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FORMULATION, CHARACTERIZATION AND STABILITY STUDY OF *AZADIRACHTA INDICA* (NEEM) FRUITS EXTRACT

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ABSTRACT

Herbal medicines have long history of use; they are renewable, cheaper source with better patient tolerance as well as acceptance. Wounding of skin is one of the common dermatological problems. Therefore, there is great need for new topical treatment that can speed up healing, minimize scarring and decreases induction of bacterial resistance. Neem trees as known they have an immunomodulatory, anti-oxidant, anti-inflammatory, antimicrobial and wound healing promoting properties. Hence this study aimed to formulate, evaluate and standardize Neem ointment and Neem gel formulae and also to study their stability. The Dried Neem fruits were extracted and then the highest antimicrobial extract was formulated two formulae of polyethylene glycol ointment and carbopol gel with concentration 3% were prepared. All the quality control tests were done for the prepared Neem formulae such as appearance, pH, content uniformity, viscosity, spreadability, washability and antimicrobial activity. Accelerated stability study test was done. Crude Neem extract, ointment and gel were standardized using high performance liquid chromatography method. This study results shows that the Neem ointment and gel quality Control results complied with the requirements stated in the official American pharmacopeia. Also the stability test results indicate stable formulae with no significant change. Furthermore, the high performance liquid chromatography results indicate the presence of major Neem marker compound. The presence of the Neem chemical constituents such as tannins, terpenoids and flavonoids with its positive antioxidant activity results may support the wound healing activity of Neem.

KEYWORDS: Neem ointment, Neem gel, Antioxidant activity, HPLC and stability study.

INTRODUCTION

Nowadays there a real need to promote the use of traditional herbal remedies by conversion of their raw material into more convenient products instead of synthesis of chemical products. Although there are many scientific approaches are being developed to deliver herbal medicines However, the challenge is to find the effective drug delivery for these herbal medicines.[1]

The topical route has a lot of merits, including the avoidance of systemic toxicity and side effects, the high concentration of drug at the target area and decreased induction of bacterial resistance. Recently the resistance to topical antibiotics has high attention of the dermatologist.[2]

A wide numbers of vehicles ranging from solid to semisolids and liquid preparations are available for topical treatment of the dermatological problems. A semisolid dosage forms are convenient in their formulation and their application.[3]

Wounds and skin damage are important issues to the dermal pharmaceutical and skin care industries. A wound occurs when the integrity of any tissue is compromised (e.g. skin breaks, muscle tears, burns or a bone fracture). A wound may be caused as a result of a fall, a surgical procedure, an infectious disease or an underlying pathological condition. Some diseases like diabetes, immunocompromised conditions, etc. lead to delay in healing. Such conditions need the use of agents to promote the healing process.[4]

MATERIALS AND METHODS

The ripe fruits of the *Azadirachta Indica* were collected from the local area. The fruits were identified and taxonomically authenticated. The carpobol 940 was gifted by General medicine Company. All the chemicals were procured from the local market in Sudan. The extract has been prepared from the dried Neem fruits by continuous extraction method (soxhlet).

Ointment preparation method

The poly ethylene glycol (PEG) was used to prepare water soluble ointment of *Azadirachta indica* fruit extracts (methanolic extract). Two grades of PEG were used, liquid PEG 400 and solid PEG 4000 in different ratios (Table 1.).

The ointment was prepared by the method of infusion. firstly specific amount of PEG 4000 was accurately weighed by electrical sensitive balance, put on the heater until melted and then specific amount of PEG 400 was added and cooled with continuous stirring. Secondly the desired amount of Neem extract was added to the ointment base.

Gel preparation method

The desired amount of carbopol 940 were weighted accurately and Sprinkled slowly into small quantity of distilled hot water (not more than 60 C°) with moderate stirring to obtain uniform dispersion and allowed to soak overnight. Desired amounts of methylparben and propylparben were dissolved in remaining amount of water by gentle heating. Specific amount of propylene glycol and neem extract were added to the above mixture .this was finally mixed with the previously soaked gel formulation. Sufficient quantity of triethanolamine (TEA) was added to neutralize the pH of the gel. Prepared formulations were filled in a suitable container and labeled accordingly. The set-up of gel preparation is given in Table 2.

