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ORIGINAL CONTRIBUTION

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Penetration study of oils and its formulations into the human hair using confocal microscopy

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Summary

Background: Laser scanning confocal microscopy (LSCM) is a nondestructive method for observing the samples in three dimensions as well in their natural environment. Therefore, it is well suited for studying human hair. This investigation is focused on evaluating the comparative penetration ability of vegetable vs mineral oils and their formulations with excipient, in human hair.

Aims: Laser scanning confocal microscopy was employed to assess thin cross sections of human hair, treated with oils and their formulations, to comprehend their penetration capability and pattern.

Methods: Hair incubated with individual oils or their formulations were labeled with the fluorescent dye was cross-sectioned into thin fragments and visualized under the LSCM.

Results: The mineral oil demonstrated better penetration through the hair than the vegetable oils. Combination of these oils with excipient, in an appropriate ratio, had a substantial influence on oil penetration in terms of the depth of penetration.

Conclusions: Our investigation proved the suitability of fluorescent-based imaging for studying the penetration of oils across human hair. This method can be employed as a potential analytical tool to study the penetration of various hair-care formulations and/or their additives, to estimate their effects on human hair.

KEYWORDS

confocal microscopy, hair-care formulations, mineral oil, penetration, vegetable oil

1 | INTRODUCTION

Popular hair-care products majorly consist of five product types, namely shampoo, hair oil, hair color, hair conditioner, and hair styling products. As per the "Indian Cosmetic Market Outlook 2018 (http://www.rncos.com/Market-Analysis-Reports/Indian-Cosmetic-Market-Outlook-2018-IM705_fig.htm)," extensive research and analysis of the cosmetic industries revealed that the share of hair-care products is maximum in the cosmetic industry, with hair oils dominates the market. The market for hair oils is growing significantly due to the consumer's preference for the benefits offered by these products. These include strengthening of hair, nourishment, fast, and better growth of hair and reduction in hair fall. Thus, several researchers have focused their attention on understanding the penetration of various ingredients, used in hair oils, into the human hair and the resulting influence on the physicochemical properties of the hair.[1,2] Diffusion of various molecules across hair sections is often investigated using fluorescent dyes. In this investigation, the penetration ability of fluorescently labeled oils and their formulations across the human hair was examined using laser scanning confocal microscopy (LSCM).

A number of techniques have been used to study the hair structure, including scanning electron microscopy (SEM), transmission electron microscopy (TEM)[3,4] atomic force microscopy[5,6] X-ray photoelectron

spectroscopy,[7] micro-diffraction,[8] secondary ion mass spectrometry,[9] and goniometry.[10] Electron microscopy techniques such as SEM and TEM require extensive sample preparation and high-vacuum conditions, which may introduce undesired artifacts within the fine details of the hair surface. AFM provides high-resolution images, as well as preserves the integrity of samples, but it is limited due to its minimum sampling area, which creates a restriction during analysis.[11] The AFM working image size is typically 20 × 20 μm, and thus many such fields of view, over the wide area of hair samples, need to be examined to gain satisfactory interpretations.[12] LSCM has provision for changing the objective lens to the ones at alternative magnifications, which overcomes the limitations faced by AFM. It provides high-resolution images, as it has an optical sectioning property, which offers a breakthrough from the traditional observation of hair in a scanning electron microscope (SEM).[13] The technique requires minimal sample preparation and the hair sample can be observed in its natural form, with almost no damage, as compared to other microscopic methods like SEM. The technique utilizes fluorescent probes (such as Nile red) for demonstrating the routes of penetration of excipients into the internal structure of hair, which varies with the hair lipophilicity and distribution of lipophilic materials within the hair.[13]

