IJBPAS, February, 2026, 15(2): 495-505

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

FORMULATION AND EVALUATION OF TOPICAL NANOEMULGEL FOR DIABETIC WOUND HEALING BY USING TRIGONELLA FOENUM GRAECUM AND AEGLE MARMELOS LEAVES EXTRACT

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Received 13th Sept. 2024; Revised 25th Nov. 2024; Accepted 10th Jan. 2025; Available online 1st Feb. 2026

https://doi.org/10.31032/IJBPAS/2026/15.2.9462

ABSTRACT

Aim: Formulation and Evaluation of Topical Nanoemulgel for Diabetic Wound Healing by using Trigonella Foenum Graecum and Aegle Marmelos Leaves extract.

Methods: O/W nanoemulsions were prepared by the ultrasonication method. F_1 to F_7 different formulations of combination of *Trigonella Foenum Graecum* [TFG] and *Aegle Marmelos Leaves* [AML] extract loaded nanoemulsions were prepared successfully and evaluated. From evaluation study best nanoemulsion formulation was selected and further it was incorporated into 1% and 1.5% Carbopol to create a gel that would make applying medication topically more convenient.

Results and Discussion: The nanoemulsion area was determined by using pseudoternary phase diagrams. *TFG seed oil* choosen as oil phase, Tween 80 and Propylene glycol used as surfactant and cosurfactant respectively. The nanoemulsion formulations were evaluated for physical appearance, particle size, zeta potential, rheology study, In-vitro permeation study. From this evaluation F_3 formulation was selected for incorporate into the gel base to form Nanoemulgel. The nanoemulgel formulations were evaluated for rheological study, drug content determination, In-vitro permeation study, Anti-microbial activity; In-vitro wound healing activity. Anti-microbial activity of prepared nanoemulgel was compared with conventional gel. The wound healing capabilities of nanoemulgel was assayed by performing In-vitro cell migration studies on L929 cells and compared with standard drug cipladine. The % of cells migrated towards the wound of nanoemulgel and cipladine were found to be 37% and 81% in 48 hrs.

Conclusion: Study concluded that topical nanoemulgel of TFG and AML Posses an effective Anti-microbial activity and it showed moderate wound healing activity.

Keywords: Wound, Diabetic wound, Nanoemulsion, Trigonella foenum graecum, Antimicrobial activity, wound healing activity

INTRODUCTION:

Ayurveda is known as the "Goddess of All Healing" in Indian culture, Numerous plant extracts and their phytoconstituents have been identified as a potentially effective substitute for medicines that promote wound healing because of their wide range of active ingredients, accessibility, and low toxicity **[1]**. Many medications nowadays are made from plants, and they work incredibly well to treat a variety of fatal illnesses. The majority of the chemicals that are separated from plants are either modifications or active components that are specifically present in that medicinal plant **[2]**. **Diabetic wound:** A collection of metabolic diseases known as diabetes mellitus (DM) are typified by impaired insulin synthesis and/or function, which results in hyperglycemia **[3]**. Actually, a diabetic wound is one type of chronic wound, but it is found in diabetic patients only, so, it is separated by other types. In this type of wound, bacteria can easily attack on the tissue part or the cells, which can contaminate easily. Most of the time, germs can still cause wounds to heal **[4]**. **Nanoemulgel:** Better skin penetration is influenced by the insertion of a nanoemulsion system poured into the hydrogel matrix, a process known as nanoemulgel, or the production of nanoemulsion based on hydrogel. Many scientists are interested in using this combination of nanomulgels to create a variety of medications which can be used to treat various types of skin conditions **[5]**.

MATERIALS AND METHODS:

Materials: TFG seed oil was gifted by Truelixir Biotech Pvt.Ltd., Dewas, Madhya Pradesh. AML Extract was purchased from Shamantak Enterprises, Pune. Tween 80, Carbopol 934, and Light Liquid Paraffin oil, Glycerin were purchased from Loba Chemical Mumbai, Propylene Glycol, Triethanolamine and Methyl Paraben were purchased from Ozone International, Mumbai. In this work, analytical reagent-grade chemicals and solvents were all employed; freshly prepared distilled water was also used.