Evaluation of prepared semisolids

a) Physical examination (organoleptic)

The Neem extract (NE) ointment and gel were prepared by the procedure mentioned above and evaluated for color, odor, consistency texture and transparency.

b) PH

The pH values of 1% aqueous solutions of the prepared ointment and gel were measured by pH meter at constant temperature. The pH meter was calibrated using buffers of pH 4 and pH 7 before measurements.

c) Homogenicity

Ointment and gel were tested for homogenicity by visual inspection after they have been set in the containers. Test was made for their appearance the presence of any aggregates.

d) Viscosity Studies

Viscosity of prepared ointment and gel was measured by using rotational Viscometer. Apparent viscosity was measured at room temperature with rotating spindle no. 6 at 100 rpm.

e) Washability

Formulations were applied on the skin and then ease and extent of washing with water were checked manually.

f) Spreadability

It was determined by two glass slides. One gram of the ointment was placed between slides. Then 50g mass was added onto the upper slide for 5 minutes. The time was noted for upper slide (movable) to separate completely from the fixed slides. Spreadability was then calculated by using the formula: S = M.L / T. Where, S = Spreadability, M = Weight tide to upper slide, L = Length of glass slide, T = Time taken to separate the slide completely from each other. The same test was done for the gel.

g) Skin irritation test

A total of 12 healthy Wistar rats of either sex of average weight 100 grams were used in this study. On the dorsal skin, an area of 4cm was shaved carefully. The animals were divided into two equal groups and labeled. One gram of Neem ointment and Neem gel were applied to the shaved area in group one respectively; same way control ointment and gel were applied to the second group for the determination of irritation characteristics.

The visual observations were recorded at regular interval of 12, 24, 48 hours for various symptoms such as scaling, lesions and erythema.

h) Drug Content uniformity

The gel and ointment content of Neem was determined using HPLC (marker compound) with UV detector at 369 nm.

i) Microbial assay

The antibacterial activities of different formulations were determined by modified agar well diffusion method. In this method, nutrient agar plates were seeded with 0.2 ml of 24 h broth culture of standard *Staph. aureus*. The agar plates were allowed to solidify. A sterile 8 mm borer was used to cut wells of equidistance in each of plates. 0.5 ml of formulations, Neem ointment and Neem gel were introduced into the wells .The plates were incubated at 37 C^{o} for 24 hours. The antibacterial activities were evaluated by measuring the zones of inhibition (in mm). Also the antibacterial activity of the formulae against the clinical isolates of staph. *aureus*. were examined by the same method.

Stability Studies of Neem formulations

The Neem ointment and gel formula were sealed in amber colored bottles with cap covered by aluminum foil and these packed formulations were stored according to the International Conference on Harmonization (ICH) guidelines. They maintained in stability chamber under controlled temperature (40 C° ± 2 Cº) and relative humidity (75% RH ± 5% RH), for three months. The formulations were evaluated before, during and after periodic interval, for changes in appearance, pH, viscosity, spread ability and wash ability.

High performance liquid chromatography (HPLC technique)

Neem extract was characterized using marker compound method.