Human hair consists of cuticle, cortex, and medulla. Hair is a protein filament that grows from the follicles present in the dermis.[14] Applying oil on hair can enhance lubrication of the shaft and help to prevent the hair breakage. Deposition of oil on the hair has a beneficial protective effect.[15] Various oils are used for human hair, which includes coconut oil, olive oil, castor oil, rice bran oil, sesame oil, mineral oils. However, most of the market oil is in the form of blends with herbs and nutrients for promoting hair growth and health. An oil formulation generally consists of two basic components—base oil and additives. The base oils contribute to about 70%-85% of the total composition, while additives make up the remaining 15%-25%. In this work, the base oils used were rice bran oil (RBO), refined til oil (RTO), and light liquid paraffin oil (LLPO), while the additives were chosen from herb extract, menthol, camphor, menthe oil, Butylated hydroxytoluene (BHT), cinnamon, vetiver oil, etc

1.1 | Vegetable vs mineral oils

Among the various substances used to fortify hair oils, rice extract functioned as a conditioning agent. The ferulic acid and esters present in rice bran oil stimulate hair growth. It also contains antioxidants, such as omega-3 and omega-6 essential fatty acids, which nourish hair and prevent premature.[16,17] Sesame or til oil is rich in vitamins such as vitamin E, vitamin B, and other B complex vitamins; and minerals like calcium (Ca), magnesium (Mg), and phosphorous (P). It has powerful antibacterial[18] and antifungal activity, due to which it is also employed as an anti-dandruff agent. It acts as a natural sunscreen when applied to hair and scalp. It prevents hair damage due to UV radiations[19] by forming a protective coat around the hair. It helps in strengthening the hair roots, acts as coolant, hair follicle moistening agent and prevents hair dryness. It has high penetration ability and helps in fast scalp circulation. It can be used to relieve aches, pains, and stiffness and is also known to benefit hair growth and reduce graying while promoting a natural shine. Mineral oil (MO) is extensively used in hair-care formulations in India, because of its nongreasy nature, and cost-effectiveness compared to vegetable oils like coconut and sunflower oils.[20] It has a high spreading capability on the hair surface, which improves gloss, and reduces split end formation.[21] Light liquid paraffin oil (LLPO) is the highly refined mineral oil used for cosmetic and medical purposes. It is a transparent liquid, odorless, and insoluble in water and alcohol. It has been widely employed in cosmetics, hair oils, baby oils, etc.

Due to the fact that vegetable and mineral oils are nonfluorescent by with excipient cannot be visualized directly by LSCM, therefore lipophilic dye, that is, Nile red was used. Dye labeled oil/oil formulations were incubated with hair sample, and a thin cross section of the treated hair was visualized under the LSCM. The images clearly showed the difference in relative penetration, when the hair was incubated with individual oils or oil formulations. Mineral oil showed a higher penetration than vegetable oils. Ingredients played an important role in oil formulation. Formulated oil exhibited higher penetration than individual base oils. In formulated oil samples, absence of RTO leads to a better relative penetration. Thus, LSCM can be a potential tool to understand penetration of newer hair-care oil formulations.

2 | MATERIALS AND METHODS

2.1 | Materials

Human hair samples comprising of strands of straight black hair of Indian origin were supplied by Emami Ltd., Mumbai, India. Different oils such as RBO, RTO, and LLPO were provided by Emami Ltd., Mumbai, India. Formulations of these oils with various excipients, in ratios, were conducted by Emami Ltd., Mumbai, India, and the resulting oil formulations were stored in refrigerator, at 4°C, for further use. Nile Red fluorescent dye, having the laser excitation of 561 nm, was used for labeling these oils and oil formulations and was purchased from Molecular Probes Inc, Invitrogen. Paraffin wax, purchased from Rarco, Mumbai, India, was used for preparing cross sections of human hair samples. Rotary Microtome, from Medimeas Instruments, Haryana, India, was used for obtaining very thin cross sections of hair samples. All experiments for visualization of oil penetration into hair samples were carried out using LSCM (Leica Microsystem, Germany).