Methods: 1) Solubility study: The solubility study of TFG (oil) and AML (extract) was determined by using different solvents.

2) Phytochemical Analysis: The Phytochemical Analysis was done for both API's by using different methods to determination the presence of different

phytochemicals like alkaloids, flavonoids, carbohydrates, saponins, amino acids, reducing sugars, etc. **[6, 7]**

3) Compatibility Study:

a] Fourier Transform Infrared Spectroscopy (FT-IR): The FTIR Spectrophotometer recorded the IR spectrum of TFG oil and AML extracts. The spectrum was evaluated over the 4000-400 cm^{-1} frequency range.

b] Pseudo-ternary phase diagram study: To create a nanoemulsion formulation with a clear look, an initial investigation was carried out. Physical homogeneity and easy flow ability using the aqueous titration method. The drug was chosen as the oil phase. As per their emulsification capacity for the system, Tween 80 and propylene glycol were chosen as surfactant and co-surfactant (Smix). Distilled water was used as an aqueous phase for the phase diagram to be constructed to determine the existence zone of the nanoemulsion. To create pseudoternary phase diagrams, a mixture with surfactant: co-surfactant used for titrations is 1:1, 2:1, 3.5:1, 4:1. The mixtures were titrated with distilled water, drop wisely using a burette until the onset of turbidity, and the end point was visually controlled against a dark background. From the end point, ternary.com software was used to compute and plot the compositions of the titrated samples on pseudo-ternary phase diagrams **[8, 9]**.

4) Preparation of Nanoemulsions of TFG (oil) and AML (extract): Different formulations of the O/W nanoemulsions were formulated by using the ultrasonication method of the above API's. Firstly, the organic phase was developed by dissolving TFG oil and AML extract (API's) in combination with Tween 80, propylene glycol and glycerin at 400 rpm. Then water is added as an aqueous phase in the organic phase to formulate coarse nanoemulsion by continuously stirring at 400 rpm and by using a 50 kHz sonicator, the coarse emulsion is subjected to ultrasonic emulsification **[10]**.

5) Manufacturing formula of nanoemulsion formulations:

Table 1: Manufacturing formula of nanoemulsion formulations

Formulation code	% Drug (D_{mix})	Light Liquid Paraffin Oil	% Tween 80	% Propylene Glycol	% Glycerin	% Water
F_1	5 (1:0)	-	40	10	8	37
F_2	5 (0:1)	5	40	10	8	32
F_3	8 (1:1)	-	40	10	8	34
F_4	8 (1:2)	-	40	10	8	34
F_5	8 (2:1)	-	40	10	8	34
F_6	8 (3:1)	-	40	10	8	34
F_7	8 (1:3)	-	40	10	8	35

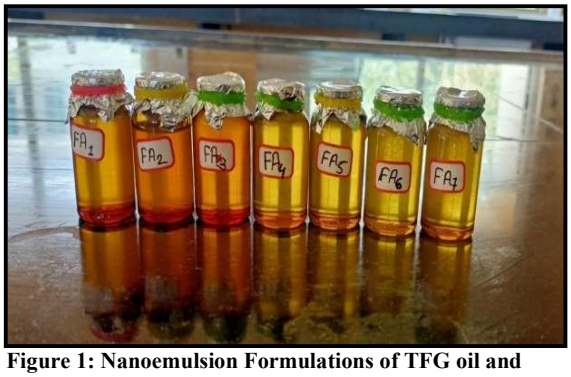


Figure 1: Nanoemulsion Formulations of TFG oil and AML Extract

6) Evaluation of Nanoemulsion Formulations:

a] Physical appearance: The prepared formulations were observed visually for their color and appearance.

