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Original Article

Ghee Based Nanostructured Lipid Carriers (NLCs) with Improved Wound Healing Effects

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Abstract

Ghee or butter oil is an excellent medium for preparing herbal cosmetics and Ayurvedic medicines formulations. However, water immiscibility restricts its use as a potential carrier system, particularly in lipid-based topical formulations. In this work, we prepared ghee-based nanostructured lipid carriers (NLCs) using olive oil as liquid lipid, Tween 60 as surfactant, and glycerol as co-surfactant by hot emulsification–ultrasonication method. Then, the loaded NLC with a mixture of ethanolic extracts of *Achillea millefolium*, *Horsetail,* and *Plantago major* L. was prepared similarly. NLCs were characterized in size, zeta potential, polydispersity index (PDI), and creaming index. The particle size of NLCs was lower than 100 nm, and the zeta potential displayed a negative charge in all formulations. The results of the creaming index showed that NLCs were stable for up to 8 weeks under refrigerated conditions. The wound-healing effects of the NLCs were evaluated using an excision model in rats. The NLCs displayed significantly increased wound contraction and decreased epithelialization period compared to standard drug phenytoin. This study showed that ghee-based formulations are much more efficacious in NLC for wound healing promotion than their macroemulsion form. The formulated system is easy to produce and apply and could be favorable for topical application in pharmaceutical industries.

Keywords: Butter oil, Lipid nanoparticles, Wound contraction, Epithelialization time, Excision model, Macroemulsion.

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1. Introduction

Nanomaterials, the particles smaller than 100 nanometres in at least one dimension, are the basic components of nanoscience and nanotechnology. The physicochemical

characterizations of nanomaterials usually are different from those of bulk materials. Due to their unique advantages, nanomaterials obtained from natural products have attracted great attention in recent years [1]. Nanoencapsulation has the potential to enhance bioavailability and stability, improve controlled release, and provide precision targeting of natural bioactive ingredients [2]. A wide range of advanced nanomaterials derived from natural products have been developed and used in drug discovery and development [3-4]. Lipid-based nanoparticles, one of the most prominent natural product-derived nanoparticles, are the most widely used delivery vehicles for pharmaceutical applications due to their very low cytotoxicity, excellent biodegradability, high encapsulation efficacy, and good physicochemical stability [5]. Two major types of lipid-based nanoparticles are solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs). Nanostructured lipid carriers (NLCs) are improved oil-in-water nano-sized emulsions composed of a solid lipid blended with a liquid lipid. These colloidal nanocarriers, as the second generation of lipid nanoparticles, have been developed to overcome disadvantages associated with solid lipid nanoparticles (SLNs), such as low entrapment efficiencies and the expulsion of the encapsulated ingredients during storage [6]. A wide range of solid lipids such as stearic acid, glyceryl monostearate, glyceryl behenate, cetyl palmitate, isopropyl myristate, Precirol ATO 5, and cocoa butter have been used in the preparation of NLCs [7-8].

The anhydrous milk fat, or ghee or butter oil, is a well-known natural product commonly used as a cooking oil and therapeutic agent in the Middle East and India [9-10]. In Ayurveda, ghee treats allergy, skin, and respiratory diseases [11]. Ghee contains up to 98% lipids in triglycerides and small amounts of free fatty acids, phospholipids, cholesterol, and fat-soluble vitamins (A and E) [12]. The fatty acid composition of ghee triglycerides shows 53.9–66.8% of saturation, and the predominant fatty acids are palmitic acid (31.7–38.3%) and oleic acid (21.6–33.7%) [13]. Using ghee as a medium for preparing various herbal formulations shows that ghee can be a suitable delivery system for lipophilic plant extracts [14]. However, the immiscibility of ghee and water is a major challenge in developing these systems. Therefore, it is essential to develop oil-in-water emulsions of butter oil. In particular, developing ghee nanoparticles can be an efficient route to novel natural product-based nanocarriers. Up to now, several emulsification methods using emulsifiers such as polyvinyl alcohol, sodium caseinate, Tween 60, and poloxamer 407 for preparing the butter oil-in-water emulsions (o/w) have been reported [15-18]. Also, a microemulsion technique for synthesizing a ghee-based SLN loaded with temozolomide using Polysorbate 80 as a surfactant has been reported [19]. Recently, a microemulsion containing cow ghee and lauroglycol as oil, labrasol as a surfactant, and Transcutol as a cosurfactant has been developed to deliver fluocinolone acetonide to the posterior eye [20]. However, to our knowledge, there are no reports regarding ghee-based NLCs in the literature. Therefore, the current study aimed to develop ghee-based NLCs and evaluate their wound-healing effects.

