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AN ASSESSMENT OF THE PIEZOELECTRIC COEFFICIENT AND THE THERAPEUTIC POTENTIAL OF IONIC LIQUID (IL) DISSOLVED HARD KERATIN FROM GOAT HORN DISCARDS

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ABSTRACT

Hard Keratin (HK) from goat horn discards was dissolved using Ionic Liquid (IL) called Tetrabutylammonium hydroxide (TBAH, 40% aqueous solution), particles of HK were extracted, evaluated for antioxidant potential and *in vitro* cell viability against A549 and HEK-293 cell lines. Different material characterization techniques viz. PFM, XRD, FESEM/EDAS, FTIR, and TGA were employed to characterize the extracted hard keratin. PFM characterization revealed a piezoelectric coefficient (d_{33}) of about 22.6 pm/V. FTIR analysis confirmed the presence of reactive Amides I, II & III at 1681, 1527, and 1249 cm^{-1} respectively. XRD spectrum demonstrated the amorphous phase of the material, and the Crystalline Index (C.I.) is 0.38. Characterization by TGA represents high thermal stability of extracted HK with a mass loss of 64.6% at 974.5°C temperature, reflective of the desired thermal stability for different applications. HK particles showed considerable antioxidant activity and inhibition of Human cell lines to an extent of 40.4% at 10 µg concentration for A549 (Lung Carcinoma cells) and 8.38% at 100 µg for HEK-293 (Human embryonic kidney cells).

Keywords: Hard Keratin (HK), Tetrabutylammonium hydroxide (TBAH), Piezoelectric Coefficient (d_{33}), PFM, XRD, TGA.

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INTRODUCTION

In India, according to the report of the National Dairy Development Board (NDDB)-2019, the livestock population of sheep and goats raised for milk and meat is 74.3 million and 148.9 million respectively (nddb. coop/information/stats/pop). Slaughterhouses' generated wastes mostly come from goat, sheep, and poultry farms.[1] These slaughterhouses daily produce tons of keratin wastes in the form of discarded animal parts viz. hoofs and horns. These are hard keratin biomaterials that are unfit for human consumption, and hence thrown out or used in landfill, can cause air, water, and land pollution with environmentally negative impacts.[2] Furthermore, the discards expand environmental threats/hazards, increase the concentration of greenhouse gases, and badly influence people's health.[3] Extraction of hard keratin from animal sources involves complex and hazardous chemicals like dithiothreitol, 2-mercaptoethanol, Urea, SDS, NaOH, and others.[4,5] Hard keratins, due to –S–S– cross-linking between cysteine residues, have low solubility and consequently high stability.[6,7] The distinguishing characteristic of keratin is the intense concentration of half-cysteine residues (roughly between 7% and 20% residues of the total amino acid), localized predominantly at the protein terminal regions[8,9], which makes its extraction difficult and warrants the use of different complex chemical solvents. A few literature reports on the dissolution of wool and feather keratin using different ionic liquids are available, the operating temperatures are high, about 130°C.[10,11] In this paper, we report, an alternative Ionic Liquid called Tetrabutylammonium hydroxide (TBAH, 40% aqueous solution) as an effective solvent for the dissolution and processing of Hard Keratin (HK) at ambient

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conditions.[12] An easy processing and extraction of HK biomaterial from direct goat horn discard and its characterization are presented. We evaluated its piezoelectric coefficient (d_{33}) using Piezoresponse Force Microscopy (PFM), and examined the anti-oxidant activity of extracted HK particles. Further, it is tested for '*in vitro'* cell viability on Lung adenocarcinoma cells (A549) and Human embryonic kidney cells (HEK-293) using MTT assay.

EXPERIMENTAL

Materials and Methods

All the chemicals used for the experiments were AR grade, purchased from FISCHER SCIENTIFIC/MOLYCHEM/MERCK/SRL, Mumbai, India. All the solutions were prepared in Reverse Osmosis (RO) water or the specified solvent mentioned in the study's protocols.

