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ORIGINAL RESEARCH

Clinical cosmeceutical repurposing of melatonin in androgenic alopecia using nanostructured lipid carriers prepared with antioxidant oils

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ABSTRACT

Background: The present work aims to formulate nanostructured lipid carriers (NLCs) exhibiting high skin deposition and high inherent antioxidant potential to repurpose the use of melatonin hormone and some antioxidant oils in the treatment of androgenic alopecia (AGA).

Research design and methods: NLCs were characterized for their size, charge, drug entrapment, antioxidant potential, physical stability, *in vitro* release, surface morphology, and *ex-vivo* skin deposition. Their merits were clinically tested on patients suffering from AGA by calculating the degree of improvement, conduction of hair pull test, histometric assessment, and dermoscopic evaluation.

Results: Results revealed that melatonin NLCs showed nanometer size, negatively charged surface, high entrapment efficiency, and high anti-oxidant potential, in addition to sustained release for 6 h. Furthermore, NLCs displayed good storage stability and they were able to increase the skin deposition of melatonin 4.5-folds in stratum corneum, 7-folds in epidermis, and 6.8-folds in the dermis compared to melatonin solution. Melatonin NLCs displayed more clinically desirable results compared to the melatonin solution in AGA patients, manifested by increased hair density and thickness and decreased hair loss.

Conclusions: The aforementioned system was shown to be a very promising treatment modality for AGA, which is worthy of futuristic experimentation.

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KEYWORDS
Nanostructured lipid carriers; melatonin; androgenic alopecia; cosmeceuticals; skin

1. Introduction

Androgenic alopecia (AGA) is a heritable process causing progressive decrease in the density of scalp hair in a gender-dependent manner. It affects 50–70% of males according to their age [1]. It was reported that free radicals cause cellular apoptotic events in the hair follicles resulting in progressive hair loss [2]. It was also further delineated that oxidative stress and dysfunction of antioxidant enzymes result in alopecia [3,4]. Therefore, formulating a suitable topical antioxidant preparation for treatment of AGA is a promising option.

Lipidic delivery systems are currently gaining attention in the treatment of dermatological diseases owing to their skin permeation potential and their ability to act as drug reservoir [5–9]. Among the promising topical lipid carriers is nanostructured lipid carriers (NLCs), which comprise a matrix of solid lipid and oil, and were proven to exhibit topical merits owing to their smaller size and occlusive properties [10]. Advantageously, antioxidant oils can be used in their formulation, thus converting them to a therapeutically functional nanocarrier [11].

In search of drugs to be loaded within the antioxidant NLCs, an ancient molecule, melatonin hormone was reported to be an excellent candidate to combat oxidative stress associated with AGA, as it was proven to be a very potent and safe antioxidant [12]. Furthermore, human hair follicles synthesize melatonin and express melatonin receptors, and it was reported that melatonin greatly influences the hair growth cycles [13], resulting in an increase in anagen hairs in the frontal and occipital areas compared with placebo [14]. Therefore, the use of melatonin in treatment of AGA would present a safe treatment that overcomes side effects encountered with the standard treatment modalities finasteride which causes gynecomastia [15], and minoxidil which causes irritation and increased heart rate [16,17].

Therefore, the presented work aimed to explore the capacity of melatonin as a promising cosmeceutical in the clinical treatment of AGA, while being loaded in a functional lipidic nanocarrier based on antioxidant oils to augment its therapeutic efficacy and facilitate its topical deposition. Till current date, there are no reports exploring the clinical efficacy of melatonin nanoparticles or any other nanoparticles in the treatment of AGA.

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Supplemental data for this article can be accessed here

2. Materials and methods

2.1. Materials

Bitter almond oil, absolute ethanol, Tween 80 (polysorbate 80), sodium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, acetone, tetrahydrofuran, methanol, and water were purchased from Al Nasr company, Cairo, Egypt. Precirol ATO 5 (glyceryl palmitostearate), Compritol 888 ATO (glyceryl dibehenate), and Labrafac Lipophile were kindly provided as gift from Gattefossé company, Lyon, France. Evening primrose oil, olive oil, and soybean oil were kindly provided as gift from Seatons company, Snaith, UK. DPPH (2,2-diphenyl-1-picrylhydrazyl), phosphotungistic acid and spectra/Por dialysis membrane were purchased from Sigma Aldrich Chemical Co., St.Louis, USA. Melatonin was purchased from Skin actives company, Gilbert, Arizona, USA.

2.2. Preparation of melatonin NLCs

Melatonin NLCs were prepared using a combined high shear homogenization/solvent diffusion technique [18,19] according to the composition shown in Table 1. Twenty-five milligrams melatonin together with 250 µl of one of four oils, namely evening primrose, olive, soybean, and bitter almond, were incorporated with 1-g solid lipid (glyceryl palmitostearate or glyceryl dibehenate) and dissolved at 80ºC in either acetone: tetrahydrofuran mixture (1:1, v/v) for glyceryl palmitostearate [20] or acetone: absolute ethanol (1:1, v/v) for glyceryl dibehenate [21]. The organic phase was poured onto hot aqueous phase including polysorbate 80 as surfactant at 75–80 ºC, and homogenized at 26,000 rpm for 2 min (Silent Heidolph crusher D91126, Schwabach, Germany), followed by overnight magnetic stirring.

2.3. Characterization of melatonin NLCs

2.3.1. Determination of the particle size, polydispersity index (PDI), and charge of NLCs

The size, PDI, and charge of the prepared melatonin NLCs were determined using Zetasizer (model ZS3600, Malvern Instruments Ltd., Worcestershire, UK) after dilution using water [22,23].

2.3.2. Determination of melatonin entrapment efficiency (EE%) in NLCs

EE% of melatonin NLCs was determined using the dialysis method [24]. NLCs were transferred to the dialysis bag, and then placed into a beaker comprising 2000 ml distilled water that was changed twice. After exhaustive dialysis of the free (unentrapped) drug in the dispersion for 2 h, an aliquot of NLCs was vortexed with methanol, and the amount of entrapped melatonin was measured at 278 nm using UV-spectrophotometry (Libra S60, Biochrom Ltd., Cambridge, UK). Furthermore, the entrapment capacity (ratio of the entrapped drug to the lipid matrix) was also calculated.