HPLC condition

Chromatographic analysis was carried out by using C18 column Phenomenex (250×4.60 mm), as the stationary phase and acetonitrile: water with 0.1ml of ortho-phospharic acid (40:60) as the mobile phase. Flow rate and injection volume were 1.0 ml/min and 20 □l respectively. The chromatographic peaks of the analytics were confirmed by comparing their retention time. The system of HPLC was Shimadzu isocratic system with automatic injector. Detection was carried out by UV detector at 369nm. All chromatographic operations were carried out at ambient temperature. Determinations were performed for the extract, ointment and gel samples. All samples were injected in triplicate. This method is carried out in the same conditions as the work of.[5,6]

Preparation of sample solutions:

To prepare stock solution of sample, 1 gm of accurately weighed NE were taken in a 100ml volumetric flask and dissolved in the mobile phase and made up to the mark. From this, the working sample solution was prepared (different concentrations0.025, 0.05, 0.075, 0.1, 1.025w/v). The supernatant was filtered through a 0.45 µm membrane then 20 µl of the filtrate was injected to HPLC automatically. The Neem ointment and Neem gel solutions were also prepared as the same way. Procedure: After setting the instrument 20µl of extract solutions, ointment and gel formula solutions were injected and the chromatograms were recorded.

Antioxidant activity

The antioxidant activity of Neem extract was evaluated using the DPPH radical scavenging assay which was determined according to the method of Shimada,[7] with some modification.

In 96-wells plate, the Neem extract sample was allowed to react with (1,1-diphenyl-2-picrylhydrazyl) stable free radical (DPPH) in dark for half an hour at 37ºC.

The concentration of DPPH was kept as (300µM). The Neem extract sample was dissolved in dimethyl sulfoxide (DMSO) while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using spectrophotometer.

Percentage radical scavenging activity by sample was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate. The total antioxidant activity of the Neem extract sample was estimated as the inhibition percentage and was calculated by using the well-established formula.

RESULTS

Evaluation of prepared Semisolid dosage forms

The ointment preparations were differ in their consistency, F3 was the proper formula contained approximately 2:1of polyethylene glycol 400 and 4000 respectively. It was semisolid stiff, while the other formulae were solid at room temperature.

The gel formula was contained carbopol 940 as gelling agent which is poly acrylic acid polymer forming a three dimensional matrix throughout a dispersion medium or hydrophilic liquid. According to the carbopol gelling agent sensitive pH nature; it is important to adjust the pH at the end by using neutralizing agent such as triethanolamine which is a basic compound. It can neutralize the acidity of the carbomer and enable the carbomer to perfect swelling. The properties of the neem gel and neem ointment are shown in table 3.

Both the Neem ointment and gel was found nonirritant and there was n ′t any sign of irritation or sensitivity. The extract content in the ointment and gel formula was 100% and 95 % respectively, The official range is 85-125% (USP, 2009). The Neem ointment and gel exhibited antimicrobial activity against both standard strains and clinical isolate strains of staph aurous (inhibition zone range 18- 20mm).

Accelerated stability study

The stability test results for neem ointment and neem gel are given in table 4 and 5.

HPLC standardization

The solutions of Neem extract, ointment and gel were injected and their chromatograms were recorded. The chromatograms of different concentrations of neem extract figure 1,2,3,4 and 5. The neem gel and neem ointment chromatograms Fig. 6 and 7. All chromatograms recorded at 369nm in acetonitrile: water with 0.1ml of ortho-phospharic acid (40:60) as the mobile phase.

Antioxidant activity of neem extract

The neem extract was able to reduce the stable, purple colored radical DPPH into discolored DPPH-H. Discoloration occurs due to the decreasing quantity of DPPH radicals in the environment. The discoloration of the DPPH therefore reflects the radical scavenging activity of the analyzed Neem extract.

Table 1: The formula of Neem ointment.

Formula Quantity (w/w)%	Polyethylene 400	Polyethylene 4000	Neem extract gm
F1	50	50	3
F2	60	40	3
F3	70	30	3
F0 (control)	70	30	-

Table 2: The formula of Neem gel.