Hard Keratin Extraction from Goat Horn

The discarded goat horns containing hard keratin (HK) were collected from the local slaughter house, thoroughly cleansed with running tap water, air dried, broken into small pieces, and stored at room temperature till further use. Horn pieces of 1 g were weighed and immersed in 5 ml of TBAH (40% in water) in a screw-capped glass bottle (10 ml) and stirred in an orbital shaker at 200 rpm for a week under ambient conditions. The HK got completely dissolved in the TBAH solvent and formed a viscous solution by the end of the week (Fig-1).

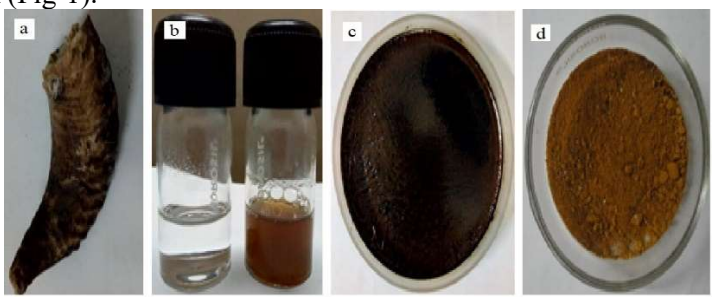

Fig.-1: Process of Dissolution of Hard Keratin (HK) from Goat Horn (a.) raw goat horn, (b.)HK dissolved TBAH Solution, (c.) Extracted HK, (d.) HK particles

From the stock solution of 1 g of hard keratin in 5 ml of TBAH, 1ml of the solution containing 0.2 g of HK was poured into a polypropylene petri dish and added 20 ml of 2 M HCl along with 5ml of Glutaraldehyde solution as a cross-linker. The petri dish containing Keratin-Glutaraldehyde solution was dried at 40°C for 12 hours. The obtained HK was pealed out and ground with mortar and pestle to make fine powder, washed with isopropyl alcohol and RO water for several times. The solvent, along with any trace chemicals left, was removed by centrifugation, the pellet dehydrated in an oven at 40°C for overnight and the acquired HK powder was stored till further use. The extracted HK was characterized by different techniques viz. FESEM-EDAX (Model: TESCAN -MIRA3 XMU), X-ray diffraction (XRD) (Rigaku Miniflex 600), FT-IR spectroscopy (FTIR), and Piezo-Force response Microscopy (PFM) (Model: MFP-3D_BIO V15, Asylum Research, CA, USA, and Igor pro V6) to understand the morphological characteristics, chemical composition and piezoelectric coefficient. The Thermo-Gravimetric Analysis (TGA) was performed using a TG Analyzer (EXSTAR/6300) with a constant heat rate of 20°C/minute, set under standard conditions. In PFM, the tip was fixed on a single particle, the voltage (known as bias voltage) was applied across the particle and its corresponding structural deformation as electro-mechanical response was recorded in terms of PFM phase data and amplitude. Electro-mechanical behavior of the HK based single particle was evaluated on nano-scale by PFM with topography, phase and amplitude as functions of applied voltage (bias voltage) in between -20 V and +20 V (Fig.-6).

Therapeutic Potential of the HK

DPPH Radical Scavenging Activity

Free radicals like hydro-peroxides and oxygen are shown to involve significantly in cytotoxicity and carcinogenesis.[13] These cancer promoters create a 'pro-oxidant condition' in the targeted tissues resulting in cancer. The anti-oxidant activities of HK particles were determined using the standard DPPH assay protocol given by Hsu *et al.*[14] with a few changes, and the results obtained were plotted in Fig.-7. First,

Hard Keratin particles of 5 different concentrations were prepared in Eppendorf tubes (200, 1000, 2000, 3000, and 4000 µg/ml) and ultra-sonicated at 100W power for 30 minutes. The reaction mixture prepared for the test contained 2500 µl RO water, 1500 µl of DPPH (0.3 mM) in methanol, and 1000 µl of desired concentration of HK particles. These test tubes were incubated for half an hour at room temperature in the dark. A blank, against each experimental tube, that contains all the chemicals without DPPH, was set up and the final absorbance was calculated by subtracting the blank absorbance from that of the experimental tubes. The DPPH free radical's reduction was inferred by using a UV-Vis spectrophotometer, which measured absorbance at 517 nm. A control reaction with 2500 µl of RO water and 1500 µl of DPPH (0.3 mM) was set up in parallel as for the other test sample. The standard used was 'Ascorbic Acid' and the % inhibition of DPPH free radical was estimated from the observed absorbance readings.