2.3.3. Measurement of the anti-oxidant potential of melatonin NLCs using DPPH assay

A stock of DPPH in methanol was prepared, kept in dark for 60 min, then its absorbance was measured at 5l5 nm, which is the maximum wavelength of absorption of DPPH [25,26]. The antioxidant potential of NLCs was assessed by adding 3900 µl of the DPPH stock solution to 100 µl of the dialyzed NLCs, then the test tubes were left in the dark for 60 min, and their absorbance values were re-measured at 515 nm against methanol. The DPPH scavenged percentage is calculated from the following equation [27]:

$$DPPH\ Scavenged\ (\%) = \frac{\text{Absorbance of stock solution} - \text{Absorbance of test solution}}{\text{Absorbance of stock solution}} \times 100\%$$

For comparative purposes, two NLCs formulations were prepared using Labrafac Lipophile oil (non-antioxidant oil) with glyceryl palmitostearate and glyceryl dibehenate.

In order to further confirm the antioxidant potential of the utilized oils, re-assessment of the antioxidant activity of NLCs was performed, using an amount of each formula equivalent to a constant amount of melatonin (0.25 mg), in order to take into consideration the antioxidant ability of the oil without the drug.

2.3.4. Physical stability of melatonin NLCs

The stability of NLCs was assessed by re-measuring their particle size, zeta potential, and PDI after storage for 3 months in the refrigerator at 2–8ºC [28].

2.3.5. *In-vitro* release of selected melatonin NLCs

A specified volume of freshly dialyzed NLCs equivalent to 2 mg of melatonin was loaded into a dialysis bag [21,28] and placed in 60 ml of phosphate buffer saline (PBS) pH 7.4, which ensured sink conditions for melatonin. Samples were horizontally shaken in a thermostatically controlled shaker (model IKA KS 4000 IC, Wilmington, North Carolina, USA) at 32°C and 50 rpm [29]. At

Table 1. Composition and characterization of melatonin nanostructured lipid carriers.

Formula code	Solid lipid	Antioxidant Oil	Particle size (nm) Mean ± S.D. $n = 3$	PDI Mean ± S.D. $n = 3$	Zeta potential (mV) Mean ± S.D. $n = 3$	EE% Mean ± S.D. $n = 3$	Entrapment capacity	DPPH% inhibition Mean ± S.D. $n = 3$
NLC1	Glyceryl palmitostearate	Evening primrose	683 ± 27.08	0.42 ± 0.07	−17.2 ± 0.53	75.6 ± 0.56	1:66	43.62 ± 0.36
NLC2		Olive	307 ± 18.31	0.38 ± 0.15	−15.1 ± 0.22	76 ± 1.25	1:66	66.22 ± 0.23
NLC3		Soybean	307 ± 3.68	0.39 ± 0.11	−16.6 ± 0.14	73.6 ± 0.56	1:68	15.46 ± 0.29
NLC4		Bitter almond	303 ± 16.24	0.72 ± 0.15	−14.6 ± 0.78	50 ± 0.60	1:100	50.90 ± 0.65
NLC5	Glyceryl dibehenate	Evening primrose	784 ± 7.30	0.44 ± 0.03	−16.2 ± 0.15	66 ± 2.26	1:76	54.70 ± 0.41
NLC6		Olive	563 ± 20.33	0.49 ± 0.02	−13 ± 0.61	74.8 ± 0.60	1:67	71.43 ± 0.32
NLC7		Soybean	616 ± 19.80	0.47 ± 0.03	−15.5 ± 0.42	61.2 ± 1.94	1:82	25.85 ± 0.15
NLC8		Bitter almond	698 ± 18.00	0.64 ± 0.11	−19.7 ± 0.00	36.4 ± 2.14	1:137	65.30 ± 0.36

certain time points, 3 ml samples were withdrawn, and the amount of released melatonin was quantified spectrophotometrically at 278 nm [30].

2.3.6. Transmission electron microscopy (TEM) for selected melatonin NLCs

The surface morphology of the selected melatonin NLCs was examined using TEM after negative staining with 1% phosphotungstic acid (JEM – 100S, Joel, Tokyo, Japan) [31–34].

2.3.7. *Ex-vivo* skin deposition/permeation of melatonin NLCs

Rat skin samples were cut into square pieces and fixed in Hanson diffusion apparatus (model 60–301-106, CA, Los Angeles, USA) in cells of 1.77 cm^2 area. The receptor medium was 7.5 ml PBS (pH 7.4) constantly mixed at 100 rpm and 32°C.

A constant volume (2 ml) of the selected melatonin NLCs formulation was placed in the donor compartment. Melatonin solution in PBS containing equivalent amount of melatonin to the NLCs formulation was used as control. After 24 h, buffer samples were collected and the skin surface was washed five times using methanol and distilled water, then dried with filter paper to remove any residue. Skin tape stripping was performed 20 times with adhesive tape, and the dermis was separated from the epidermis using a scalpel. Tape strips and skin specimens were placed each in 20 ml methanol followed by sonication for 4 h to extract melatonin from each skin layer [35]. Samples were filtered and analyzed using a validated UPLC method developed in our laboratory (Agilent 1290 infinity, Waldbronn, Germany), using a mobile phase of acetonitrile:water 75:25 at a flow rate 0.6 ml/min and 10 µl were injected into a C18 column and analyzed at 223 nm.

2.4. Clinical efficacy of melatonin NLCs in treatment of AGA

The clinical study included 40 male patients suffering from AGA, divided into 2 groups. Group I (20 patients) were treated with topical melatonin solution in PBS (pH 7.4), while group II (20 patients) were treated with topically applied NLC2 formulation. Patients were included from the dermatology outpatient Clinic of Minia University Hospital. Inclusion criteria were male patients, aged 18–50 years suffering from mild-to-moderate AGA (stages I-V Hamilton-Norwood classification), who have not used topical, systemic, or intralesional therapy for AGA during and 6 months prior the clinical study. A written approval was obtained from each patient, and the clinical study was approved by the research and research ethics committees of the faculties of medicine and pharmacy (Minia and Ain Shams universities), respectively, for experimental and clinical studies (REC ASU- 176), following the guidelines of the declaration of Helsinki for human subject experimentation.