In the present study, to develop new natural nanopharmaceuticals, we prepared a ghee-based, nanostructured lipid carrier (Blank NLC) using olive oil as liquid lipid together with Tween 60 as surfactant and glycerol as co-surfactant. Furthermore, a loaded NLC with a mixture of ethanolic extracts of *Achillea millefolium, Horsetail,* and *Plantago major* L. (AHP-NLC) was prepared. The prepared NLCs were characterized in terms of size, zeta potential, polydispersity index (PDI), and creaming index, and their wound healing effects were studied using an excision wound model in rats.

2. Materials and Methods

2.1. Materials

Tween 60, glycerol, and chloral hydrate were obtained from Merck Company and used as received. Olive oil was purchased from Barij Essence Pharmaceutical Co., Iran. Ethanol (99%) was purchased from Ghatran Shimi Tajhiz Co., Iran. Ghee was purchased from a local market and used without further treatment.

2.2. Preparation of Plant Extracts

The above-ground parts of *Achillea millefolium, horsetail,* and *Plantago major* L. were collected from East Azerbaijan province, Iran's natural habitats, then dried under shade and subsequently powdered. The powder of dried samples (50 gr) was subjected to successive extraction with ethanol (99%) (300 cc) at room temperature. After 48 h, the solutions were filtered, and the solvent recovered under reduced pressure at 40°C. The yields of *Achillea millefolium, horsetail,* and *Plantago major* L. extracts were 2.6 gr (5.2%), 4.9 gr (9.8%), and 5.2 gr (10.4%), respectively. The crude extracts were stored at 4°C until use.

2.3. Preparation of Nanostructured Lipid Carriers (NLCs)

Nanostructured lipid carriers (NLCs) were prepared by a hot homogenization technique employing a high-speed homogenizer and an ultrasonication method [21]. The aqueous phase, including Tween 60 and glycerol, was heated to 45 °C. The oil phase, consisting of *Achillea millefolium, horsetail,* and *Plantago major* L. extracts, ghee oil, and olive oil, was heated to the same temperature and then slowly added to the aqueous phase while being stirred, and then the resulting colloidal system was homogenized using a high shear homogenizer (Heidolph Silent Crusher M, Germany) at 11,000 rpm for 15 min. Then, the formed emulsion was sonicated (work time 60 s, rest time 15 s) using a probe sonicator (Vibracell VCX130; Sonics, USA) at an amplitude of 60 kHz for 10 minutes. The resulting AHP-NLC was kept at room temperature for 24 hours, and the nanoparticle's size was measured. Blank NLCs were prepared as stated above without adding *Achillea millefolium, Horsetail,* and *Plantago major* L. extracts to the formulation. The compositions of the NLCs are shown in **Table 1**.

2.4. Preparation of Macroemulsions

Ghee and *Achillea millefolium, Horsetail,* and *Plantago major* L. extracts macroemulsion (AHP-Ghee emulsion) were prepared with the same composition as AHP-NLC (**Table 1**).

Table 1: Compositions for NLC formulations.

Formulations (g)	Ghee	Olive oil	T60	Glycerol	Water (mL)	*Achillea millefolium*	*Horsetail*	*Plantago major* L.
Blank-NLC	0.90	0.60	1.50	1.50	25.50	0.00	0.00	0.00
AHP-NLC	0.90	0.60	1.50	1.50	25.32	0.06	0.06	0.06

Note: T60: Tween 60; AHP-NLC: *Achillea millefolium*, *Horsetail*, and *Plantago major* L. coloaded NLC.

The aqueous phase, including Tween 60 and glycerol, was heated to 45 °C.

The oil phase, consisting of *Achillea millefolium*, *horsetail*, and *Plantago major* L. extracts, ghee, and olive oil, was heated to the same temperature and then slowly added to the aqueous phase and the resulting mixture was stirred for 20 min at 2,000 rpm at 45 °C. *Achillea millefolium*, *Horsetail*, and *Plantago major* L. extracts emulsion (AHP emulsion) were prepared by adding 0.06 g of each of the three extracts to the aqueous phase (1.5 g Tween 60, 1.5 g glycerol, and 26.82 g water). The mixture was stirred for 20 min at 2,000 rpm at 45 °C.