2.2 | Preparation of hair sample

Straight, black human hair samples were utilized during the entire study. The length of the selected hair sample was kept at 10 ± 0.2 cm, and a total of four hair strands were used for studying the penetration of each test sample. Initially, the hair samples were cleaned with distilled water, followed by drying with a hair drier to facilitate grease-free samples. A block of paraffin wax containing different hair samples was prepared, using specified L molds for every analysis. Very thin cross sections (block size ~60 μ) were obtained from the individual blocks of the hair strands treated with each test

TABLE 1 Preparation of oil formulations using various ratios of base oils and additives

Sr No.	Code of oil formulations	Ingredients	Formula base
1	NRO 786	Herb extract, Menthol, Camphor, Mentha oil, BHT	RBO:RTO:LLPO (65:05:30)
2	NRO 786 B—1	Herb extract, Menthol, Camphor, Mentha oil, BHT	RBO:LLPO (69:31)
3	NRO 791	Herb extract, Menthol, Camphor, Mentha oil, BHT	RBO:RTO:LLPO (25:05:70)
4	NRO 791 B—1	Herb extract, Menthol, Camphor, Mentha oil, BHT	RBO:LLPO (29:71)
5	NRXT 264	Herb extract, Menthol, Camphor, Mentha oil, Clove, Cinnamon and vetiver oil, BHT	RBO:RTO:LLPO (65:05:30)
6	NRXT 264 B—1	Herb extract, Menthol Camphor, Mentha oil, Clove, Cinnamon and vetiver oil, BHT	RBO:LLPO (69:31)
7	NRXT 270	Herb extract, Menthol Camphor, Mentha oil, Clove, Cinnamon and vetiver oil, BHT	RBO:RTO:LLPO (25:05:70)
8	NRXT 270 B—1	Herb extract, Menthol Camphor, Mentha oil, Clove, Cinnamon and vetiver oil, BHT	RBO:LLPO (29:71)

LLPO, light liquid paraffin oil; RBO, rice bran oil; RTO, refined til oil.

sample, using a sharp stainless steel blade of the Rotary Microtome. The samples were fixed on a glass slide with cover-slip, using nail polish, and placed inverted on stage for microscopic visualization.

2.3 | Preparation of oils formulations

Different oil formulations were prepared by mixing additives with the base oils, in different ratios, as stated in Table 1. Two formulations, namely Himani Ayurvedic Navratna Oil (Oil formulation codes of NRO) and Himani Ayurvedic Navratna Extra Thanda Oil (Oil formulation code of NRXT) were prepared. Formulations 1-4, as stated in Table 1, contained BHT, mentha oil, menthol, and camphor were referred as Himani Ayurvedic Navratna Oil. On the other hand, formulations 5-8 as stated in Table 1, contained BHT, vetiver oil, cinnamon oil, clove oil, capsaicin, menthol, and camphor and were referred as Himani Ayurvedic Navratna Extra Thanda Oil. RTO was present at the concentration of 0%-5% v/v in all the formulations, whereas the ratio of rice bran oil and light liquid paraffin oil varied between 65%-70% and 25%-30%, v/v, respectively. The resulting oils/oil formulations were labeled using Nile red and applied on hair samples for analysis using LSCM.

2.4 | Fluorescent labeling of test samples

The oils or oil formulations were incubated with Nile red for uniform fluorescent labeling. About 100 µL of the stock solution of the dye (1 mg/mL) was added to the individual sample of oil/oil formulations. The dye was vortex with the oils/oil formulations and incubated for 1 hour, under continuous stirring with rotor-spin (Tarson Product Pt. Ltd., Mumbai, India), at 50 rpm. Thereafter, the hair samples were incubated with labeled oils/oil formulations and further incubated for additional 1 hour at room temperature (25°C). Thin cross sections of the fluorescently labeled hair samples were mounted on glass slides to evaluate the oil penetration into the hair.

2.5 | Laser scanning confocal microscopy

Laser scanning confocal microscopy analyses were performed with a Leica SP8 microscope (Leica Microsystem, Heidelberg, Germany), equipped with a UV laser, argon laser, and helium-neon lasers. The investigations of the hair cross sections were performed using a 63× oil objective (NA = 1.4), which provides the best numerical aperture.[22] The treated hair cross sections were initially studied in transmission mode, without fluorescence excitation, using yellow light (wavelength = 561 nm), in order to prevent bleaching of the fluorescent dye. Thereafter, the fluorescent dye labeled oils/oil formulations were excited at 561 nm, with an emission band of 570-632 nm, having gain value of 782. The laser intensity was kept constant at 4.96 during all the analyses. Specimens were captured using a combination of emitted and transmitted light. The resultant images were overlaid, in some cases, to determine the location of the fluorescent signal. Z-stack images were procured at the maximum laser intensity. Parameters for image acquisition in the bright field have been stated in Table 2.