In each group, all patients were instructed to apply the provided NLCs formula or melatonin solution daily on affected scalp areas for a total period of 3 months. Assessment of the clinical response was done at 16th week (i.e. 1 month after the end of treatment). Patients were clinically assessed using:

2.4.1. Photography

Photography assessment was performed by taking digital photos for the scalp before and at the 16th week of treatment. An improvement grade expressed as the mean of grades provided by three professional independent blinded observers was provided for each patient through comparing the degree of baldness before and after treatment. Score was given from: −1 = worse than baseline, 0 = no improvement, 1 = minimal improvement (< 20%), 2 = mild improvement (20–39%), 3 = moderate improvement (40–59%), 4 = good improvement (60–79%), and 5 = excellent improvement (80–100%) [36].

2.4.2. Hair pull test

Hair pull test was performed by gentle grasping of few hair strands (about 50–60 hairs) from the vertex between thumb, index, and middle finger, and these extracted hairs were counted, before and at the 16th week [37]. This test provides an estimation of how much hair is being lost [38].

2.4.3. Histometrical assessment

Difference in microscopic measurement of the diameter of hair shaft was assessed through pulling out hairs from the vertex of 20 patients (10 from each group) before and after treatment, and then they were placed on a glass slide mounted with immersion oil. A computer-assisted program was employed for measurement of mean hair shaft thickness by obtaining five measurements for each hair shaft [39].

2.4.4. Dermoscopic examination

Dermoscopy was carried out on a fixed area on the scalp of patients (10 cm from the glabella and a fixed area in the right parietal region) using Dermlite dermoscope. Baseline and post-treatment photographs (at 16th week) were provided together with dermoscopic evaluation regarding yellow dots (i.e. sebaceous debris), diameter of hair shaft, and hair density [37]. A score of 0 indicated no improvement while a score of 1 indicated improvement of AGA.

2.5. Statistical analysis of data

One-way ANOVA and Tukey Kramer post-test were used to analyze non-clinical data using Graphpad® Instat software, La Jolla, California, USA. For clinical experiments, quantitative data were expressed as range and mean ± S.D. whereas qualitative data were expressed as number and percent. Statistical analysis included ANOVA, paired *t*-test, independent samples *t*-test, and Chi-square test. *p* values less than or equal 0.05 were statistically significant.

3. Results and discussion

3.1. Preparation of melatonin NLCs

NLCs were prepared by combined high shear homogenization/solvent diffusion technique since it was the optimum method of preparation based on our preliminary assessment, taking into the consideration the large amount of lipid utilized for NLCs formulation Polysorbate 80 at 2% concentration was

sufficient to coat the nanoparticles and avoid their coalescence owing to steric stabilization [40].

3.2. Determination of the particle size, PDI, and charge of NLCs

As demonstrated in Table 1, the particle size of NLCs ranged from 303 to 784 nm. Their PDI values ranged from 0.38 to 0.72, indicating polydispersity of the particles, which might be attributed to the use of high shear rather than high-pressure homogenization technique for the preparation, especially at the large amount of solid lipid used [41,42]. The relatively large particle size compared to other NLCs formulations reported in the literature could be ascribed to the large amount of solid lipid used (1000 mg), which was necessary to achieve good encapsulation for melatonin, based on our preliminary screening results.

It can be observed that NLCs prepared using glyceryl dibehenate as solid lipid displayed significantly larger particle size compared to those prepared using glyceryl palmitostearate ($p < 0.05$). This could be attributed to the difference in the viscosity of the two solid lipids, since molten glyceryl dibehenate viscosity was reported to be higher than that of glyceryl palmitostearate, leading to larger particle size resulted from the homogenization inefficiency [41]. It could also be attributed to glyceryl dibehenate's longer chain length which is mainly based on behenic acid (C22) than glyceryl palmitostearate which is mainly based on stearic acid (C18) [43]. The absence of a correlation between oil type and particle size could be ascribed to the complex nature of each oil, being composed of different fatty acids, resulting in different interaction modes with the solid lipid and surfactant molecules.

All formulations exhibited a negative charge, with values ranging from −11.8 to −19.7 mV, indicating acceptable stability [44] as a result of the electrostatic repulsion between nanoparticles [45] despite that these values are lower than the value normally required for steric stabilization (−30 mV). The origin of the negative charge on NLCs is attributed to the presence of ionizable fatty acids of triglycerides in glyceryl palmitostearate and glyceryl dibehenate in addition to those present in the oils [21,43,45]. The difference in the magnitude of negative charge among NLCs formulations could be ascribed to the different fatty acid contents of the solid and liquid lipids.

3.3. Determination of EE% of melatonin NLCs

As shown in Table 1, the EE% of melatonin NLCs varied from 36% to 76%. An overall high EE% of melatonin in NLCs could be ascribed to its lipophilicity (log p 1.2), which favors its incorporation within lipidic matrices. This came in accordance with several authors who attempted encapsulation of melatonin in lipidic formulae [46]. Moreover, melatonin was successfully incorporated in solid lipid nanoparticles and in NLCs at an EE% of 72% [47,48] concurring with our results, and further confirming melatonin's lipid affinity.

On further inspection of the results, it can be noted that NLCs prepared using glyceryl palmitostearate exhibited higher EE% than those prepared using glyceryl dibehenate ($p < 0.05$), suggesting higher affinity for melatonin in the former lipid, similar to what was encountered with other authors [49,50]. The comparable EE% of melatonin within NLCs, except for almond oil-based NLCs, suggested that they exhibited comparable crystal lattice imperfections within the solid lipid core, which is capable of encapsulating almost equivalent amount of melatonin in evening primrose, olive, and soybean oil-based NLCs. The low EE% encountered with almond oil NLCs could be attributed to a lower affinity between melatonin and the oil dictated by the log p of melatonin. This came in accordance with other authors who attempted the encapsulation of different parabens within glyceryl palmitostearate almond oil NLCs [51].

