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RESEARCH ARTICLE

Formulation of Nano-sized dispersion from Calendula Officinalis: A Natural gateway to preparation of a Hair Dye

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ABSTRACT:

Hair dyes have been used to cover gray hair or to give an appealing look. In the present study, the extract of *Calendula Officinalis* was used in preparing a hair dye. This represents a natural gateway in development of hair dye to avoid damage caused by synthetic dyes. The yellow coloring principle of the extract, Calendulin, was converted into nano-sized formula ensuring hair fiber penetration. Nine formulae were prepared by solvent evaporation technique, based on a 3^2 factorial design using Design Expert® program, independent variables being: speed and time of homogenization; three levels: low, medium and high. Polyvinyl alcohol (0.5% w/v) was used as stabilizing agent. Formulae were evaluated by determination of Zeta size, potential and polydispersity index. Response surface modeling enabled choosing the optimal formula (least particle size, polydispersity index and highest zeta potential), which was evaluated by transmission electron microscopy and dyeing effect as a gel containing 0.5% Carboxymethyl cellulose. F7, produced by operating the homogenizer at 26000rpm for 10 minutes proved to be optimal which succeeded to dye white hair and lighten up the black hair as evidenced by optical microscope images. Hence it was recommended to use F7 in covering the white hair.

KEYWORDS: *Calendula officinalis*, nano-formula, Response surface modeling, natural hair dye, grey hair.

INTRODUCTION:

Today most of the human beings pay special attention to their beauty in which hair play a major important role. Nearly 70% of people exceeding 50 years of age have got hair associated problems including balding and graying of hair. Occasionally, these aging symptoms can take place earlier[1].

Many reasons can account for hair graying as genetics, stress, deficiency of nutrition and disease. Premature hair graying is hereditary and it has been reported that at the age of fifty, half of the population of the world will have half of their hairs going grey[2]. Consequently, there is a high market demand for hair care[1].

At the age of 40 years, graying starts on head skin, starting initially from the temples, followed by beard, moustache and finally up to the chest.

Generally, premature depigmentation in adults is mainly due to a variety of other factors, as illness, some specific drugs, shock,etc[3].

Throughout human history, people wished to change their hair appearance as it was a way for social status differentiation. Hair dyes have been used long ago since the age of Ancient Egyptian when Ramses II used henna to strengthen red hair color. With Ancient Greeks, a rinse of potassium solution was used to bleach hair, followed by its rubbing with an ointment consisting of yellow flower petals and pollen[4]. Since the Second World War, hair dyes have progressed in the field of discovery and application. There is a high frequency of using hair color cosmetics specially by females[5]. However, the hair fiber structure may exhibit severe damage due to the chemical nature and mechanism of action of such hair[6].

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Color formation happens due to complex reactions involving the effect of an oxidizing agent present[7] and alkalinzer. The reaction takes place in an alkaline medium where hair cuticle opens, hence, allowing dye molecules penetration into the cortex. This results in formation of a colored complex possessing an elevated molar mass. Consequently, the molecules that had formed in the hair fail to exit hair cuticle[6]. Hair cuticle opening results in severe hair damage. In addition, many chemical hair dyes have been reported to possess several toxicities, predisposing to cancers and teratogenesis[8].

Natural dyes are colors originating from plant, animal or insect substance without chemical processing[9]. Long ago, natural organic substances were mixed with metals such as copper and iron, to obtain more permanent or richer color degrees. Plants as *Lawsonia inermis, Eclipta alba., Aloe barbadensis, Hibiscus rosasinensis, etc*,[10] were utilized as principle ingredients for hair coloring in hair care products. Natural dyes are also considered as mordants due to the presence of tannins. Tannins generate an attraction between hair and dyes which improves fastness and dye color[1].

Natural dyes are ecofriendly, with low toxicity and are less allergenic. They consist of catechins, flavonoids, ascorbic acid, etc, with natural anti-oxidant property[11]. Plant extracts are used to manipulate several hair disorders such as dandruff, premature graying and head lice[12]. Such plant extracts serving as natural hair dyes solve the problem of the destruction of the scalp and hair cuticle¸ hence, are safe for use. Natural colors include many pigments such as carotene (Golden), lutein (yellow) and anthocyanins (red)[11].