2.5. Characterization of the NLCs

Mean particle size (Z-average), polydispersity index (PDI), and zeta-potential of the NLCs were measured at 25 °C by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

2.6. Assessment of the Stability of NLCs

5 mL of NLC was transferred into a glass tube (internal diameter 1 cm, height 10 cm) with a stopper and was stored at various temperatures (5 and 25 °C) for ten weeks. The total height of the NLC (H_t) and the height of the cream layer at the top of NLC (Hc) were measured at 2, 4, 6, 8, and 10 weeks. Creaming index (CI) was calculated as [22]:

$$CI(\%)=H_s/H_t \times 100 \quad (1)$$

$$H_s=H_t-H_c \quad (2)$$

2.7. Animals

Thirty adult male Wistar rats (200–250 g) of approximately three months of age were purchased from the Islamic Azad University of Marand animal house and were used for wound healing studies. The animal experiments were performed according to the guide for the care and use of laboratory animals published by NIH. Rats were housed in polypropylene cages under standard environmental conditions (23±2 °C, 60±5 % humidity, 12 h light/ dark cycle). During the experiment, the animals had free access to a standard pellet diet and water ad libitum. All experimental procedures used in this study were approved by the ethical committee of Razi Students Research Center with approval number 1865/107973/30877708/1399.

2.8. Evaluation of Wound Healing Activity (Excision Wound Model)

Wound healing activity was carried out as described by Morton and Malone with some modifications [23]. Animals were anesthetized by intraperitoneal injection of chloral hydrate (0.3 ml of 10% solution per 100 g of body weight), and their back hair was shaved. The

shaved region was sterilized with ethanol before making the wound. A full-thickness circular wound of about 250 mm^2 was made on the depilated area by removing the skin of the dorsum with circular motions using a scalpel, toothed forceps, and pointed scissors. The wounds were left undressed and exposed to the open environment. The rats were randomly divided into six groups of five animals in each group and placed in a separate cage. Group 1 was considered the control group and treated with saline solution. Group 2 was positive control and treated with 1% phenytoin ointment as a standard healing agent. Groups 3-6 were treated topically with AHP emulsion, AHP-Ghee emulsion, Blank-NLC, and AHP-NLC respectively.

The treatments were carried out once daily for 23 consecutive days, and 1 ml of each treatment was applied topically. A digital camera monitored the progressive changes in the wound area, and photographs of the lesions were taken on predetermined days, i.e., 0, 4, 7, 10, 13, 17, and 22. Then, the wound area was calculated using ImageJ software. The wound contraction was expressed as a reduction of the original wound size percentage. The wound contraction percentage was calculated using the following equation [24].

$$\text{Wound contraction (\%)} = \frac{\text{Initial wound size} - \text{Specific day wound size)}}{\text{Initial wound size}} \times 100 \quad (3)$$

The time needed for complete epithelialization was recorded in days unit and considered the healing time. The time to obtain 50% wound closure (WC_{50}) was calculated by plotting the percentage of wound contraction against days [25].

2.9. Statistical Analysis

The results were represented as Mean ± standard deviation (SD) and were evaluated using the Student's t-test. Values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. NLCs Characterization

As illustrated in **table 1**, all NLCs were prepared with 5%w/w of the lipid phase. In these preparations, ghee as solid lipid and olive oil as liquid lipid were used in a ratio by weight of 1.5:1. Tween 60 as emulsifier and glycerin as co-emulsifier agents were utilized in the 1:1 w/w ratio. Also, the ratio of oil phase: an emulsifier is 1:1. Accordingly, 0.2%w/w of each of the *Achillea millefolium*, *Horsetail*, and *Plantago major* L. extracts were added to 5%w/w of lipid matrix to prepare AHP-NLC formulation.

As illustrated in **Figure 1 (A)**, the particle size of blank-NLC and AHP-NLC was 59.82 nm and 87.73 nm, respectively. Also, the polydispersity index (PDI) and zeta potential of blank-NLC were 0.414 and − 6.21 mV, and those of AHP-NLC were 0.348 and −9.83 mV, respectively (**Figure 1 (B)**). The zeta potential measurements indicated that NLCs acquired negative surface charge due to negatively charged residual carboxylic groups in olive oil and ghee matrices.

