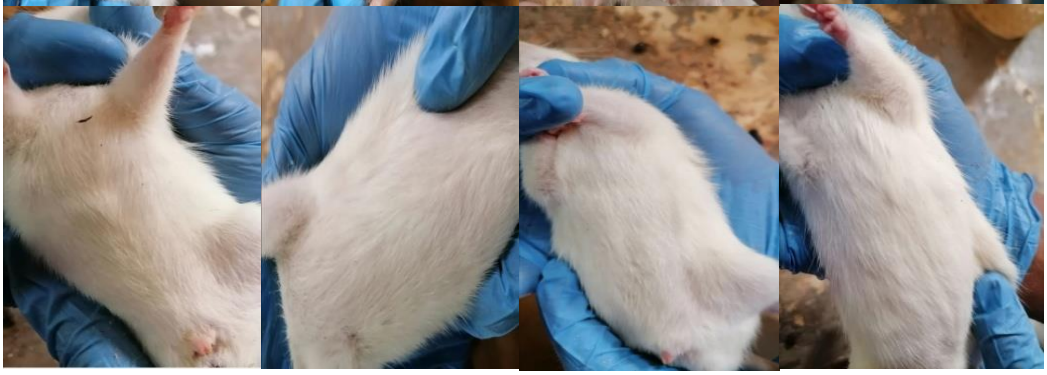


Figure 3: Hair growth promoting effects of keratin extract in mice (n=4); CD and SD, control and sample groups in day 0, 7, 14 and 21.

Hair growth initiation and completion time and quality of hair result were considerably changed upon treatment with keratin extract solution. As shown in Fig (3), in the control group, hair growth was initiated in the denuded area in the second week except one mice, but in wool keratin extract solution-treated groups, hair growth was initiated in the first week, that mean keratin extract solution caused premature switching of hair follicle from resting telogenic phase to active anagenic phase that agree with Md Rashedunnabi Akanda, Hak-Yong Kim et al who extracted keratin and applied on the synchronized depilated dorsal skin of telogenic C57BL/6 mice which support their results by histo-photometric analysis (H&E staining).

Complete hair growth was observed on the third week in the control group area but in wool keratin extract solution-treated groups were observed in the second week. Also the results showed that the intensity of hair growth was higher in treated group at all time than control group. The hair was hard, long thick and healthier in treated group whereas a weak, short, light and pale hair was observed in control group. These results clearly emphasized the effect of keratin solution in promoting hear growth with a healthy manner.

Table (3): Comparison between control groups animals and keratin extract treated group's animals:

Animals / **Time**	**Control groups animals**	**Keratin extract treated groups animals**
Hair growth initiation time	In the second week	In first week
Hair growth completion time	In the third week	In the second week
Quality of hair	Weak, short, light, pale	Hard, long, thick, healthy

3.7. Skin irritation study:

Albino rats were selected to detect the irritation of keratin extract solution. There was no erythematic or edema of skin. This indicated that the keratin extract solution was a safe agent for topical administration.

4. Conclusion:

Dissatisfaction regarding hair growth is one of the most important and frequent complications in human beings. This is not a life-threatening condition, but it plays a critical role in determining self-image, psychological well-being, and social perception and endangers certain inherent physiology of the skin. This study concluded that topical application of keratin extract from wool reduced the time required for hair growth initiation and completion and improved the quality of hair. The results indicated that keratin extract from wool represents a promising biomaterial for the management and applications of hair follicle disorders. But further studies and characterization are needed

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