b] Particle size and zeta potential measurement: nanoemulsion particle size additionally zeta potential were measured at 21° C temperature.

c] Rheology study: The nanoemulsion viscosity of various formulations was evaluated by a Brook-field type rotary viscometer with spindle 62 at 10 rpm for 3 min.

d] *In-vitro* permeation study: Studies of *in-vitro* drug release were conducted using a Franz diffusion cell. Nanoemulsion formulations were drawn from each formulation (F_1-F_7) and applied the surface cellophane membrane, which placed in between the donor and receptor compartments. As a dissolution medium phosphate buffer pH 6.8 was used. The dissolution cell temperature was maintained at 37°C. This entire assembly was held on a magnetic stirrer. Samples (2ml) were removed at appropriate time intervals and diluted with the same solvent up to 10 ml, replacing them with new dissolution media in equal quantities. Samples were spectrophotometrically evaluated at 288 nm and the permeation drug cumulative was calculated **[11, 12, 13]**.

7) Preparation of carbopol gel: Carbopol gels were prepared using distinct concentrations, 1%, 1.5% W/V carbopol in triple distilled water. Carbopol was dispersed into the distilled water for 2 hours, to socked completely. After that triethanolamine and methyl Paraben were added into it, and the gel was homogenized for 2 hours at 600rpm. After that carbopol gel was subjected to expulsion the trapped air bubbles from the prepared gel by two cycles of sonication for 15 min **[14, 15, 16]**.

8) Manufacturing formula for carbopol gel:

Table 2: Manufacturing formula for carbopol gel

Formulation code	Carbopol 934 (%)	Triethanolamine (%)	Methyl Paraben (%)	Water
G_1	1	1	0.3	Q.S
G_2	1.5	1	0.3	Q.S

9) Evaluation of carbopol gel:

a] pH determination: Prepared gel was brought into the 250 ml beaker. A digital pH meter was immersed in it and recorded the readings.

b] Rheology study of carbopol gel: The Brook-field type rotary viscometer was used with spindle 64, the viscosity of carbopol 934 was evaluated at 10 rpm for 3 min.

10) Preparation of Nanoemulgel Formulation:

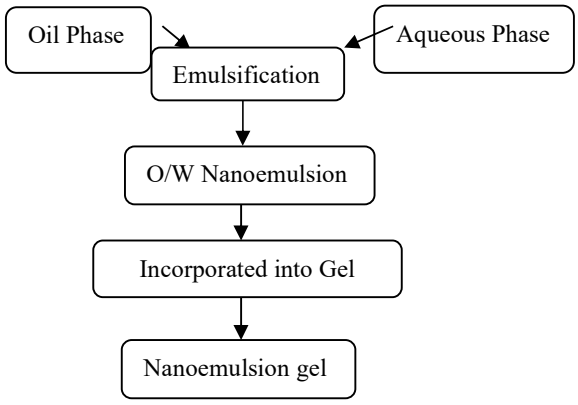


Figure 2: Flow Chart of Preparation of Nanoemulsion Gel

11) Evaluation of Nanoemulsion gel:

a] Physical appearance: The prepared nanoemulsion gel formulation evaluated visually for its color, homogeneity, grittiness, and phase separation.

b] pH determination: 250 ml of beaker were taken and Nanoemulgel was poured into the beaker, and a digital pH meter was immersed in it and the reading of pH meter was recorded. A similar procedure was done three times and the mean was calculated.

c] Rheology study: The Brook- field type rotary viscometer was used to determine the viscosity of the nanoemulsion gel with spindle 64; the viscosity was evaluated at 10 rpm for 3 min.

d] Swelling Index: On porous metal foil, 1 g of gel was collected and then placed separately in a 50 ml beaker with 10 ml of 0.1N NaOH. The sample was then removed at different time intervals, dried it, and reweighted.

e] Drug content determination: An equivalent of 10 mg of drug from the nanoemulgel formulation was dissolved into 10 ml solvent then filtered by whatman filter paper. Further dilutions were built by using the same solvent. Measured absorbance by using UV spectrophotometer and determined the drug content.