2.6 | Quantitative measurements

Comparative analyses of all the samples were carried out with respect to control samples, that is, hair incubated with only Nile red

TABLE 2 Parameters for image acquisition in the bright field using confocal microscope

SL. no.	Parameters	Values
1	Step size	0.5 μ
2	Format	512 × 512
3	Pixel size	100 μm
4	Zoom size	3 times
5	Objectives	63×
6	Laser excitation	561 nm
7	Laser emission	570-632 nm
8	Laser intensity	4.96 W/cm^2
9	Scan speed	600 Hz
10	Gain value	782

dye. Relative penetration for each test samples was calculated with respect to control sample. Images were processed and quantified based on the mean fluorescent intensity (MFI) of the selected regions of interest (ROI), the analyses being conducted using LAX software of Leica Microsystem, Germany.

3 | RESULTS AND DISCUSSION

The human hair by itself exhibits a high amount of auto-fluorescence. Therefore, in the current investigation, we determined the specific parameters for operating the Confocal Laser Scanning Microscope (stated in Table 2), at which the hair auto-fluorescence was negligible and the fluorescence of the hair samples could be measured.

3.1 | Microscopic visualization of human hair

Laser scanning confocal microscopy was employed for visualizing the hair samples. The bright-field images, of the longitudinal surface morphology and the transverse cross section of the human hair, were visualized using a 60× oil immersion lens. The captured images (Figure 1) clearly show the longitudinal surface morphology of human hair sample (Figure 1A). A very thin cross section (60 μm) of the hair sample was viewed under bright field. The ROI around the cross section, as depicted in Figure 1B, was considered during the penetration studies of fluorescently labeled oils/oil formulations.

3.2 | Observation of control sample as hair treated with only fluorescent dye

Figure 2 depicts the bright field image of the cross section of hair treated with fluorescent dye, that is, Nile red alone. A lack of fluorescent signal was observed whenever the hair samples were incubated with only the dye (Figure 2B). Once the oil/oil formulations were labeled with Nile red and observed under the microscope, the fluorescent signal could be observed. Thus, hair strands incubated with the dye, alone, were used as the control during the studies. The mean fluorescence intensities of the hair cross sections treated with fluorescence dye were calculated, and the relative oil penetration was expressed as a percentage.

3.3 | 3D Visualization of hair samples treated with oil/oil formulations

The optical sectioning (z-scan) of hair samples incubated with fluorescently labeled oils/oil formulations revealed a 3D image that clearly demonstrated the uniform distribution of fluorescently labeled oil/oil formulations inside the human hair (Figure 3). Representative image clearly shows that half of its slice view represents cross-sectioned hair structure in bright field, while the remaining half exhibited uniformly penetrated fluorescent oil (in red color). This confirmed the oil penetration capability of the hair structure.

3.4 | Penetration of vegetable oils vs mineral oil in human hair

Confocal microscopy images of the untreated human hair were compared with those of hair incubated with Nile red, and fluorescently

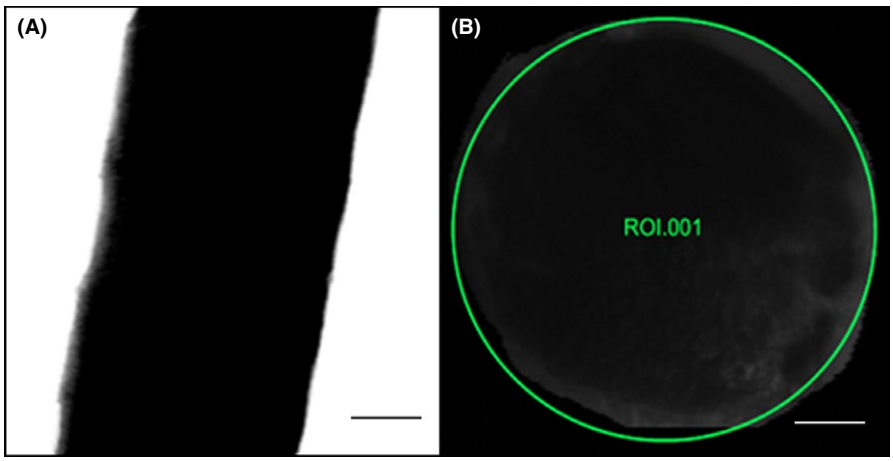

FIGURE 1 Images of human hair: A, Longitudinal (left), B, Transverse view of hair (right), scale bar = 40 μm