MTT Assay for Cell Viability

MTT Assay[15] was performed to evaluate the *in vitro* cell viability with two independent experiments on the two cell lines viz. A549 (Lung cancerous cells) and HEK-293 (Human Embryonic Kidney cells), using six different concentrations viz. 0 µg, 5 µg, 10 µg, 25 µg, 50 µg, and 100 µg of the extracted HK compound in triplicates. Cells were trypsinized and the number of viable cells in the cell suspension was analyzed using a trypan blue assay. The cell count was taken by hemocytometer, and a 100 µl culture medium with a cell density of 5.0 X 10 ^{3} cells was loaded per well in a 96 well titer plate and incubated at 37^{0}C undisturbed overnight. After incubation, 100 µl of fresh medium (added after aspiration of the old medium) with the chosen test compound concentration, was added to the designated wells of the titer plate. After 24 hours, the solution containing the drug/test particles was discarded, a fresh medium with a 100 µl volume of MTT solution (0.5 mg/mL) was added to the wells, and all the plates were again incubated for 3 hours at 37^{0}C. At the end of the incubation time, the reduction of the MTT salt by the mitochondria to the chromophore formazan crystals was observed in the metabolically active cells. The formazan crystal precipitates were solubilized in DMSO and the optical density was determined on a microtiter plate reader at 570 nm. The percent growth inhibition was calculated[16] from the following expression:

$$\% \text{ Inhibition} = \frac{100\ (\text{Control} - \text{Treatment})}{\text{Control}}$$

Statistical Analysis

To evaluate the effect of HK particles on the viability of the two cell lines, Student's T-test was performed, and IC50 was calculated using the "Quest Graph™ IC50 Calculator" of *AAT Bioquest, Inc*.[17] The statistically significant variations were identified on the basis of the 95% confidentiality limits allowed for biological data ($p < 0.05$).

RESULTS AND DISCUSSION

FTIR Spectrum depicting the characteristic functional groups of the HK protein extracts from goat horn is presented as Fig.-2. The strong vibration of amide I band originating from the C=O stretch was observed at 1681 cm^{-1}, while the peak for amide II vibration at 1527 cm^{-1} was attributed to N-H bend and C-N stretch vibrations. Amide III, indicated by the band stretching at 1249 cm^{-1}, is the effect from C-O, C-N stretching vibrations, and also O=C-N, N-H bending vibrations.[18,19,20]

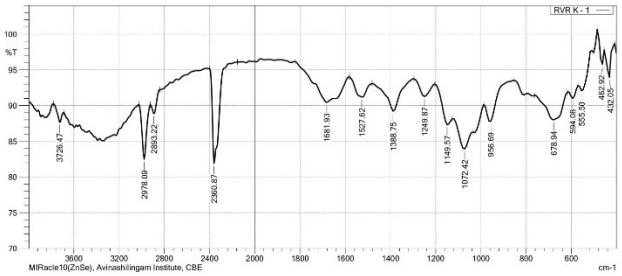

Fig.-2**:** FTIR Spectrum of the Extracted Hard Keratin (HK)

X-ray Diffractometric (XRD) spectrum obtained for the sample of HK is presented in Fig.-3. The α-helix structure is shown in peak at 2θ=17.8° of the test sample of HK.[21] Hence, it is inferred that some crystallinity

is retained after the regeneration process. The low crystallinity index (C.I.) of 0.38 indicates the predominantly amorphous state of the HK extracted in this study.[22] The crystallinity index was calculated by the equation, C.I. = Sc / St, where 'Sc' represents the area of the crystalline domain and 'St', the area of the total domain, respectively.