Calendula officinalis L. (marigold) is an Asteraceae/Compositae family member, originating fromthe Mediterranean and Central Europe regions. The extract obtained from its flower is used to prepare cosmetics[13]. Calendula extract consists of terpenes and terpenoids asfaradiol, bisabolol, arnidiol, chamazulene, and esters), carotenoids (having structures of lycopene and rubixanthin), flavonoids (as quercetin, kaempferol aglycones and isorhamnetin) and polyunsaturated fatty acids ascalendic acid[14].

The main coloring principle in calendula is calendulin, which is a carotenoid present at a percentage of (0.8–1.0%) in dried petals. In addition, there are other coloring compounds like lycopene, rubixanthin, and violaxanthin[15]. The extract qualification of flavonoids and carotenoids is usually done using thin layer chromatography[16].

The use of *Calendula officinalis L.* for medical applications dates to the Twelfth century. The plant is active as angiogenic, vascular regenerator, analgesic, antimicrobial agent, antioxidant[17,18], found in flowers especially in pollen proteins[19]. In cosmetic products, calendula is used in formulating products for sensitive skin in addition to products with soothing effect which are recognized as safe for use[13].

Kishimoto et al reported the carotenoid composition in petals of *C officinalis L* as a function of orange or yellow petal color[20].

Today, nanotechnology has invaded the cosmetic field. Several nanocarriers were used in hair cosmetic products as liposomes, polymeric carriers and metallic nanoparticles. They were utilized for repair of hairs, hair masks, hair protection against heat and light and hair coloration[21].

The present study aimed at formulating the color imparting ingredient of calendula extract to a novel nanoparticle based natural hair dye. The concept of utilizing a natural source to develop a product usually targets the human safety and wellbeing as part of sustainability goals. This would allow an improvement in the uptake of the color imparting ingredient by the hair through its transformation into a nano-formulation having strong penetration capabilities[22]. This would avoid the use of an alkaline medium to open the hair cuticle to allow dye penetration into the hair fiber. Consequently, hair damage is avoided. Also, the study was targeted to cover the white hairs with a yellow color.

MATERIALS AND METHODS:

Materials:

Plant Sources: *Calendula officinalis L.* collected from the garden (Sekem botanical garden, Belbeis) and authenticated by the botanist. Polyvinyl alcohol (PVA), (Aqualon, USA); Petroleum ether, 95% Ethanol, (analytical reagent grade, El-Nasr Company for Pharmaceuticals, Egypt): Carboxymethylcellulose Sodium 2000Cps, (CP Kelco, Georgia, USA).

Equipment:Rotary evaporator (Biobase 1 KA RV10 basic Rotavap, China); UV/Vis spectrophotometer (A&E Lab, UK); Electric balance (Sartorius AG balance, Germany); Ultrasonic cleaner (KS-300EL, China); high-speed homogenization (HeidolfDiax 900, Germany); magnetic stirrer (Wisestir MSH-20A, Daihan Scientific Co., Korea); Malvern Nano ZS® Zetasizer (Malvern Instruments Ltd. Malvern, UK); Transmission electron microscope (JEOL, model JEM-2100, Japan)

Preparation of Plant Extracts:
About 500g of fresh petals were crushed, dried and subjected to cold maceration with 96% ethanol for 72 hours. The extract was decanted and concentrated using rotary evaporator at a temperature not exceeding 60°C. The extract was stored in desiccator till the following processing[23].

Determination of Color Intensity:
As a preformulation test, the choice of the solution concentration used to prepare nanoparticles was based on color intensity calculations. Solutions of calendula extract in petroleum ether were prepared using 100mg, 300mg and 500mg extract. The intensity of color was calculated based on Glories' methodology[24]. Solution absorbance was determined at wavelengths of 420, 520 and 620nm using UV/Vis spectrophotometer. Color intensity was calculated based on the equation 1:

Color intensity = Abs (420nm) + Abs (520nm) + Abs (620nm)[25].eq.(1)
The solution with the highest color intensity was chosen for preparation of nanoparticles.