It is evident that the increase of particle size of AHP-NLC compared to that of blank-NLC can be due to the encapsulation of plant extracts in the lipid matrix [26].

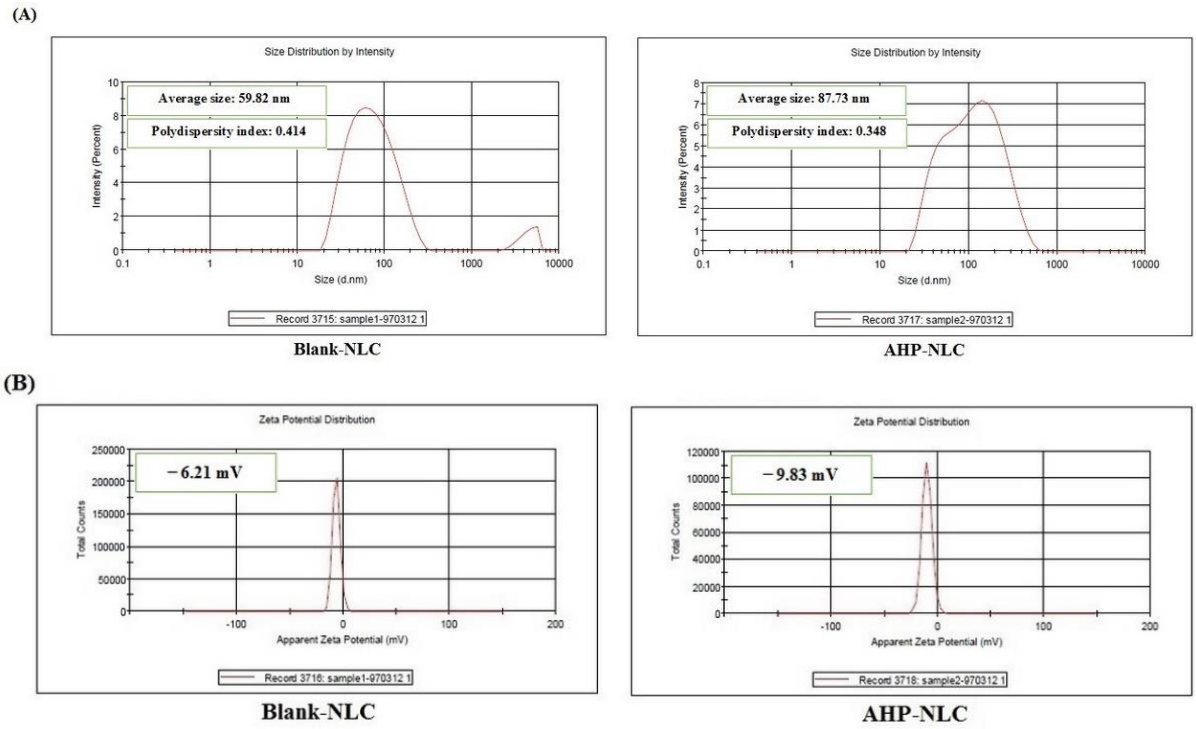

Figure 1. Size distributions by intensity (A) and zeta potential distributions (B) of NLCs.

However, the polydispersity index (PDI) and zeta potential of AHP-NLC are lower than those of blank-NLC. These results indicate that AHP-NLC is more stable than blank-NLC.

3.2. Stability of NLCs

The creaming index (CI) is a key parameter to evaluate the physical stability of NLCs [27]. CI percentages of NLCs after storage periods of 2, 4, 6, 8, and 10 weeks at 5 and 25 °C are shown in **Figure 2**.

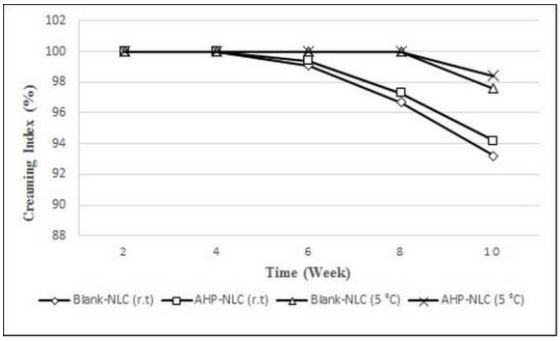

Figure 2. Creaming index of NLCs after storage for ten weeks.