Formula Quantity (w/w)%	Carbopol 940	Propylene glycol	Methyl parben	Propyl parben	Triethanolamine	Distilled water	Neem extract (gm)
F1′ Gel	2	5	0.15	0.05	Q.S	Q.S	3
F0′ control	2	5	0.15	0.05	Q.S	Q.S	-

Table 3: Shows the prepared semisolids properties

Property / Formula	Organoleptic properties	Viscosity cps	Spreadability mg.cm/s	pH
Neem Ointment	Semisolid, stiff opaque, homogenous light brown and had distinct odor	11950	53.57	6.8
Neem Gel	semisolid, translucent, glossy, smooth, homogenous dark brown and had distinct odor	11900	62.50	**7**

Table 4: Accelerated stability of Neem ointment

Parameter	Initial	After One month	After Two month	After Three month
Organoleptic properties	Semisolid translucent smooth, glossy, homogenous, dark brown, non greasy and distinct odor	No change	No change	No change
PH	6.8	6.7	6.7	6.7
Viscosity	11950cps	11871cps	11894cps	11949cps
spreadability	53.57	68.18	65.21	53.57

Table 5: Accelerated stability of Neem gel.

Parameter	Initial	After One month	After Two month	After Three month
Organoleptic properties	Semisolid translucent smooth, glossy, homogenous, dark brown, non greasy and distinct odor	No change	No change	No change
PH	7	6.9	6.9	6.9
Viscosity	11900cps	11876cps	11884cps	11899cps
spreadability	62.50	68.18	65.78	62.50

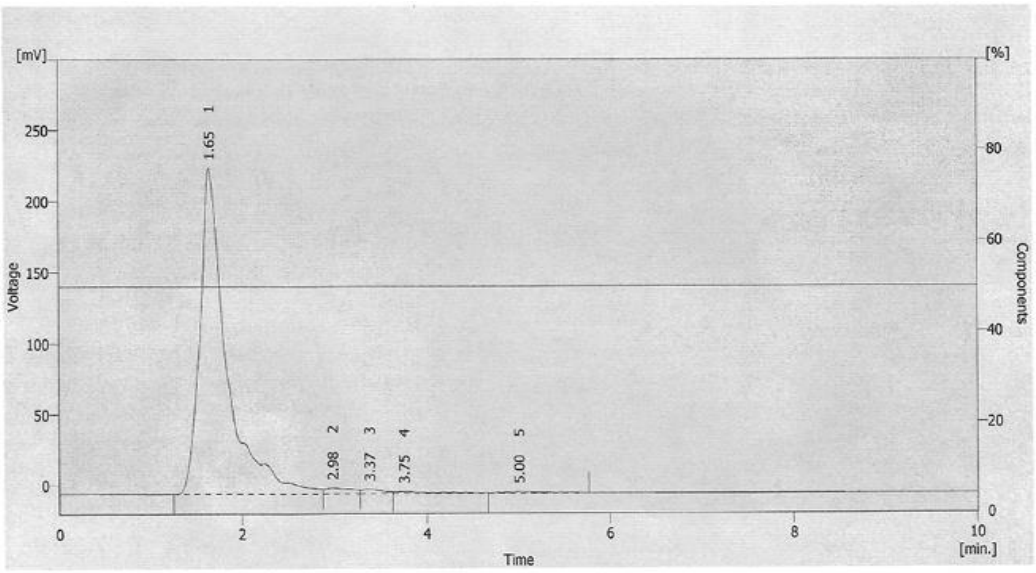

Fig. (1): HPLC chromatogram of neem extract.