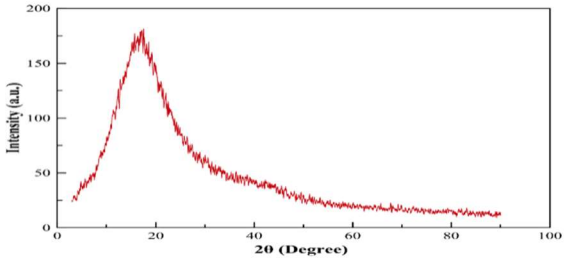

Fig.-3: XRD Spectrum of the Extracted Hard Keratin (HK)

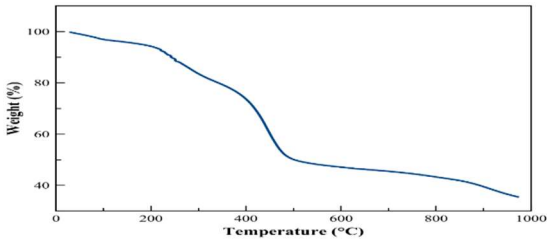

Fig.-4: TGA Analysis Depicting the Thermal Degradation of Hard Keratin (HK)

The Thermo gravimetric behaviors of the HK particle were analyzed from the TGA curves within the temperature range of 28°C to 1000°C. The initial weight of the HK which was around 3.82 mg, was subjected to TGA in a controlled way to measure the change in weight w.r.t. temperature, and the final weight was found to be 1.35 mg at 974.5°C. TG curve depicted three steps of decomposition: In the first step, at about 220°C, there was a 7% loss in mass due to the evaporation of moisture and volatile chemical compounds absorbed by the HK during its processing (Fig.-4). The sample started degrading exponentially from 220°C to 550°C (2nd step), effecting a mass loss of 50% which could be attributed to the greater percentage related to degradation of its structures and denaturing of the keratin[23,24] and in the third stage, a linear degradation till 974.5°C causing a mass loss of 2.47 mg (64.7%) was noted. The leftover mass is the hard keratin part that is stable at a very high temperature of more than 974.5°C, and hence would be an ideal substance for applications requiring high thermal stability. FESEM/EDS results are presented in Fig.-5a and 5b, and Table-1. The data demonstrates the particles as of spherical morphology with mesoporous nature and the mean particle size as about 935 nm. The elemental analysis of the extracted HK indicated 'Carbon' to the extent of 67.84% followed by 'Oxygen' at about 27.42%, 'Chlorine' at 3.73%, 'Sulphur' at about 0.56%, and 'Silica' at 0.45% (Table-1).

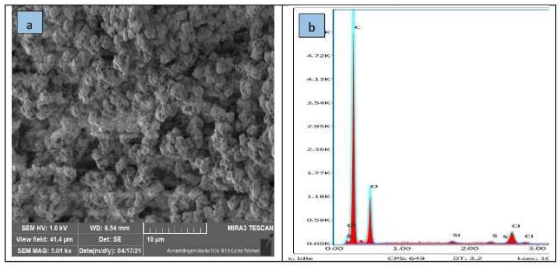

Fig.-5**:** SEM of Extracted Hard Keratin (HK) Sample (a) and EDAS of HK Sample (b)

A Piezoresponse Force Microscopy (PFM) analysis was performed to determine the local piezoelectric response of the Hard Keratin (HK) particles at the nano-scale, and the data obtained was used to determine the piezoelectric co-efficient using the formula $S = d_{33}E$. The topology of amplitude and phase are as shown in Fig.-6a and 6b, and the PFM with phase and amplitude in response to bias voltage is shown in Fig.-6c and Fig.-6d. The phase and amplitude values derived from the PFM data indicated the material polarization

and local strain responses. The phase images (Fig.-6c) displayed dark and bright regions of the polarization directions.