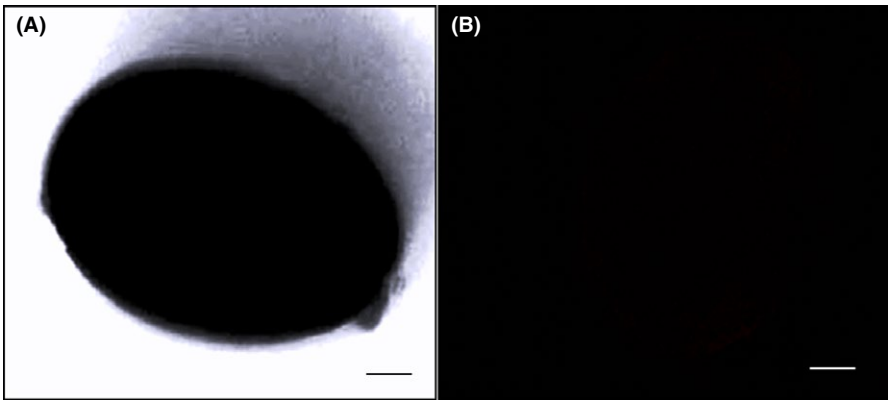

FIGURE 2 Laser scanning confocal microscope image for A, Hair cross section in bright field, B, Nile Red dye without oil, scale bar = 40 µm

labeled RBO, RTO, and LLPO. The bright-field images for hair cross sections and the fluorescent signal for the labeled oils were overlaid to compare the penetration of different oils across the hair sections. Figure 4 demonstrates the penetration of various oils in human hair samples. Initially, the hair in bright field (Figure 4A) was used to select the region of interest (ROI), and the same was considered for hair samples incubated with oils. False colors were used to understand the penetration pattern of different oils, viz. green color was used for RBO (Figure 4B), red color for RTO (Figure 4C), and pink color for LLPO (Figure 4D). Overlaying the colors of the individual oils into a single ROI (Figure 4E) revealed the histogram corresponding to the intensity of all fluorescence.

Histogram of the individual samples was plotted to quantify their fluorescence intensity. The fluorescence signal was quantified in terms of mean value. Mean value is the ratio of pixel sum to the sum processed pixel. Sum processed pixel corresponds to all the color dotted points which give the fluorescent signal in the selected region of interest. The Sum pixel corresponds to the all points of drawn ROI. LLPO (purple) showed a uniform distribution of fluorescent signal with highest intensity (mean value = 29.71), in comparison with the vegetable oils, that is, RBO (green color) and RTO (red color). Among the vegetable oils, RTO demonstrated uniform fluorescence signal of higher intensity (mean value = 21.05) than RBO (mean value = 8.49). Mean value of individual fluorescent oils that had penetrated across the hair cross section were calculated with selected ROIs and have been listed in Table 3. The mean value represents the number of processed pixels (representation of dye color point distribution quantified by instrument). The relative penetrations, expressed as a percentage, were calculated for individual oils with respect to the control sample (hair incubated with dye only).

As seen from Table 3, mineral oil or LLPO had better penetration capacity (approx. 75%) than vegetable oils, that is, RBO (approx. 14%) and RTO (approx. 65%). Among the vegetable oils, RTO (65.22%) exhibited approximately five times higher penetration than RBO (13.78%). Therefore, it was concluded that the individual oils had differential penetration depending on their intrinsic properties. RBO exhibited less penetration capability, whenever applied individually, while RTO by itself was a good penetrant. These experiments were extended to oil formulations.