Design of Experiment:
The design of the experiment for preparation of Calendula nanoparticle formula (CNF) followed a 3^2 factorial design. The effect of two factors speed and time of homogenization (high-speed homogenizer operated at 25ºC) was studied. Each factor was set at three levels: low, medium and high coded as -1, 0 and 1. The choice of the factors was based on preliminary studies carried out before implementing the experimental design. The levels for the speed of the homogenizer were 12000, 20000 and 26000rpm. The time intervals of homogenizations were 3, 6 and 10 minutes. These factors were thought to be influential on the formulation and optimization of nanoparticles. The factors studied with their respective levels and constraints are shown in table 1**.** Nine formulae shown in table 2 were randomly performed. The responses measured were zeta size (ZS), zeta potential (ZP) and polydispersity index (PDI) for each formula.Design-Expert software (V. 11.0.0, Stat-Ease Inc., Minneapolis, USA) was used for the application and evaluation of the factorial designs. Numerical optimization[26,27] was performed according to the constraints listed in table 1. This method is based on the utilization of desirability functions.

Table 1. 3^2 Full Factorial Design factors, levels and constraints for formulation of CNF

Factors (Independent Variables)	Levels		
	-1	0	1
A: Speed (rpm)	12000	20000	26000
B: Time (min)	3	6	10
Responses (Dependent Variables)	Constraints		
R1: ZS (nm)	Minimize		
R2: PDI	Minimize		
R3: ZP (mV)	Maximize		

Table 2. Suggested Formulae for preparation of CNF

Formula	Factorial Design Factors	
	Speed	Time
F1	-1	0
F2	-1	-1
F3	0	-1
F4	-1	1
F5	0	1
F6	0	0
F7	1	1
F8	1	0
F9	1	-1

Statistical Analysis:
Means of obtained responses were compared by ANOVA factorial at $\alpha = 0.05$ significant level. All experiments and characterizations were performed in triplicates.

Preparation of CNF:
Nanoparticles have been prepared using solvent evaporation method[28,29]. An amount of 500mg of the calendula extract was weighed and dissolved in 20ml of petroleum ether by sonication at 25ºC. The solution was poured onto 80ml of 0.5% PVA solution (the ratio of the organic to aqueous phase of 1:4 has been chosen based on previous trials). The mixture was exposed to high-speed homogenization according to the stated design of experiment. At the end of the homogenization period, the formed nanoparticle dispersion was transferred to a magnetic stirrer operated at 500rpm for 2hours at 25ºC to remove the residual organic solvent.The resulting dispersion was exposed to further investigation.

Evaluation of CNF:
a. Particle Size,and polydispersity index (PDI):
The average particle size (PS) and polydispersity index (PDI) of the nanoparticle dispersion were measured through dynamic light scattering (DLS) using Malvern Nano ZS® Zetasizer. 0.1ml of each sample was diluted with double distilled water to 5ml to get a weakly opalescent solution, which was sonicated prior to measurements The dispersions were examined using glass cuvettes at 25°C. Each sample was determined in triplicate and the mean was calculated. The PDI values gave an indication to the preparation homogeneity[30,31].

b. Determination of Zeta potential (ZP):
The zeta potential of CNF was measured using Zetasizer Nano. The process was based on laser multiple angle particle electrophoresis analysis. The same dilution of dispersion as stated in the size and PDI determination was carried out. All measurements were done at room temperature (25ºC) and repeated three times[3032].

c. Response Surface Modeling (RSM):
The optimal CNF formula was chosen based on a Response Surface Model (RSM). The model decided

the optimal formula from the prepared 9 formulae, based on the responses (dependent variable): ZS, ZP and PDI to each of the studied factors (independent variables): Speed and time of homogenization, each at the stated levels (low, medium and high)[33].

The goals of each factor were set to minimize the particle size (PS) and PDI[34] and to maximize the Zeta potential (ZP)[35] which supported nanoparticle stability.A desirability of not less than 0.7 was considered optimal.

d. Transmission electron microscope (TEM):

The morphology of CNFof the optimal formula chosen based on the RSM was identified using transmission electron microscopy equipped with super twin lens. A droplet of CNF was placed on a carbon-coated copper grid. Excess fluid was got rid of using filter paper.Lastly, the sample was dried for 15 minutes at room temperature and the morphology of particles was investigated[36,37].

Formulation of CNFGel:

The optimized CNFwas incorporated in 0.5% CMC Na (2000cp) to form a suitable hair dye gel.

Dyeing Process:

The optimized formula in gel form was tested for dyeing both natural black and white hair got from a human volunteer (Caucasian race). As suggested, the formula was intended to provide covering of white hair color without exposing the hairs to the damaging effect of bleach and alkaline media. Afterwards, the different hair categories were inserted into the gel formulation for one hour. These hair categories were: white hairs and black hairs. That was followed by removing the remaining gel by washing with water and mild shampoo and rinsing.