There were no signs of creaming until 4th week, and after that, room temperature stored NLCs showed thin cream layers with a 99.1-99.4 % creaming index at six weeks. All NLCs stored at 5 °C showed no serum layer separation until the eighth week. However, the room temperature stored in Blank-NLC and AHP-NLC showed creaming indexes of 96.7 % and 97.3 % after 8 weeks of storage. After ten weeks of storage, the room temperature stored NLCs showed creaming indexes of about 93.2 %–94.2 %, whereas NLCs stored at 5 °C only showed slight creaming (creaming indexes of about 97.6 %–98.4 %). As shown in **figure 2**, the creaming rate of AHP-NLC is somewhat slower than that of blank-NLC at both room and refrigerated temperatures. These results correspond with polydispersity index (PDI) and zeta potential, demonstrating that AHP-NLC is more stable than blank-NLC.

3.3. Wound Healing Evaluation

In the present study, wound healing was evaluated by wound contraction and epithelialization time measurements. The photographs of wound area contraction of rat groups at different time intervals (0, 4, 7, and 13 d) are presented in **Figure 3**. Also, the details of the influence of the NLCs and other formulations on wound area contraction of different groups over 22 days are presented in **Table 2**.

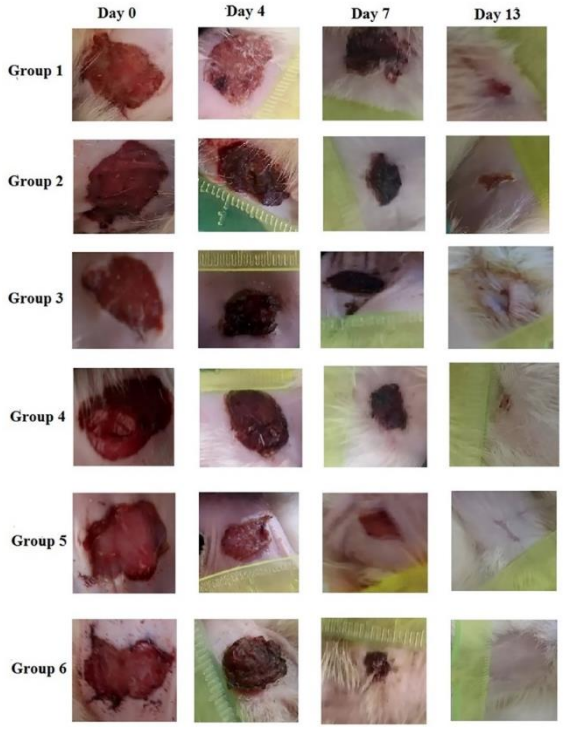

Figure 3. Photographs of the macroscopic appearance of wound closure at different times.

All test-treated rats and the treated group showed a significant reduction in wound size compared to the control group animals ($P<0.05$). The higher wound closure rate of AHP-emulsion than standard drug phenytoin on days 7 ($p = 0.01$) and 10 ($p = 0.064$) following previous studies in which the extracts of *Achillea millefolium, Horsetail,* and *Plantago major* L. exhibited good wound healing activity. Based on previous studies, antibacterial phytoconstituents like 1,8-Cineole, β-thujone, trans-nerolidol, isospathulenol, and cubenol constitute the main components of the essential oils in Iranian *A. millefolium* species, which can accelerate the healing process [28]. Also, the presence of flavonoids and phenolic compounds in *A. Millefolium* extracts promote wound healing activity [29]. *Plantago major* L is mainly known for its wound-healing properties in almost all parts of the world [30]. It is indicated that the polyphenol-rich ethanolic extract of *Plantago major* L accelerates wound healing by stimulating cell proliferation [31]. On the other hand, the wound-healing effects of *Horsetail* can be attributed to the content of antiinflammatory phytoconstituents such as β-sitosterol, campesterol, and isofucosterol.

Table 2: Percentage wound contraction in rats on different days.