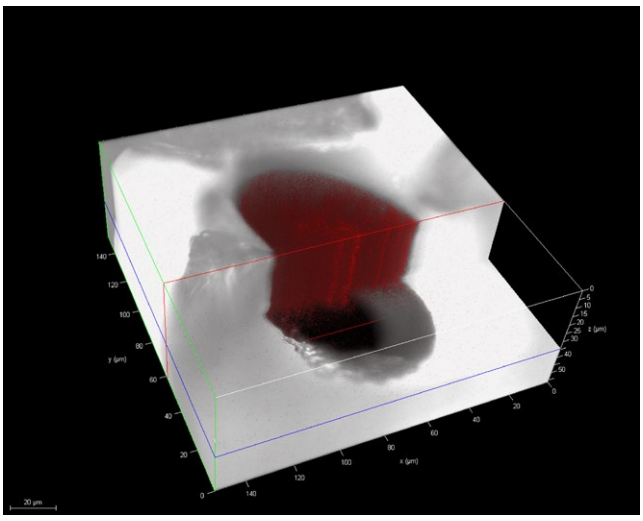

FIGURE 3 3D (z-scan) visualization of fluorescently labeled oil on hair cross section, scale bar = 40 µm

3.5 | Penetration of oil formulations labeled with Nile red in human hair

The fluorescence intensity of the labeled oil formulations that had penetrated across the hair cross sections was captured and quantified for the mean fluorescence intensity (MFI) in the region of interest (ROI). From the Figure 5A-H, the ROI was drawn based on the cross section of hair sample. The oil penetration ability for the individual oil formulations was calculated from their mean value of the ROI. The relative oil penetration was calculated with respect to control hair samples.

The formulated oils were classified into two main categories, Himani Ayurvedic Navratna oil (Figure 5 upper panel) and Himani Ayurvedic Navratna extra thanda oil (Figure 5 lower panel). The formulation compositions and the ratios of the base oils used therein have been stated in Table 1. ROI of each hair cross section was used for the quantification of MFI.

In case of oil formulation, the formula base of the vegetable vs mineral oil and its proportion ratio enhances the oil penetration behavior. From the Table 4, it can be observed that the oil formulations without RTO, such as NRO 786-B1, NRO 791-B1, NRXT 264-B1 and NRXT 270-B1, exhibited better oil penetration, corresponding to a

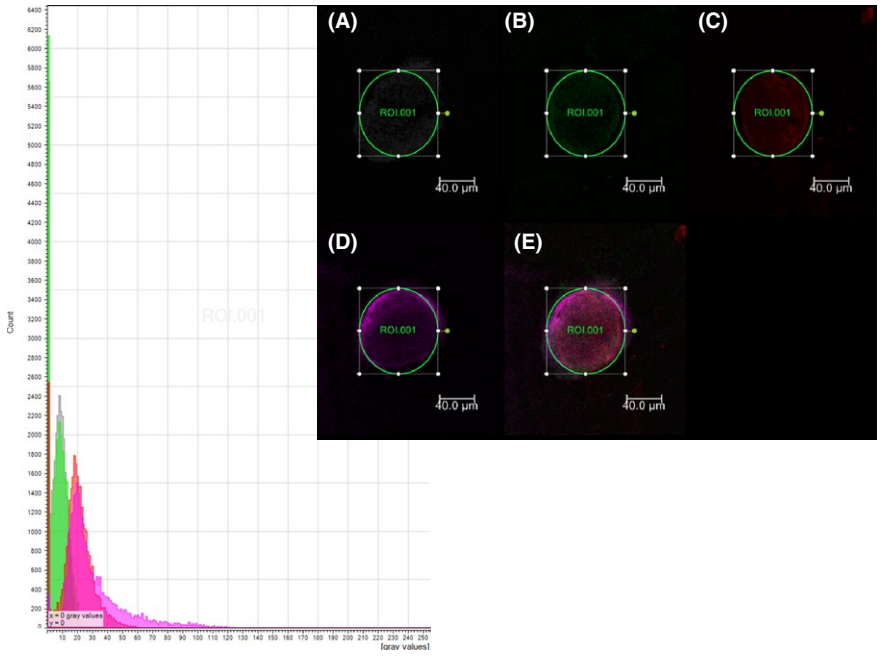

FIGURE 4 Confocal microscopy of A, Hair; B, Hair treated with RBO (green); C, Hair treated with RTO (red); D, Hair treated with LLPO (pink) and E, Overlay, scale bar = 40 µm