Color uptake by the hairs was investigated through hair examination under the optical microscope. Also, microphotographs of the examined hairs were taken (Optical Microscope with image analyzer). The dyed hairs (both white and black) were compared to the untreated white and black hairs, as well as dyed hairs using a chemical permanent dye[38].

RESULTS:

The initial concentration for preparing the CNF was chosen based on color intensity calculation.The following table 3 shows the results of the color intensity testing based on equation 1.

Table 3. Color intensities of different concentrations of Calendula extract solution in petroleum ether

Concentration mg/ 20 ml	Color intensity
100	0.466
300	1.443
500	1.669

Evaluation of CNF:

The suggested formulae based on the design of experiment were 9, where the preparation process followed a random order of preparation. The resulting dispersions were evaluated based on measuring ZS, PDI and ZP. The results were summarized in table 4.

Table 4. Recorded responses for CNF formulae

Formula	Speed	Time	ZS (Nm)	PDI	ZP (mV)
1	0	0	331.1 (±2.5)	0.385	-7.12 (±1.19)
2	0	1	329 (±1.1)	0.36	-7.6 (±0.1)
3	1	0	320 (±4.6)	0.38	-10.7 (±0.64)
4	0	-1	329 (±5.6)	0.36	-7.3 (±0.495)
5	1	-1	331.1 (±4.2)	0.385	-7.59 (±0.57)
6	1	1	318.65 (±2.26)	0.386	-16.45 (±0.38)
7	-1	1	339 (±2.102)	0.4035	-10.7 (±2.18)
8	-1	-1	350 (±3.25)	0.4	-7.59 (±0.94)
9	-1	0	339 (±1.14)	0.4035	-11(±0.71)

The RSM was used to compare the effect of changing the factors on the obtained responses (ZP, ZS and PDI). The responses of the factorial designs were analyzed individually using two-factor interaction models to describe the relation between the response under question and the studied factors. The interactions and the relations between the factors and the responses were represented in (figure 1), so as to allow selection of the optimal formula to resume the study.

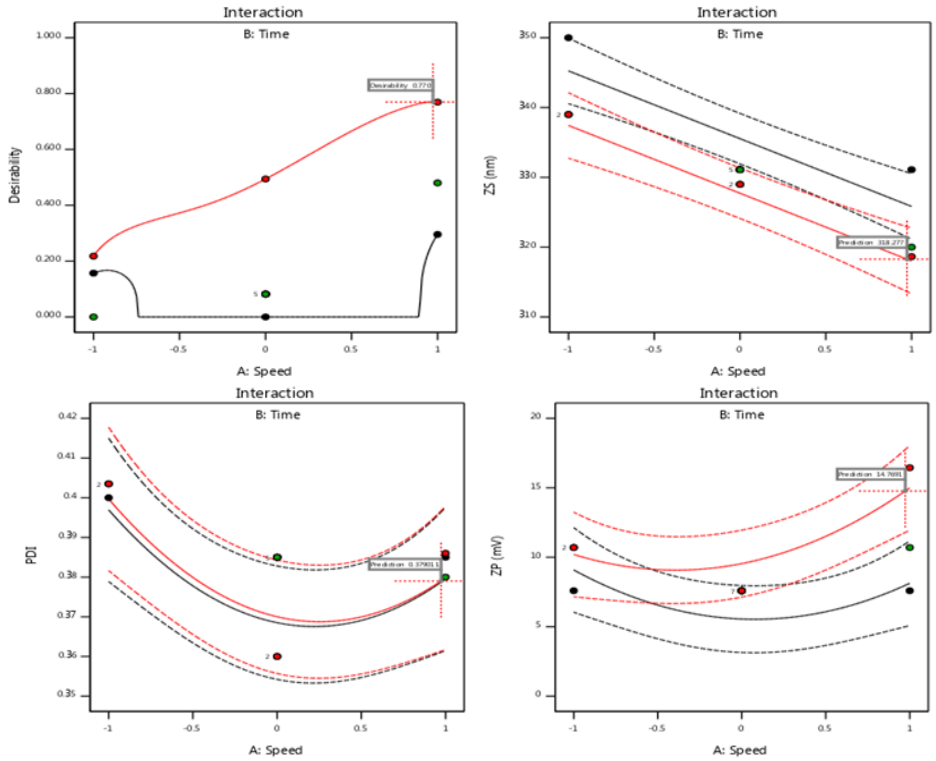

Figure1. Interaction between the factors and the responses

Also, (figure 2) represents the 3D surface used for screening of the influences of the studied factors (speed and time) over the responses (ZS, PDI and ZP). The RSM suggested that F7 was the optimal formula with desirability 0.77.