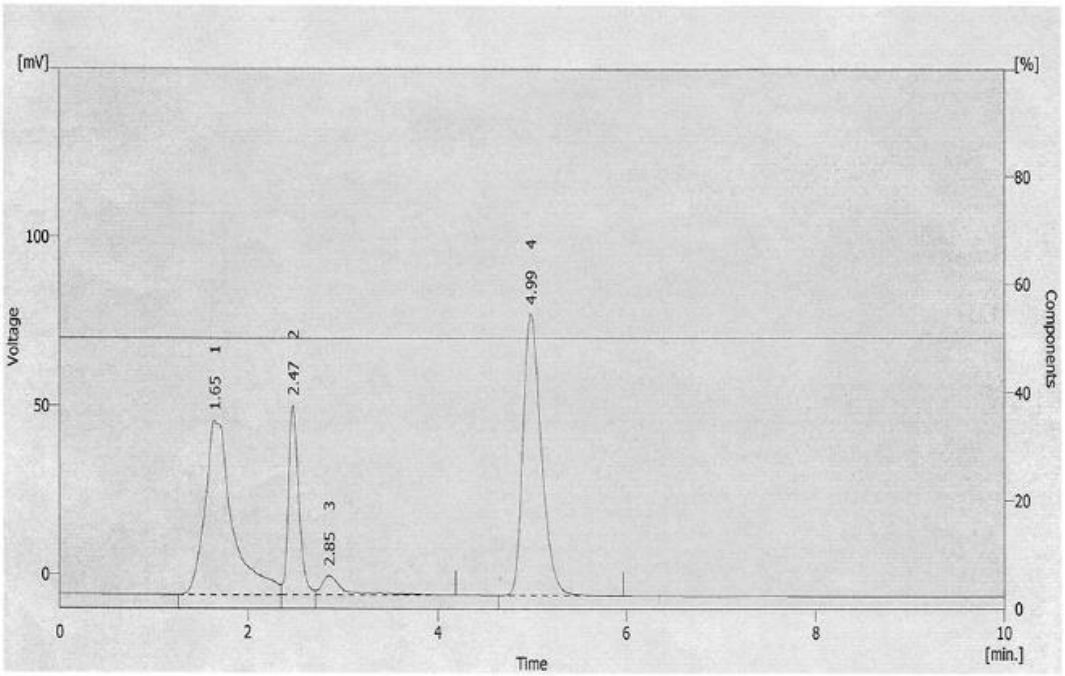

Fig. (2): HPLC chromatogram of neem gel.

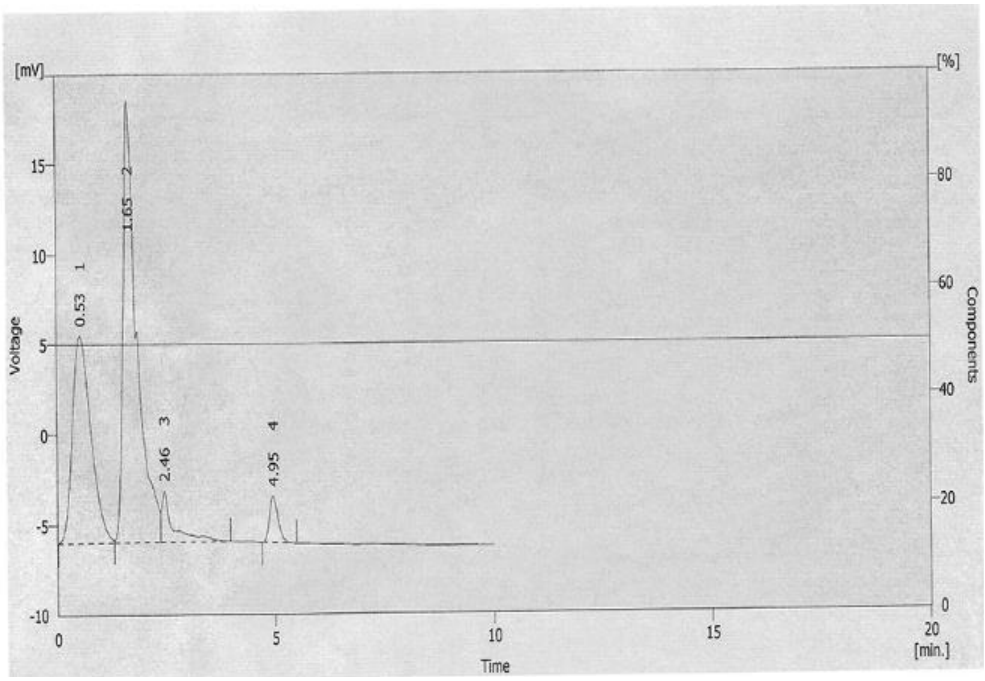

Fig. (3): HPLC chromatogram of neem ointment.