relative penetration of almost 80%. Combinations of RBO and LLPO, containing the ingredients included in Himani Ayurvedic Navratna oil, demonstrated the highest penetration of around ~88%. While, inclusion of even 5% (v/v) RTO in the oil formulations, as in case of Himani Ayurvedic Navratna Extra Thanda oil, that is, NRXT 270, resulted in a lower penetration of only ~53%. This clearly indicates that, combination of base oil with ingredients resulted, a more effective penetration across the hair cross section, as compared to the base oils alone. While RBO, by itself, demonstrated a very low penetration (~14%) across the human hair, its combination with other oils or ingredients greatly improved the oil penetration. On the other hand, RTO alone exhibited a good penetration of ~65%. However, its combinations demonstrated a lower penetration across the hair samples. Formula base ratio of RBO:LLPO in the formulation also shows a tremendous behavior in relative oil penetration. A compositions consisting, 30% LLPO and 70% RBO of the formula base exhibited almost 60%-70% relative oil penetration. However, when the ratios of these base oils were reversed (LLPO:RBO = 70:30), the penetration of the resulting mixture was merely 53%-56%. Overall, it was concluded that the oil formulations included in Himani Ayurvedic Navratna oil improved the

TABLE 3 Relative oil penetration of vegetable vs mineral oil on hair cross section

Parameters	RBO	RTO	LLPO
Mean values (gray values)	8.49	21.05	29.71
Sum processed pixel	27 716	27 716	27 716
Pixel sum	235 505	583 505	823 481
Relative penetration (%)	13.78	65.22	75.36

LLPO, light liquid paraffin oil; RBO, rice bran oil; RTO, refined til oil.

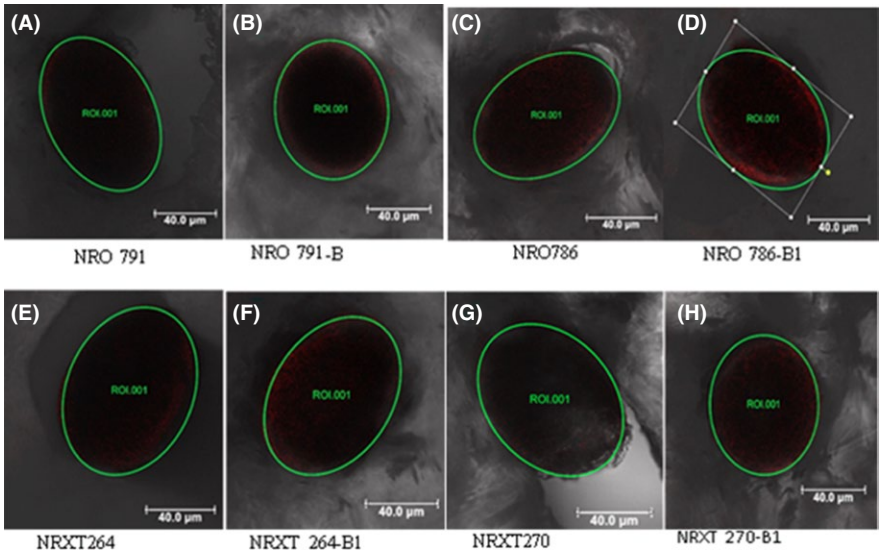

FIGURE 5 Penetration of oil formulation across the hair cross sections: ROI of each samples categorized as Flexi chasse formulations for Himani Ayurvedic Navratna Oil (lower panel) and Flexi chasse formulations for Himani Ayurvedic Navratna Extra Thanda Oil (upper panel) represents the fluorescent red color signal in hair cross section, scale bar = 40 µm