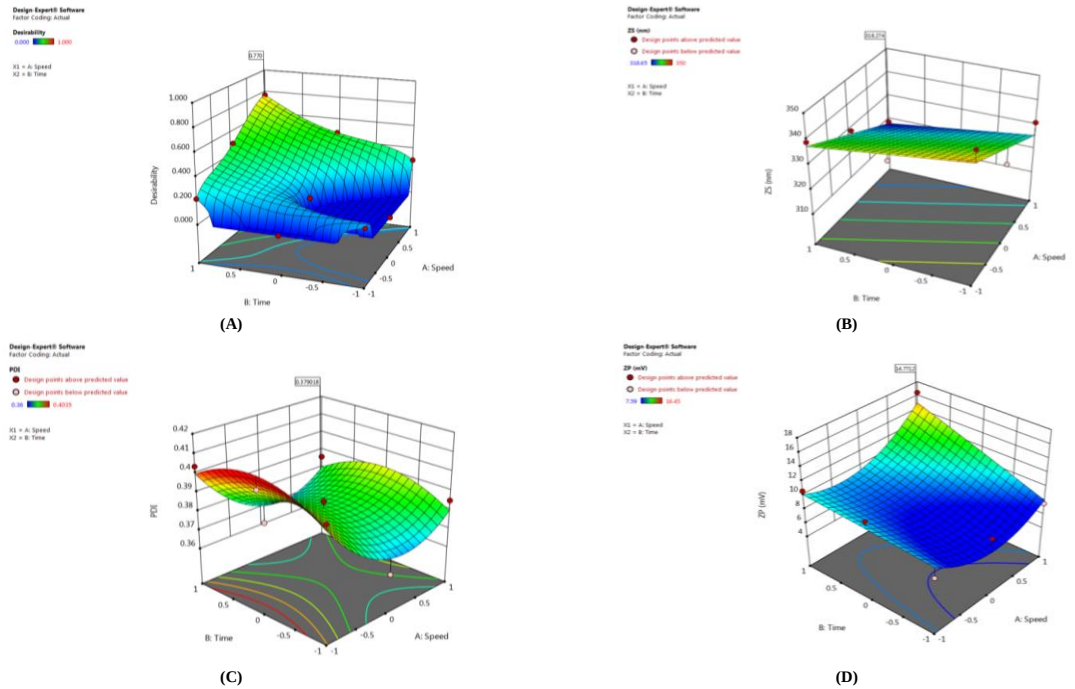

Figure 2. 3D surface of Desirability (A) and influences of speed and time on ZS (B), PDI (C) and ZP (D) Hence, F7, obtained by operating the homogenizer at the highest speed (26000 rpm) for 10 minutes was chosen as the optimal formula to be utilized for further investigation.

Transmission electron microscopy (TEM):
The surface morphology of the optimal formula F7 was demonstrated using TEM and was shown in (figure 3). Microphotographs of F7 revealed the spherical nature of the surface of the nanoparticles.

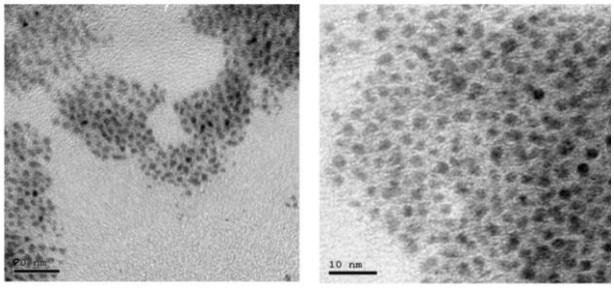

Figure 3. TEM of the optimized CNF formula (F7)

Dyeing process:
The dyeing process proved the efficiency of the optimal formula F7 in covering the white color of the hair, and producing a color similar to that obtained from synthetic dyes. A microphotograph of the white hair which was dyed with F7 is shown in (figure 4 A) compared to the color of the synthetic dye on a hair in (figure 4 B). Also, the dyed hair with F7 (figure 4 A) could be compared to a white hair (figure 4 C). F7 did not cover the black color of the hair, however, the black dyed hair with F7 seemed to acquire a yellow highlight as shown in (figure 4 D) compared to a black untreated hair shown in (figure 4E).