Table-1: Elemental Composition of Hard Keratin (HK) – EDAX Analysis

Element	Weight %	Atomic %
C K	67.84	75.30
O K	27.42	22.85
Cl K	3.73	1.40
S K	0.56	0.23
Si K	0.45	0.21

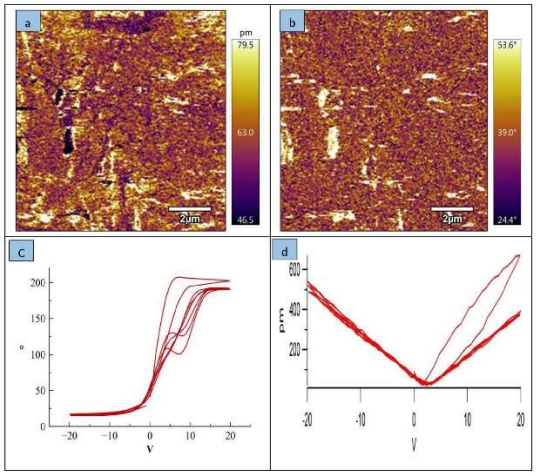

Fig.-6: Piezoresponse Force Microscopy (PFM) Characterization. (a) Amplitude image, (b) Phase image, (c) Phase Loop, and (d) Amplitude Loop

The applied electric field generated the phase hysteresis loop from which the ferroelectric properties of the material could be deduced. It was inferred from Fig.-6c that the phase toggles between 190° and 200° of the hysteresis loop at 20 V, and beyond this voltage, the phase loop gets saturated. Baji *et al.* (2011) explained that the stress induced by the tip is caused by 180° switching of the hysteresis loop, resulting in the domain rearrangement that affects the piezoelectric response.[25] The amplitude signal from the HK particle exhibits the butterfly loop which is characteristic of the piezoelectric property. The variation of strain (S) is equal to the changes in the amplitude response under the influence of electrical field (E), and the mean piezoelectric coefficient (d_{33}) of the HK particles, calculated using the formula $S = d_{33}E$[26-29], is 22.6 pm/V.

Antioxidant Activity

Natural vitamins C and E and synthetic anti-oxidants such as butylated hydroxyanisole, butylated hydroxytoluene, ethoxyquin etc. inhibit carcinogens by modifying their activation, mutagenicity, and effect detoxification.[30] DPPH scavenging in percent was determined by the expression:[31]

$$\text{DPPH Scavenging (\%)} = [(Ac - At)/Ac] \times 100$$

Where 'Ac' and 'At' refer to the absorbance values of the 'Control' and the 'Test' respectively. The IC50 value was calculated with the % scavenging activity against the HK particles' concentration from the plotted graph and was found to be 11.40 mg, which is significantly low ($P<0.05$) as compared to the chemical standard of 10.98 µg; yet useful as a biological material and serves as a good substitute for the synthetic chemical.

MTT Assay for A549 and HEK-293 Cell Lines

Electrical stimulation of varying intensities was demonstrated to effectively inhibit cancer cell proliferation, and is considered as a preferred alternative to other traditional approaches. Electrical cues of mild intensities affect the proliferation of cancer cells without the aid of chemical medication and also lower the multidrug resistance in the cells. From the results of our experiments, we could infer that the ultrasonically activated

'Hard Keratin' particles released piezoelectric stimuli that may have arrested the cancer cellular cycle at the G0/G1 interphase by interfering with Ca^{2+} ion balance and up-regulating the expression of Kir3.2 gene governing the inward rectifier K^+ channels. Further, cytoskeletal organization mediating cell multiplication could also have been affected.[32,33]

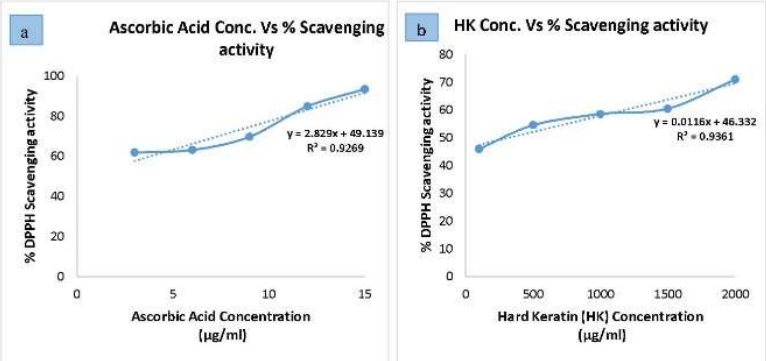

Fig.-7: DPPH Assay: (a) Ascorbic Acid – Standard (b) Hard Keratin (HK)