TABLE 4 Relative penetration of labeled oil formulation on hair cross section

Oils/ Oil formulations	Mean value	Relative penetration (Dye control; %)
NRO 786	4.88349728	69.55
NRO 786-B-1	12.09065	87.77
NRO 791	3.43203097	56.67
NRO 791-B-1	7.2072313	79.36
NRXT 264	3.43203097	56.69
NRXT 264-B-1	8.10788778	81.76
NRXT 270	3.15278413	52.82
NRXT 270-B-1	7.81003673	80.96

penetration percentage by ~5%-10% as compared to the oil formulations included in Himani Ayurvedic Navratna Extra Thanda oil, which was attributed to the different formulation excipients contained in them. Himani Ayurvedic Navratna Extra Thanda oil contained additonal ingredients such as clove, cinnamon and vetiver, as comapared to the Himani Ayurvedic Navratna oil. The extra thanda oil was thus, formulated as a stronger variant of navratna oil, to impart an extra cooling effect. Among all the oil formulations, the maximum relative penetration (87.77%) was observed for the formulation NRO 786-B1, while the least relative penetration (52.82%) was seen in case of NRXT 270. Navratna oil containing RBO:LLPO in the ratio of 69:31% v/v, without RTO, and labeled as NRO 786-B1, exhibited a uniform fluorescence across the hair cross section (Figure 5D). Whereas, Himani Ayurvedic Navratna Extra Thanda oil containing RBO:RTO:LLPO in the ratio of 25:5:70% v/v and labeled as NRXT 270, showed a very less fluorescence across the hair cross sections (Figure 5G). This suggested a reduced penetration of oil formulations in presence of RTO and the ability of clove, cinnamon and vetiver excipients to make it extra thanda in oil formulations. Til oil having the property of forming protective coating layer which prevents from UV radiations.[20] Whenver, til oil itself applied on hair it may form such layer on the hair and shows a uniform labeling signal. While presence of til oil on oil formulations may prevent the other excipient permeation on hair. This could be a possible reason for relatively low penetration percentage in case of oil formulations consisting of base formula of even 5% of til oil. The formed protective coating may reduce the diffusion of other ingredients in oil formulations. Thus til oil had an inhibitory effect on oil formulations, while the formulations devoid of til oil showed varying penetration depending on the additional ingredients contained in them.

4 | CONCLUSIONS

Laser scanning confocal microscopy was successfully employed as a nondestructive technique to observe the human hair in its natural state, where in both, the hair surface and the internal hair structure could be visualized. Three different oils (RBO, RTO, and LLPO) and their formulations were studied to understand their penetration across the human hair. Comparative analyses were carried out for all the hair samples treated with individual oils/oil formulations, and the results were compared with the control hair sample incubated with Nile red, alone. It was observed that among the individual oils, that is, RBO exhibited only 14% penetration, while LLPO and RTO possessed better penetration percentages of 75% and 65%, respectively. Combinations of these oils with various ingredients resulted in improved penetration of the formulations. Combination of RBO and LLPO, without RTO, resulted in a relative oil penetration of ~80%-90%. Inclusion of even 5% v/v of RTO leads to almost 25%-30% decrease in oil penetration percentage. Ingredients such as clove, cinnamon, and vetiver oil played a significant role in the extra thanda oil formulations. Formulation of these ingredients with RBO and LLPO resulted in relative penetration of almost 80%, as observed in case of NRXT 264-B-1 and NRXT 270-B-1. In case of navratan oil, absence of these ingredients resulted in 10% increase in the relative penetration. Therefore, it was concluded that oil formulations possessed a higher penetration capability than the individual base oils. RTO, alone, demonstrated almost 65% penetration across the human hair. However, its inclusion in oil formulations, even at a low concentration of 5% v/v volume, lowered the relative penetration of the formulations. Thus inclusion of formulation ingredient, along with the base oils, had a significant influence on oil penetration behavior across the human hair. Overall, the study indicated the suitability and ease of visualizing hair treated with fluorescently labeled oils/oil formulations using LSCM, thus suggesting it to be a rapid, elegant and nondestructive analytical tool for observing human hair in its natural environment.

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CONFLICT OF INTEREST

The authors declare there are no competing interests.

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