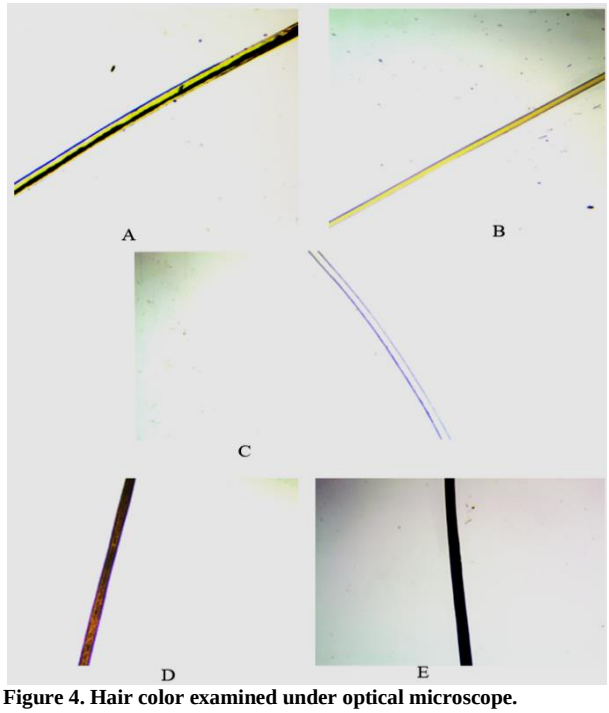

Figure 4. Hair color examined under optical microscope.
A: human white hair dyed with F7; B: human hair dyed with synthetic dye; C: human white hair; D: human black hair dyed with F7; E: human untreated black hair

DISCUSSION:

It was evident from the calculation results, based on equation 1, that the highest intensity belonged to the solution of the highest concentration (500mg extract/20 ml petroleum ether). That solution was used to prepare the CNF for dye formulation.

Statistical analysis showed that the ZS differed significantly between formulae based on speed ($p < 0.0001$) and time ($p = 0.0155$) of homogenization. High speed was responsible for mechanical and hydraulic shear, leading to size reduction [39]. Similarly, homogenization speed is an image for the power of operation of the homogenizer and the force imparted to the dispersion. Increasing the homogenizer speed reflected an increase in the power utilized in size reduction[40]. The high speed resulted in rapid dispersion of the organic phase causing reduction of the size of the organic phase globules. Consequently, dispersed phase particles with reduced sizes were obtained upon evaporation of the organic phase[41].

Formulae differed significantly in their PDI based on chosen speed ($p=0.0325$), but did not show significant difference with respect to time of homogenization ($p > 0.05$). That finding goes in accordance with the results of Mula*et al*, where the use of high speed resulted in narrow range for size distribution[42]. Regarding ZP, formulae differed significantly based on the time of homogenization ($p=0.0119$), but did not show significant difference with respect to speed of homogenization ($p > 0.05$). Allowing sufficient time for homogenization permitted proper coating of PVA on the nanoparticles, hence augmenting ZP with the increase of time of homogenization[39].

Hair dying results supported the proposed idea of enhancing color penetration into the hair through converting the coloring principle of calendula into nano-sized particles. Nanoparticles had the capability of penetrating the hair fibers[43] deep to the inside, without causing any damage to the cuticle of the hair. That finding was also reinforced by the lightening of the black color of the hair (figure 4D). Thus, it was possible to overcome hair damage brought about by using alkaline media utilized in synthetic dyes[3].

In conclusion, it was possible to avoid hair fiber damage caused by use of alkaline media associated with synthetic hair dye. That was achieved by formulating the coloring principle in Calendula Officinalis as a nano-sized dispersion, which permitted its penetration into hair fiber without the need to open the hair cuticle. F7 with the optimal particle size, polydispersity index and zeta potential succeeded in penetrating the hair shaft as evidenced in the hair photographs without the need to

open the hair shaft with alkali. Hence, the product represented a safe and sustainable pathway for dyeing white hair rather than coloring dark hair.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation**.**

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