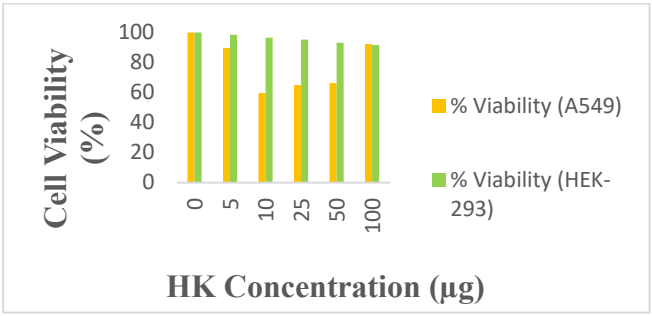

Fig.-8: Cell Viability of A549 Lung Carcinoma cells and HEK-293 Kidney cells in percent upon treatment with Hard Keratin (HK)

MTT assay is an estimation of the cellular metabolism from the number of mitochondria of cells expressed.[34] The results of this test for A549 lung cancer cells after 24 hours of incubation time showed the highest inhibition of the metabolic activity to the extent of 40.4% at the concentration of 10 µg. With other concentrations of 25 µg, 50 µg, and 100 µg, the obtained inhibitions were 35.06%, 33.8%, and 7.7% respectively, all with reference to untreated cells (Fig.-8). In contrast to the general expectation, a reduction in the inhibition of cell metabolic activity was observed with the increase in HK concentration and is worth to be further investigated. One possible explanation would be that at higher levels, HK particles could have aggregated and become ineffective.[35] At low concentrations, HK particles could presumably penetrate into the cells, disrupt the membrane potential & structure of mitochondria, and thus inhibit the viability of cancerous cells and their cell division. The IC50 values calculated for HEK-293 (normal) and A549 (Lung cancer) cell lines were 32.97 µg and 104.24 µg respectively, with 59.6% viability for HEK 293 cell lines as against 96% for A549 cell lines (Normal) at 10 µg concentration of Hard Keratin (HK) particles. The obtained difference between the control and the experimental cell lines was evaluated by Student's T-test and found to be considerable at $p < 0.05$. HK particles did not show any significant inhibition of the normal HEK-293 human embryonic kidney cells, and hence have the advantage of therapeutic application.

CONCLUSION

Hard Keratin (HK) was extracted by keeping goat horn pieces in the Ionic Liquid (IL) of Tetrabutylammonium hydroxide (TBAH, 40% aqueous solution) at ambient conditions. Hard Keratin (HK) particles were characterized using PFM to estimate the mean piezoelectric coefficient, and is found to be 22.6 pm/V, the highest of all those reported to date. Different techniques for analysis such as FESEM/EDAS, XRD, FTIR, and TGA were utilized to determine various physical and chemical properties. Morphologically, the HK particles are spherical with mesoporous nature and the mean particle size is about

935 nm. XRD characterization reveals amorphous phase with crystallinity index (C.I.) lower than 0.38. FTIR spectral analysis confirmed the presence of Amide I, II and III at 1681, 1527, and 1249 cm^{-1} respectively. TG analysis represented a mass loss of 64.6% only up to 974.5°C temperature, indicating high temperature stability of the residual fraction. The extracted HK particles are tested for their antioxidant potential, *in vitro* cell viability against HEK-293 and A549 human cell lines. The cytotoxicity tested on A549 lung cancer human cell lines indicated significant inhibition to 40.4% at 10 µg concentration of HK, while the inhibition was minimal, about 8.38% only for the normal (HEK-293) cell lines.

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