3.4. Measurement of the anti-oxidant potential of melatonin NLCs using DPPH assay

Melatonin NLCs were formulated using antioxidant oils, namely olive, soybean, almond, and evening primrose oils. Olive oil contains at least 30 phenolic compounds such as simple phenols (tyrosol and hydroxyl tyrosol), flavonoids, aldehydic secoiridoids, lignans, and vitamins as vitamin E, which are known to possess potent free radical scavenging effects [52,53]. Almond oil contains caffeic, vanillic, ferulic acids, p-coumaric, kaempferol, quercetin, delphinidin, isorhamnetin, cyaniding, and procyanidin B2 and B3 in addition to vitamin E, all of which were proven to be strong antioxidants [54,55]. Evening primrose oil contains gallic acid together with methyl- and ethyl-esters, caffeic, protocatechuic and ellagic acids (−)−epicatechin, (+)−catechin, quercetin, (−)−epicatechingallate, procyanidins, penta-O-galloyl-β-D-glucose, which were proven to be potent anti-oxidants [56,57]. Soybean oil contains six isoflavones, which are considered major phenolic antioxidant compounds, namely genistin, daidzin, daidzein, glycitin, glycitein, and genistein [58]. All of these aforementioned components within the chosen NLCs oils serve as natural antioxidants proven to prevent the formation of free radicals.

Upon comparing different NLCs formulations in Table 1, it was obvious that their antioxidant potential (measured by taking constant volume of NLCs) is arranged in the following order: olive oil NLCs > almond oil NLCs > evening primrose oil NLCs > soybean NLCs. The olive oil NLCs displayed significantly higher antioxidant activity ($p < 0.05$) compared to all other antioxidant oils, which is probably attributed to its high content of phenolic compound hydroxyl tyrosol; which is considered to be the most potent antioxidant [52,59]. Labrafac lipophile NLCs exhibited low DDPH% inhibition of 12.41% and 19.21% for glyceryl palmitostearate and glyceryl dibehenate NLCs, respectively, owing to the lack of phenolic antioxidants in the composition of labrafac lipophile, and its DPPH% inhibition would be totally attributed to its melatonin content. All other oils displayed variable DPPH scavenging activity based on their phenolic compounds' content. It was also obvious that glyceryl dibehenate-based NLCs displayed significantly higher DPPH% inhibition than glyceryl palmitostearate-based NLCs ($p < 0.05$), which is suggestive of better preservation of antioxidant activity of the oils upon their incorporation within glyceryl dibehenate solid lipid phase rather than glyceryl palmitostearate.

Similar to what was obtained in the previous section, when a volume of NLCs containing a constant amount of melatonin was used for testing the antioxidant potential, olive oil containing formulations also displayed the highest DPPH% inhibition ($p < 0.05$). Melatonin solution in water at an amount equivalent to 0.25 mg (control) displayed DPPH% inhibition of 18.15%, suggesting that the utilization of antioxidant oils in the preparation of NLCs was a positive added value in terms of potentiation of antioxidant activity. Results are displayed in supplementary 1.

3.5. Physical stability of melatonin NLCs

NLCs displayed mostly insignificant changes in size, PDI, and charge suggesting their stability, which might be attributed to the negative charges present on NLCs surface causing repulsion between the particles, hence preventing aggregation [60]. Data are shown in supplementary 2.

3.6. In vitro *release study on the selected melatonin NLCs*

Upon inspection of the combined results of size, zeta potential, polydispersity, DPPH% inhibition, and storage stability, it can be observed that all NLCs formulations displayed favorable particle size in nanometer range and comparable zeta potential values. Formulae NLC7 and NLC8 displayed the lowest EE% values for melatonin and hence were excluded from the release experiment. Furthermore, formula NLC3 which displayed the lowest DPPH% inhibition was excluded from the release study as well. Moreover, NLC5 which exhibited the highest significant increase in particle size upon storage was also excluded from this study. To recapitulate, formulae NLC1, NLC2, NLC4, and NLC6 were selected for conduction of the release experiment.

As shown in Figure 1, NLCs showed sustained release of melatonin for 6 h, and 100% release was achieved after that time. This sustained release is attributed to the affinity of the lipophilic melatonin to the hydrophobic triglycerides fatty acids chains present in glyceryl palmitostearate and glyceryl dibehenate, resulting in a slow release pattern [61]. Owing to the importance of increased drug amount released topically within short period of time, formula NLC2 which displayed the highest released amount of melatonin after 30 min was chosen for further characterization.

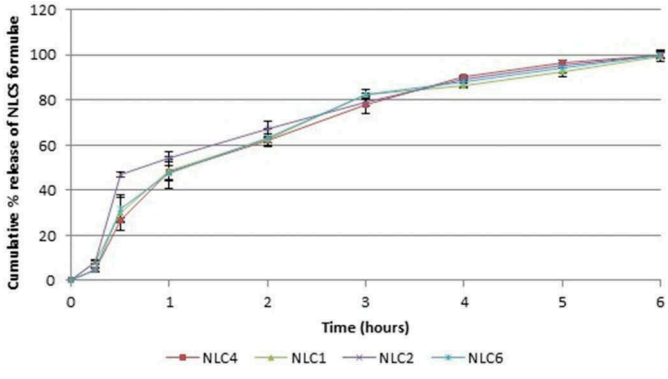

Figure 1. Cumulative % released of melatonin from the selected nanostructured lipid carriers NLCs formulae.

3.7. Determination of NLCs morphology using TEM

As shown in Figure 2, formula NLC2 displayed nearly spherical shape with a smooth surface, similar to what was encountered with other authors [29,62].

3.8. Ex vivo *skin deposition/permeation of melatonin NLCs*

The percentage of melatonin accumulated into the stratum corneum, epidermis, and dermis or permeated through the skin (reaching the receptor compartment) for both NLCs and melatonin solution was shown in Table 2 and Figure 3. Only a small amount of melatonin permeated to the PBS solution, suggesting topical rather transdermal traits of the NLCs. NLCs significantly increased the skin deposition of melatonin compared to the solution, reaching 4.5-folds in stratum corneum, 7-folds in epidermis, and 6.8-folds in the dermis ($p < 0.05$). This could be attributed to the lipidic characteristics of NLCs and the melatonin lipid-to-water partition coefficient which promote the partitioning in the lipidic stratum corneum. In addition, the nanometer size of the NLCs formulation supports their penetration to deeper skin layers [63]. NLCs were reported to ensure close contact with the stratum corneum superficial junctions, allowing the diffusion of active agents. Following the application of NLCs to skin surface, water evaporates and hence, they form an adhesive layer causing skin occlusion and increasing drug's thermodynamic activity.