f] *In- vitro* Permeation Study: The Franz Diffusion (FD) cells were used for the *In-vitro* drug permeation investigations. The formulation was applied to the surface of the cellophane membrane. As a dissolution medium, phosphate buffer pH 6.8 was used. This entire assembly was held on a magnetic stirrer, and a magnetic bead continually stirred the solution. The sample was removed at appropriate time intervals, diluted with the same solvent, and replaced with an equal amount of fresh dissolution media. Samples were spectrophotometrically evaluated at 288 nm, and the cumulative drug permeation was calculated **[17, 18]**.

g] Anti-microbial study: The bactericidal activity of the nanoemulgel, plain drug, and conventional gel was investigated using agar well diffusion method studies. This study was done by using solidified agar media, which wells contain plain drug, nanoemulgel, and conventional gel and the bacterial suspension was poured into the Petri dish. Incubated for 24 hr and the zone of inhibition was observed **[19]**.

h] Kinetic release study: The *in-vitro* release curves of models were subjected to a linear regression analysis in order to ascertain the release kinetics: zero order, first order, and second order by using rate law. Matrix models, Korsemeyer-Peppas model were determined by using PCP-Disso-V_3 Software **[20]**.

i] *In-vitro* Wound Healing Activity (Scratch assay): The wound healing capabilities of nanoemulgel was assayed by performing *invitro* cell migration studies on

L929 cells by a previously described method. Briefly, $2x10^5$ cells/mL were seeded in 6 well plates and cultured overnight. Cells were then washed with Delbucco's phosphate buffered saline (DPBS), and a scratch was made with a sterile 200 µl tip. The detached cells were treated with 100 µl of sample N-RW and 5 µg/ml of the positive control, Cipladine, and incubated for 24 hrs. The cell migration and morphological changes of cells were observed in the images taken by an inverted microscope, with a digital camera, the experiments were performed in triplicate (n 1/4 3). The width of the scratch and wound closure at different time intervals (48 hrs.) was analyzed by SAGLO software **[21]**.

RESULTS AND DISCUSSION:

1] Solubility study: The best solubility of TFG oil and AML extract was found in solvent that was formed by combining of methanol and chloroform in 9.5: 0.5 ratios.

2] Phytochemical analysis:

a] Alkaloids: Green color was formed.

b] Flavonoids: greenish yellow color was appeared.

c] Saponins: After 15 min. Foam layer was formed on the mixture.

d] Carbohydrates: At the junction of two liquids violet color ring were indicated.

e] Reducing sugars: Green color was appeared.

f] Amino acids: A dark violet color was appeared.

g] Proteins: The colors were changed to violet color, which confirmed the presence of proteins.

3] UV λmax: The wavelengths of the TFG oil and AML extracts were determined by using the UV-spectrophotometer, and the λmax was found to be 288 nm and 282 nm.

4] Fourier Transform infrared spectroscopy (FT-IR):

a] FT-IR of TFG oil:

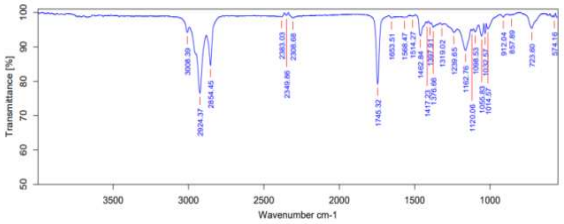


Figure 3: FT-IR spectra of TFG oil

b] Interpretation of FT-IR spectra:

Table 3: Interpretation of FTIR spectra of TFG Extract (oil)

Functional Groups	Actual IR Range	Observed IR Range of TFG oil
C-H	3000-2850	2924.37
C=O	1750-1730	1745.32
C=C	1680-1600	1653.51
C-O	1300-1000	1162.76
H-C-H	1465-1460	1462.84
C-Cl	785-540	723.60

c] FT-IR of AML extract:

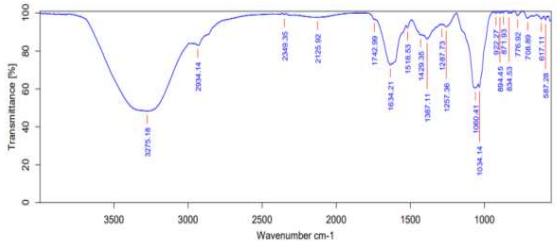


Figure 4: FT-IR spectra of AML extract

d] Interpretation of FT-IR spectra:

Table 4: Interpretation of FTIR spectra of AML Extract

Functional Groups	Actual IR Range	Observed IR Range of AML (extract)
O-H	3400-3200	3275.18
C-H	3000-2850	2934.14
C≡C	2250-2100	2125.92
C=O	1750-1730	1742.99
C=C	1680-1600	1634.21
C-O	1300-1000	1060.41
C-Br	<667	617.11

5] Pseudo Ternary Phase Diagram:

In present composition of nanoemulgel formulation, the proportion 4:1 gives the best or more regions, therefore this ratio of surfactant were selected for the preparation of the nanoemulgel.

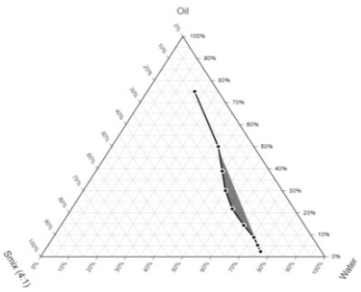


Figure 5: Pseudo ternary phase diagram of 4:1 ratio

Evaluation of Nanoemulsion:

1] Physical appearance: Formulations were observed visually, which were transparent yellow in color.

2] Particle size and zeta potential: The particle sizes of the formulations were in range of 1-100 nm. Negative zeta potential shows greater stability.

Table 5: Particle size and Zeta potential of Nanoemulsion Formulations

Sr. No.	Formulation Code	Particle size (nm)	Zeta potential (mv)
1.	F_3	7.39	-15.7
2.	F_6	8.64	-14.5

3] Rheology Study: The viscosity of nanoemulsions was determined by a Brookfield viscometer using spindle number 62, which was found to be between 4021 to 4481 cp.

4] *In-vitro* permeation studies: The *In-vitro* permeation study showed best permeation through the cellophane membrane of the formulations. F_3 formulations flux was found to be 70.83 $\mu g/cm^2/hr$.

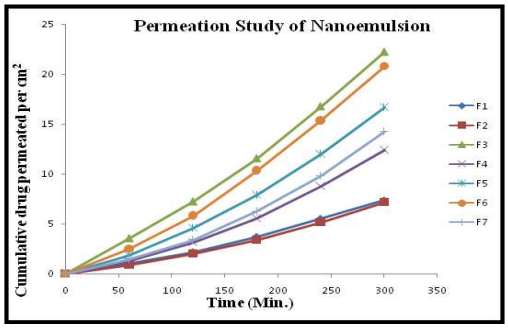


Figure 6: In-vitro Permeation Study of Nanoemulsion Formulations

Evaluation of Gel:

1] pH Determination: The pH of Gel was found to be 6.3 and 6.5. This pH of plain gel in range 6 to 6.5, shows a normal pH range of the skin, which does not produce any skin irritation.

2] Rheology study of gel: The viscosity of plain gel was determined by a Brookfield viscometer using spindle number 64, which was 5361 and 5619 cp. The viscosity of gel depends on the conc. of carbopol 934.