Group	Percentage wound contraction (%)					
	Day 4	**Day 7**	**Day 10**	**Day 13**	**Day 17**	**Day 22**
1	21.42 ± 4.38	41.10 ± 1.25	72.70 ± 2.17	84.00 ± 3.88	95.02 ± 1.16	99.77 ± 0.11
2	47.56 ± 5.83*	69.70 ± 6.61*	81.63 ± 9.73	93.93 ± 4.56*	98.40 ± 2.60	100.00
3	41.72 ± 11.61	79.90 ± 3.72*#	92.51 ± 1.36*	97.90 ± 1.40*	100.00*	100.00
4	30.87 ± 9.84#	81.02 ± 5.52*	93.13 ± 2.01	99.20 ± 0.90*	100.00*	100.00
5	40.20 ± 8.12*	86.58 ± 5.33*#	95.57 ± 1.52*#	100.00*#	100.00*	100.00
6	51.01 ± 2.76*	91.00 ± 0.87*#	98.37 ± 2.12*#	100.00*#	100.00*	100.00

Values expressed as mean ± standard error of the mean; *$p<0.05$ compared to the control group; #$p<0.05$ compared to the phenytoin group; Student's t-test made the comparisons.

Table 3: Bioactive phytoconstituents in *Plantago major* L., *Horsetail* and *Achillea millefolium* extracts [29-32].

	***Plantago major* L.**	***Horsetail***	***Achillea millefolium***
Alkaloid	Indication and Plantagonin	Nicotine, Palustrine, and Palustrinine	Achilleine and Moschatine
Flavonoids	Apigenin 7-glucosid, Baicalein, Hispidulin, Hispidulin 7-glucuronide, Homoplantaginin, Luteolin glucosides, Nepetin 7-glucoside, Plantaginin and Scutellarein	Kaempferol glucosides, Quercetin3-O-glucoside, Apigenin, Apigenin 5-O-glucoside, Luteolin, Luteolin 5-O-glucoside, Genkwanin 5-O-glucoside, Isoquercitrin	Apigenin, Luteolin, Quercetin, and Kaempferol with their corresponding glycosides, Artemitin, Casticin, Centaureidin, Chrysoeriol, Jaceidin, Dihydroquercetin, Salvigenin, Rutin, Axillarin and Nevadensin
Phenolic compounds	Caffeic acid, Chlorogenic acid, Plantamajoside and Acteoside	Caffeoy-meso-tartaric acid derivatives, methyl esters of protocatechuic and caffeic acids, 5-Caffeoylshikimic acid, Onitin and oniti-9-O-glucoside, Equisetumosides A-C	Chlorogenic acid, Caffeic acid, Quinic acid, Caffeoylquinic acid derivatives, Cynarin, *p*-Coumaric acid, Ferulic acid, and Choline
Lipids	Myristic acid, Palmitic acid, Stearic acid, Arachidic acid and Behenic acid	Cholesterol, Epicholestanol, 24- methylene cholesterol, Isofucosterol, Campesterol and ß-Sitosterol	β-Sitosterol, Stigmasterol, Campesterol and Cholesterol
Terpenoids	Loliolid, Oleanolic acid, Ursolic acid, 18 ß- Glycyrrhetinic acid, sitosterol, Asperuloside, Aucubin, Catapol, Gardoside, Geniposidic acid, Majoroside, 10-Actoxymajoroside, 10-Hydroxymajoroside and Melittoside	Isobauerenol, Taraxerol, Germanicol, Ursolic acid, Oleanolic acid and Betulinic acid	Achillin, Achillinins A-C, Leucodin, Millifolides A-C, Seco-tanapartholides A and B

Furthermore, β-sitosterol increases angiogenesis, which is believed to enhance *Horsetail*'s effects on wound healing (**Table 3**) [32].

The group treated with AHP-Ghee emulsion showed a slightly greater wound closure than the AHP emulsion group from day 7 to 10, and a significant difference was found on day 13 ($P < 0.05$). This remarkable difference can be attributed to the synergistic effect of ghee and plant extracts, and the same effects have been reported for similar ghee-based formulations [33]. It has been proven that ghee alone shows wound healing activity, and its activity is almost parallel to antibiotics [34]. It is due to the antimicrobial activity of fatty acids and the antioxidant properties of vitamins A and E present in ghee [35].