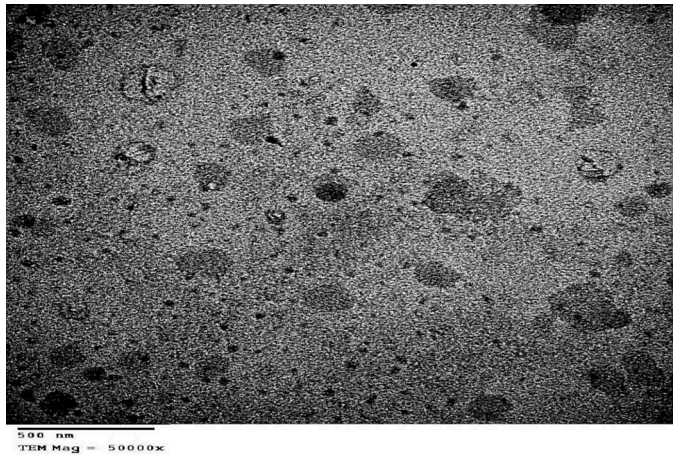

Figure 2. Negative stain electron micrograph of formula NLC2 at a magnification of 50000×.

Table 2. *Ex vivo* skin deposition/permeation data on the selected formula NLC2 compared to melatonin solution.

Skin layer	NLC2 Mean ± S.D. $n = 3$	Melatonin solution Mean ± S.D. $n = 3$
SC	12.51% ± 0.35	2.76% ± 0.04
Epidermis	31.50% ± 0.38	4.46% ± 0.07
Dermis	8.23% ± 0.44	1.21% ± 0.14
Receptor compartment	3.07% ± 0.26	0.58% ± 0.05

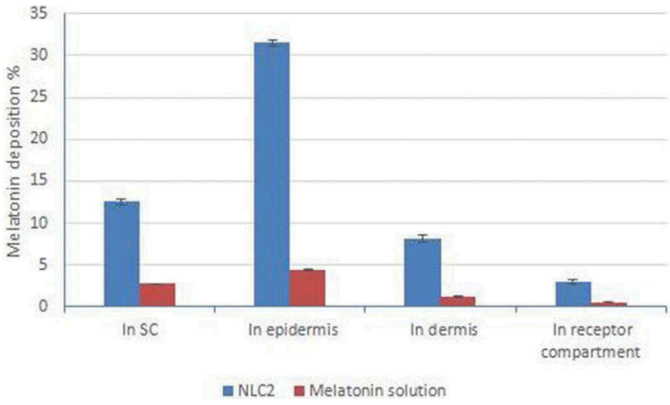

Figure 3. *Ex vivo* skin deposition/permeation of formula NLC2 compared to melatonin solution.

Stratum corneum hydration minimizes corneocyte packing, increases inter-corneocyte gaps and allows drug partitioning into the stratum corneum lipids [64].

3.9. Clinical efficacy of melatonin NLCs in treatment of AGA

According to Hamilton Norwood classification, the baldness of patients ranged from type I to V baldness indicating mild-to-moderate hair loss. Assessment of the clinical response was done in terms of follow-up photography/scoring, and assessment of hair loss and hair diameter. No side effects were reported by patients receiving NLCs or melatonin solution indicating their safety and ensuring patient compliance.

As shown in Table 3, improvement was evident in a total of 8 patients (40%) of group I compared with a total significant improvement in all 20 patients (100%) in group II ($p < 0.05$). Therefore, it can be concluded that group II treated with NLC2 displayed significantly better improvement scores for patients than group I patients treated with melatonin solution ($p < 0.05$). Figure 4 displays photographic evidence of improvement in a representative patient from group I and another patient from group II.

Table 3. Comparison between groups I and II regarding dermatologist's assessment after treatment.

Improvement degree	Group (I)* $N = 20$ N (%)	Group (II)** $N = 20$ N (%)
Minimal	7 patients (35%)	
Mild	1 patient (5%)	2 patients (10%)
Moderate	-	4 patients (20%)
Good	-	12 patients (60%)
Excellent	-	2 patients (10%)
No improvement	12 patients (60%)	-

* treated with melatonin solution,
** treated with melatonin NLCs.

Hair pull test is considered a noninvasive diagnostic method which rapidly estimates hair loss severity [65]. As evident in Table 4, the mean number of epilated hairs was significantly decreased in groups I and II after treatment ($p < 0.05$). However, the extent of decrease in hair loss was significantly higher in group II patients treated with NLCs formulation than group I patients treated with melatonin solution ($p < 0.05$).

Regarding the histometric assessment of the hair shaft, as evident in Table 4 and Figure 5, the extent of hair diameter

Table 4. Comparison between groups I and II regarding hair pull test and hair shaft thickness before and after treatment.

Groups	Hair pull test		Hair shaft thickness (μm)	
	Before $N = 20$	After $N = 20$	Before $N = 10$	After $N = 10$
Group I * Range	7–10 hairs	5–8 hairs	40.99–54.22	44.52–56.76
Mean ± S.D	7.9 ± 0.97	6.6 ± 0.82	48.54 ± 4.67	51.54 ± 3.90
Group II ** Range	6–10 hairs	1–5 hairs	40.66–52.44	71.99–93.22
Mean ± S.D	7.7 ± 1.08	2 ± 1.03	44.98 ± 3.60	83.25 ± 7.39

* treated with melatonin solution,
** treated with melatonin NLCs.

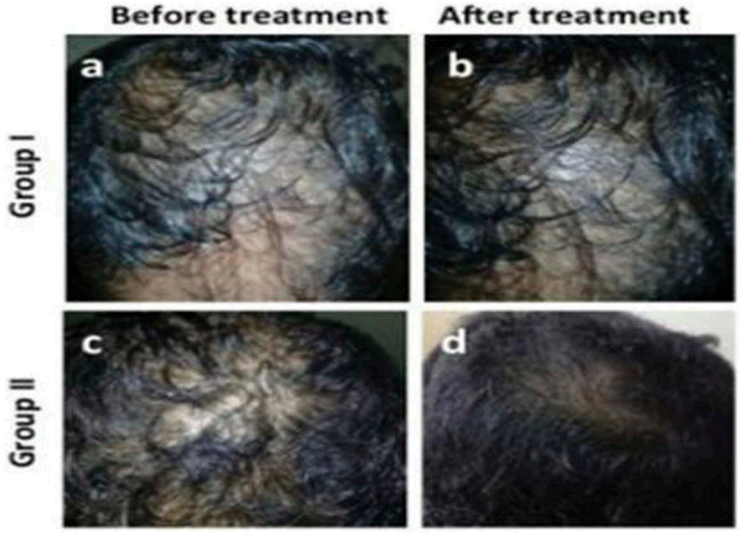

Figure 4. Two male patients with androgenic alopecia before treatment (a, c). At 16th week of treatment (b, d), the improvement was minimal in group I after topical treatment with melatonin solution (b) and excellent in group II after topical treatment with NLC2 formula (d).