DISCUSSION

The main goal of this study was to formulate a simple, effective, compatible, inexpensive formula. The preformulation studies had been done to choose the most proper formula according to the physiochemical properties of the Neem extract. The water miscible polyethylene glycol ointment and carpbopol hydrogel were chosen because they are compatible with the wound nature and condition.[8]

The addition of 2% carbopol 940 in the gel formula will enhance the honeycomb structure; this structure was stronger than irregular fibrous network structure. Hence the viscosity property of the gel system will increased.[9] The high concenteration of carbopol in the solution would enhance free –COOH group which formed hydrogen bonding with the surface. However it will enhance the bioadhesive properties of Neem gel, so it will prolong the residence time of the gel on the skin.[10]

The penetration rate of drugs through the stratum corneum can be increased with appropriate vehicles and transdermal penetration enhancers. The propylene glycol can alter rheology and drug delivery characteristics of topical Neem gel owing to its viscosity and due to the change in solvent-polymer and solvent-solvent interactions. By this way it can increase the solubility of the active ingredient and as a result enable the gel formula to deliver maximum amount of the drug.[11]

The neutralizer triethylamine (TEA) has a Binding ability to poly acryl acid (PAA) polymers better than Tromethamins (TRIS) and NaOH so it gives a higher degree of polymer chain expansion.[12] The ointment and gel had very slightly acidic to neutral pH which was Compatible with normal human skin physiology.[13]

The distinct odor of the Neem ointment and gel refer to the presence of Neem terpenes compounds.[14] The Neem formulae have a suitable viscosity, at rest‘‘(in its container) and as they are sheared during application.[15] The spreadability plays an important role in patient compliance and helps in uniform application of gel and ointment to the skin.[16] The antimicrobial activity of the neem ointment and neem gel indicated the good release and diffusion of the Neem extract from the formula.[17]

As the results presented in Tables (1) and (2) shown no significant changes in physicochemical properties of the formulae even after its exposure to accelerated conditions of temperature (40 ± 2C^{o}) and humidity conditions (75 ± 5%RH), hence, the formulae were found to be stable after subjecting to accelerated stability conditions.[18]

The methanolic extract of neem fruits, ointment and gel were standardizing by marker compound method. The chromatograms of the different neem extract solution reveal the presence of different retention times which represent different compounds .the peak area of each compound increased as the concentration of the neem extract increased.

The chromatograms of the neem extract, ointment and gel for the same concentration reveal peaks which appear at the same retention times (fig. 1, 2 and 3).[6,19]

The single sharp peak at retention time 4.99 is reported by Kumar,[20] as immunostimulant glucosamine neem compound.

The methanolic neem extract had radical scavenging activity. This antioxidant activity mainly depends on the hydroxyl groups existing in the phenolic compounds, such as flavonoids, alkaloids, terpenoids and their derivatives. All these compounds were present in the neem fruits crude extracts as shown in the phytochemical screening results.[21]

The neem phenolic compounds possess astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialization, so improve wound healing process.[22]

CONCLUSIONS

The study deals with the preparation and evaluation of Neem ointment and Neem gel for wound healing. They were prepared with concentrations 3% (w/w) by fusion method using different excipients. The Neem formulae were evaluated for their quality and stability. The extract and the formulae were characterized using HPLC technique.

The gel and ointment had acceptable appearance, pH, viscosity, spreadability and drug content values. Also they were stable and had no change in their tested parameters.

Hence, the synergistic effect of Neem antimicrobial and antioxidant activity as well as being able to increase the rate of wound contraction accelerated the wound-healing process. This result supports the traditional use of neem for wound management. Both Neem formulas has high wound healing effect as it promotes wound contraction and shortens epithelisation period more than the crude extract.

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