The wound contraction process in the blank-NLC proceeded slightly faster than that of the AHP-Ghee emulsion group. The highest wound contraction rate was observed in rats treated with AHP-NLC. On day ten, the percentage of wound contraction was significantly ($P<0.05$) greater than all the other groups. However, on day 7, this group showed a significantly ($P<0.05$) greater wound contraction than the control, phenytoin, AHP emulsion, and AHP-Ghee emulsion groups.

The average epithelialization period in all groups and their WC_{50} values are shown in **Figure 4**.

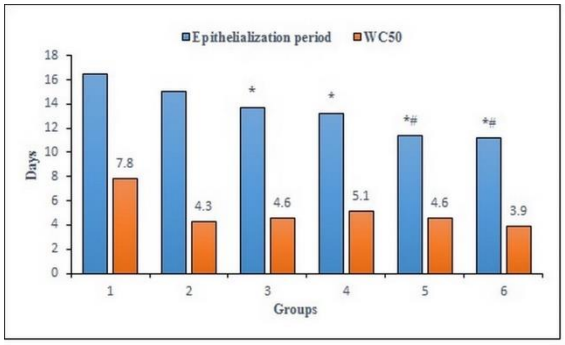

Figure 4: Epithelialization time and WC_{50} values of wound contraction in days. Values expressed as mean ± standard error of the mean; *p<0.05 compared to the control group; #p<0.05 compared to the phenytoin group; Student's t-test made the comparisons.

The time for complete closure of wounds was obtained as 16.50 ± 0.57, 15.00 ± 0.82, 13.67 ± 0.58, 13.25 ± 0.96, 11.40 ± 0.55, and 11.20 ± 2.17 for control, phenytoin, AHP-emulsion, AHP/Ghee-emulsion, blank-NLC and AHP-NLC respectively. The wound treated with the standard drug phenytoin showed a decreased (although not quite significant) epithelialization period compared with the control ($P=0.058$). However, the epithelialization period was significantly shorter in all other groups than in control rats ($P<0.05$). Meanwhile, the epithelialization period significantly differed between AHP-NLC and all other groups except blank-NLC-treated rats ($P<0.05$). As shown in **figure 4**, the WC_{50} values of all treated groups (3.9-5.1 days) were lower than those observed in the control group (7.8 days), and the AHP-NLC group showed the best WC_{50} (3.9 days). These results are consistent with those obtained in wound contraction since AHP-NLC exhibited the highest wound contraction rate.

4. Conclusion

Some medicinal plants possess bioactive compounds with antioxidant, anti-inflammatory, antimicrobial, and angiogenic properties that can promote wound healing. Plant-derived nanoformulations constitute one of the most promising alternatives to conventional drugs in the management of wounds. They not only increase the skin penetration of active phytochemicals because of their high surface area-to-volume ratio but also produce a sustained and controlled release of therapeutics. To our knowledge, no studies have been published on the wound-healing effects of ghee-based nanoformulations. Therefore, this study aimed to develop ghee NLCs to improve the wound-healing effects of ghee-based formulations. Several ghee-based formulations containing natural and plant-derived products have been reported to possess wound-healing properties. The present study used a mixture of *Achillea millefolium*, *Horsetail* and *Plantago major* L. extracts as an active ingredient in formulation. Our study showed that the herb extract blend emulsion

(0.2% of each extract) had better wound-healing effects than phenytoin.

Ghee (3%) and olive oil (2%) improved the emulsion's wound-healing activity. This remarkable difference can be attributed to the synergistic effect of ghee and plant extracts. However, the blended formulation of three extracts was much more efficacious in NLC than its conventional emulsion form. The studies indicated that the positive synergistic effect between ghee and a mixture of *Achillea millefolium*, *Horsetail*, and *Plantago major* L. extracts was significantly improved in NLC formulation. The accelerated wound healing by NLC can be attributed to the nano-sized droplets of NLC, which form a continuous film upon evaporation of water molecules.

On the other hand, these nanoparticles with a large surface-to-mass ratio ensure good surface contact with the stratum corneum, increasing the amount of bioactive phytochemicals penetrating the skin. The results showed that the nanosizing of ghee formulations significantly improved their wound-healing activities. Our finding suggests that ghee-based NLCs have significant potential for use as an alternative formulation for wound healing and other topical applications.

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Conflict of interest

The authors declare to have no conflict of interest.

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