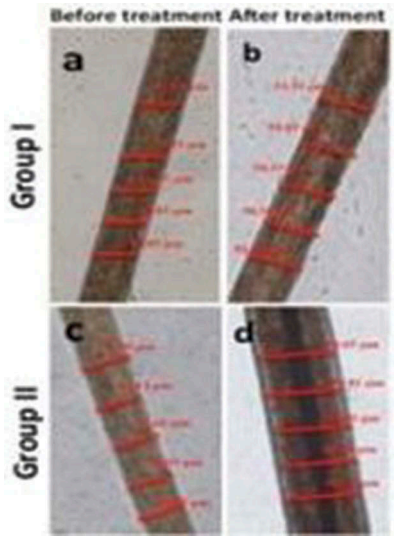

Figure 5. Hairs from male patients with androgenic alopecia before therapy (a, c). At 16th week of treatment (b, d), there is significant increase in the hair shaft diameter in group II aftertopical treatment with NLC2 formula (d) with also a significant increase in hair shaft diameter in group I after topical treatment with melatonin solution but to a smaller extent (b) (magnification × 400).

increase was significantly higher for group II patients compared to group I patients ($p < 0.05$).

Upon dermoscopic examination of patients before receiving treatment, they displayed an increase in the thin vellus hairs with variability in the diameter of more than 20% of the hair shafts, along with the presence of yellow dots, positively correlating with the severity of the disease [66]. As displayed in Figure 6, the post-treated patients of group II displayed significantly higher increase in hair density and hair shaft diameter with dramatic reduction or disappearance of yellow dots than in group I patients ($p < 0.05$).

As can be collectively deduced from the results, melatonin encapsulation in antioxidant NLCs maximized its therapeutic potential in AGA, as could be inferred from the clinical assessment criteria. The promising behavior of NLCs in treatment of AGA could be ascribed to their ability to facilitate the delivery of melatonin to hair follicles, besides interacting with skin lipids as well to allow for drug diffusion and depot formation inside the skin [67]. Furthermore, the small size of NLCs, their lipophilic nature, their sustained release properties, and enhanced skin deposition compared to the conventional solution form improved the drug–skin bioavailability by improving the drug's penetrability.

4. Conclusion

In the current work, the pharmaceutical as well as clinical merits of melatonin NLCs in the treatment of AGA were proven, hence, opening a wide range of possibilities for the use of nanoparticles in the treatment of dermatological diseases. Furthermore, the current work proved that cosmeceuticals can effectively replace conventional drug therapy, hence overcoming side effects of the latter. Futuristic work requires clinical work on larger number of patients, with more comprehensive studies on the mechanistic aspects of how a cosmeceutical treats certain diseases such as AGA.

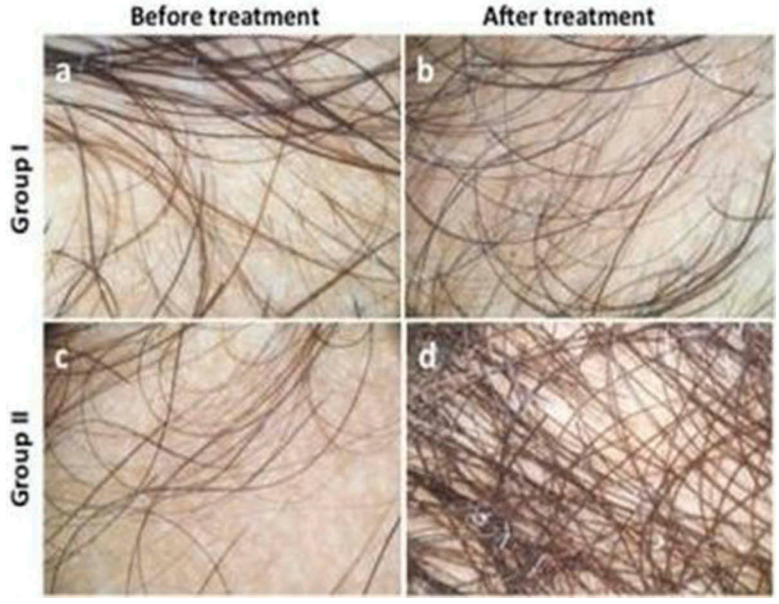

Figure 6. Dermoscopic features of scalp of male patients with androgenic alopecia before therapy showing yellow dots, variation in the hair shaft thickness, and presence of hair follicles with single and double hair (a, c). At 16th week of treatment (b, d), there is increase in the hair density, hair shaft thickness, and decrease in yellow dots after treatment in groups II, with no dermoscopic improvement in group I after topical application of melatonin solution (b) (magnification × 10).

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Declaration of interest

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Author contributions

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References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Norwood OT. Male pattern baldness: classification and incidence. South Med J. 1975;68:1359–1365.
2. Naito A, Midorikawa T, Yoshino T, et al. Lipid peroxides induce early onset of catagen phase in murine hair cycles. Int J Mol Med. 2008;22:725–729.
3. Mosley JG, Gibbs CC. Premature grey hair and hair loss among smokers: a new opportunity for health education. BMJ. 1996;313:1616.
4. Prie BE, Iosif L, Tivig I, et al. Oxidative stress in androgenetic alopecia. J Med Life. 2016;9:79–83.
•• **Describes how can androgenic alopecia be caused by oxidative stress and hence benefit from antioxidant treatment.**
5. Nasr M, Mansour S, Mortada ND, et al. Vesicular aceclofenac systems: a comparative study between liposomes and niosomes. J Microencapsul. 2008;25:499–512.
6. Nasr M, Mansour S, Mortada ND, et al. Lipospheres as carriers for topical delivery of aceclofenac: preparation, characterization and in vivo evaluation. AAPS PharmSciTech. 2008;9:154–162.
7. Bseiso EA, Nasr M, Sammour O, et al. Recent advances in topical formulation carriers of antifungal agents. Indian J Dermatol Venereol Leprol. 2015;81:457–463.
• **A review summarizing novel nanocarrier techniques for topical delivery in treatment of skin fungal infections.**
8. Nasr M, Abdel-Hamid S. Lipid based nanocapsules: a multitude of biomedical applications. Curr Pharm Biotechnol. 2015;16:322–332.
9. Abdelgawad R, Nasr M, Moftah NH, et al. Phospholipid membrane tabulation using ceramide doping "cerosomes": characterization and clinical application in psoriasis treatment. Eur J Pharm Sci. 2017;101:258–268.
10. Loo CH, Basri M, Ismail R, et al. Effect of compositions in nanostructured lipid carriers (NLC) on skin hydration and occlusion. Int J Nanomed. 2012;8:13–22.
•• **An important reference detailing the effect of formulation variables on the physical stability of nanostructured lipid carriers.**
11. Lacatusu I, Badea N, Ovidiu O, et al. Highly antioxidant carotene lipid nanocarriers: synthesis and antibacterial activity. J Nanopart Res. 2012;14:902.
12. Reiter RJ, Mayo JC, Tan DX, et al. Melatonin as an antioxidant: under promises but over delivers. J Pineal Res. 2016;61:253–278.
13. Fischer TW, Slominski A, Tobin DJ, et al. Melatonin and the hair follicle. J Pineal Res. 2008;4:1–15.
14. Fischer TW, Burmeister G, Schmidt HW, et al. Melatonin increases anagen hair rate in women with androgenetic alopecia or diffuse alopecia: results of a pilot randomized controlled trial. Br J Dermatol. 2004;150:341–345.
15. Ramot Y, Czarnowicki T, Zlotogorski A. Finasteride induced gynecomastia: case report and review of the literature. Int J Trichology. 2009;1:27–29.
16. Leenen FH, Smith DL, Unger WP. Topical minoxidil: cardiac effects in bald man. Br J Clin Pharmacol. 1988;26:481–485.
17. Rossi A, Cantisani C, Melis L, et al. Minoxidil use in dermatology, side effects and recent patents. Recent Pat Inflamm Allergy Drug Discov. 2012;6:130–136.
18. Mukherjee S, Ray S, Thakur RS. Design and evaluation of itraconazole loaded solid lipid nanoparticulate system for improving the antifungal therapy. Pak J Pharm Sci. 2009;22:131–138.
19. Ranpise NS, Korabu SS, Ghodake VN. Second generation lipid nanoparticles (NLC) as an oral drug carrier for delivery of lercanidipine hydrochloride. Colloids Surf B Biointerfaces. 2014;116:81–87.
20. Agrawal Y, Petkar KC, Sawant KK. Development, evaluation and clinical studies of Acitretin loaded nanostructured lipid carriers for topical treatment of psoriasis. Int J Pharm. 2010;401:93–102.
21. Shamma RN, Aburahma MH. Follicular delivery of spironolactone via nanostructured lipid carriers for management of alopecia. Int J Nanomed. 2014;9:5449–5460.
22. Fadel M, Samy N, Nasr M, et al. Topical colloidal indocyanine green-mediated photodynamic therapy for treatment of basal cell carcinoma. Pharm Dev Technol. 2017;22:545–550.
23. Abdel-Salam FS, Ammar HO, Elkheshen SA, et al. Anti-inflammatory sunscreen nanostructured lipid carrier formulations. J Drug Deliv Sci Technol. 2017;37:13–19.
24. Verma S, Doshi A. Formulation, development and characterization of nanostructured hetrolipid matrix of levofloxacin hemihydrate for ocular drug delivery. J Pharmacol Clin Toxicol. 2014;2:1–5.
25. Gonzalez-Paredes A, Clarés-Naveros B, Adolfina Ruiz-Martinez M, et al. Delivery systems for natural antioxidant compounds: archaeosomes and archaeosomal hydrogels characterization and release study. Int J Pharm. 2011;421:321–331.
26. Mishra K, Ojha H, Chaudhury NK. Estimation of antiradical properties of antioxidants using DPPH assay: a critical review and results. Food Chemistry. 2012;130:1036–1043.
27. Nasr M. Development of an optimized hyaluronic acid-based lipidic nanoemulsion co-encapsulating two polyphenols for nose to brain delivery. Drug Deliv. 2016;23:1444–1452.
28. Das S, Ng WK, Kanaujia p, et al. Formulation design, preparation and physicochemical characterizations of solid lipid nanoparticles containing a hydrophobic drug: effects of process variables. Colloids Surf B. 2011;88:483–489.
29. Gaba B, Fazil M, Khan S, et al. Nanostructured lipid carrier system for topical delivery of terbinafine hydrochloride. Bulletin of Faculty of Pharmacy, Cairo University. 2015;53:147–159.
30. Mohammed MM, Hamadi SA, Aljaf AN. Formulation of melatonin as a cream and studying the release, diffusion, and stability of the cream. AJPS. 2009;6:43–55.
31. Nasr M, Abdel-Hamid S. Optimizing the dermal accumulation of a tazarotene microemulsion using skin deposition. Drug Dev Ind Pharm. 2016;42:636–643.

32. Nasr M, Abdel-Hamid S, Moftah NH, et al. Jojoba oil soft colloidal nanocarrier of a synthetic retinoid: preparation, characterization and clinical efficacy in psoriatic patients. Curr Drug Deliv. 2017;14:426–432.
33. Yousry C, Fahmy RH, Essam T, et al. Nanoparticles as tool for enhanced ophthalmic delivery of vancomycin: a multidistrict-based microbiological study, solid lipid nanoparticles formulation and evaluation. Drug Dev Ind Pharm. 2016;42:1752–1762.
34. Abdel-Salam FS, Mahmoud AA, Ammar HO, et al. Nanostructured lipid carriers as semisolid topical delivery formulations for diflucortolone valerate. J Liposome Res. 2017;27:41–55.
35. Bsieso EA, Nasr M, Moftah NH, et al. Could nanovesicles containing a penetration enhancer clinically improve the therapeutic outcome in skin fungal diseases? Nanomedicine (Lond). 2015;10:2017–2031.
36. Abdallah MA, El-Zawahry KA, Besar HA. Mesotherapy using dutasteride-containing solution in male pattern hair loss: a controlled pilot study. J Pan-Arab League of Dermatologists. 2009;20:137–145.
37. Jha AK, Udayan UK, Roy PK, et al. Platelet-rich plasma with microneedling in androgenetic alopecia along with dermoscopic pre- and post-treatment evaluation. J Cosmet Dermatol. 2018;17:313–318.
38. Dhurat R, Saraogi p. Hair evaluation methods: merits and demerits. Int J Trichology. 2009;1:108–119.
39. El-Domyati M, Hosam W, Moftah NH, et al. Hair follicle changes following intense pulsed light axillary hair reduction: histometrical, histological and immunohistochemical evaluation. Arch Dermatol Res. 2017;309:191–202.
40. Al-Qushawi A, Rassouli A, Atyabi F, et al. Preparation and characterization of three tilmicosin-loaded lipid nanoparticles: physicochemical properties and in-vitro antibacterial activities. Iran J Pharm Res. 2016;15:663–676.
41. Pathak p, Nagarsenker M. Formulation and evaluation of lidocaine lipid nanosystems for dermal delivery. AAPS PharmSciTech. 2009;10:985–992.
42. Lason E, Sikora E, Ogonowsko J. Influence of process parameters on properties of nanostructured lipid carriers (NLC) formulation. Acta Biochim Pol. 2013;60:773–777.

•• **An important review summarizing details of attempts for production of NLCs in addition to their properties.**

43. Fang YP, Lin YK, Su YH, et al. Tryptanthrin-loaded nanoparticles for delivery into cultured human breast cancer cells, MCF7: the effects of solid lipid/liquid lipid ratios in the inner core. Chem Pharm Bull (Tokyo). 2011;59:266–271.
44. Khalil RM, Abd El-Bary A, Kassem MA, et al. Influence of formulation parameters on the physicochemical properties of meloxicam-loaded solid lipid nanoparticles. EJP. 2013;12:63–72.
45. Huang Z, Hua S, Yang Y, et al. Development and evaluation of lipid nanoparticles for camptothecin delivery: a comparison of solid lipid nanoparticles, nanostructured lipid carriers, and lipid emulsion. Acta Pharmacol Sin. 2008;29:1094–1102.
46. Dubey V, Mishra D, Asthana A, et al. Transdermal delivery of a pineal hormone: melatonin via elastic liposomes. Biomaterials. 2006;27:3491–3496.
47. Priano L, Esposti D, Esposti R, et al. Solid lipid nanoparticles incorporating melatonin as new model for sustained oral and transdermal delivery systems. J Nanosci Nanotechnol. 2007;7:3596–3601.
48. Sabzichi M, Samadi N, Mohammadian J, et al. Sustained release of melatonin: a novel approach in elevating efficacy of tamoxifen in breast cancer treatment. Colloids Surf B Biointerfaces. 2016;145:64–71.
49. Khames A. Preparation and characterization of sildenafil loaded solid lipid nanoparticles: drug delivery system suitable for nebulization. Der Pharmacia Lettre. 2017;9:98–112.
50. Palei NN, Das MK. Preparation and characterization of lornoxicam loaded solid lipid nanoparticles made from different lipids. Int J Pharm Pharm Sci. 2013;5:438–442.
51. Cerreto F, Paolicelli p, Cesa S, et al. Solid lipid nanoparticles as effective reservoir systems for long-term preservation of multidose formulations. AAPS PharmSciTech. 2013;14:847–853.
52. Owen RW, Giacosa A, Hull WE, et al. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer. 2000;36:1235–1247.
53. Kalogeropoulos N, Tsimidou MZ. Antioxidants in Greek virgin olive oils. Antioxidants (Basel). 2014;3:387–413.
54. Matthaus B, Ozcan MM, Juhaimi FA, et al. Effect of the harvest time on oil yield, fatty acid, tocopherol and sterol contents of developing almond and walnut kernels. J Oleo Sci. 2018;67:39–45.
55. Amarowicz R, Troszynska A, Shahidi F. Antioxidant activity of almond extract. J Food Lipids. 2005;12:344–358.
56. Schmidt Š, Niklová I, Pokorný J, et al. Antioxidant activity of evening primrose phenolics in sunflower and rapeseed oils. Eur J Lipid Technol. 2003;105:427–435.
57. Kiss AK, Derwińska M, Granica S. Quantitative analysis of biologically active polyphenols in evening primrose (*Oenotheraparadoxa*) seeds aqueous extracts. Pol J Food Nutr Sci. 2011;61:109–113.
58. Mujić ŠE, Jokić S, Sarić Z, et al. Isoflavone content and antioxidant properties of soybean seeds. Croat J Food Sci. 2011;3:16–20.
59. Vilaplana-Pérez C, Auñón D, García-Flores LA, et al. Hydroxytyrosol and potential uses in cardiovascular diseases, cancer, and AIDS. FrontNutr. 2014;1:1–11.
60. Das S, Ng WK, Tan RB. Are nanostructured lipid carriers (NLCs) better than solid lipid nanoparticles (SLNs): development, characterizations and comparative evaluations of clotrimazole-loaded SLNs and NLCs? Eur J Pharm Sci. 2012;47:139–151.
61. Reddy LH, Murthy RSR. Etoposide-loaded nanoparticles made from glyceride lipids: formulation, characterization, in vitro drug release, and stability evaluation. AAPS PharmSciTech. 2005;6:158–166.
62. Mishra A, Imam SS, Aqil M, et al. Carvedilol nano lipid carriers: formulation, characterization and in-vivo evaluation. Drug Deliv. 2016;23:1486–1494.
63. Gokce EH, Korkmaz E, Dellera E, et al. Resveratrol-loaded solid lipid nanoparticles versus nanostructured lipid carriers: evaluation of antioxidant potential for dermal applications. Int J Nanomed. 2012;7:1841–1850.
64. Baroli B. Penetration of nanoparticles and nanomaterials in the skin: fiction or reality? J Pharm Sci. 2009;99:21–50.
65. Vujovic A, Del Marmol V. The female pattern hair loss: review of etiopathogenesis and diagnosis. BioMed Res Int. 2014;2014:1–8.
66. Hu R, Xu F, Han Y, et al. Trichoscopic findings of androgenetic alopecia and their association with disease severity. J Dermatol. 2015;42:602–607.
67. Kaur G, Bedi PMS, Narang JK. Targeting alopecia with topical nanocarriers. WJPPS. 2017;6:326–333.