Evaluation of Nanoemulsion Gel:

1] Physical appearance:

Table 6: Physical appearance of nanoemulgel and conventional gel formulations

Formulation code	Color and appearance	Phase separation	Grittiness	Homogeneity
Nanoemulgel	Transparent yellow	No	No	Homogeneous
Conventional gel	Transparent yellow	No	No	Homogeneous

2] pH determination:

Table 7: pH of the Gel formulations

Formulation code	pH
Nanoemulgel	6.2
Conventional gel	6.6

3] Rheology study: The viscosity of the gel formulations was determined by a

Brookfield viscometer using spindle number 64. The viscosity of gel varies depending on the concentration and nature of gelling and emulsifying agents. The viscosities of nanoemulgel and conventional gel were found to be 4945 and 5120 cp.

4] Swelling index: The swelling index of nanoemulgel and conventional gel was found to be 4.2 and 5.

5] Drug content: The drug content of nanoemulgel and conventional gel was found to be 95.6% and 89.8%, respectively.

6] *In-vitro* Permeation Study: *In-vitro* Permeation of the nanoemulgel through the cellophane membrane was determined, and the flux of the nanoemulgel and conventional gel was found to be 62.06 and 59.19.

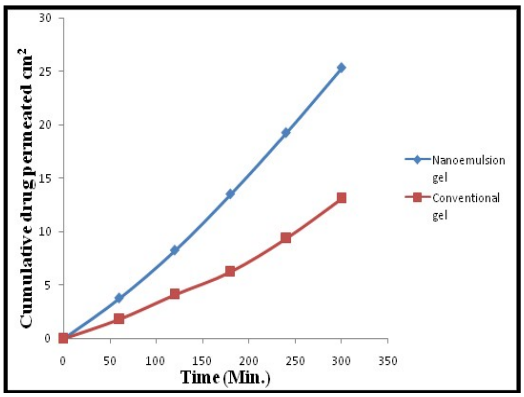


Figure 7: In-vitro permeation study of nanoemulgel formulation

7] Anti-microbial activity: Nanoemulgel formulation inhibits bacterial growth (fig. No.8.18). Most of the bacterial growth was inhibited by the nanoemulsion gel as compared to the conventional gel as well as the plain herbal drug (oil).

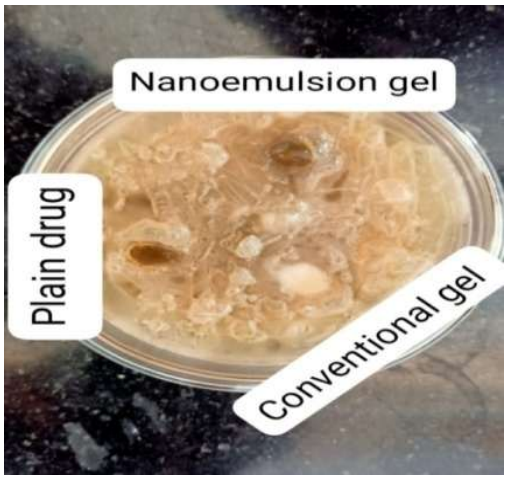


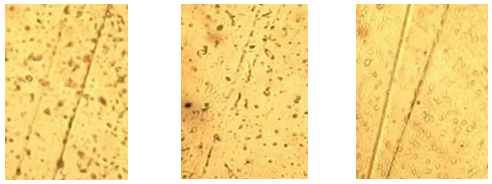
Figure 8: Anti-microbial activity of components

8] Release kinetic study: The study of release kinetics is important, for better understanding the efficiency of the nanoemulgel. Determining the release characteristics is aided by choosing an appropriate kinetic model to fit the nanoemulgel release data. According to the data on the profile of nanoemulgel, it best fits to zero order kinetic model (R^2 = 0.9961). High linearity of the plots was achieved. It means that the nanoemulgel is released quickly with constant rate, dependent on the initial drug concentration in the nanoemulgel. The nanoemulgel release profile could also be best explained by the Korsmeyer-Peppas model, regression line is characterized by a higher R^2 value (R^2 = 0.9949).

9] *In-vitro* Wound Healing Activity (Scratch Assay): The wound healing capabilities of the nanoemulgel sample were assayed by performing *In-vitro* cell migration studies on L929 cells.

Table 8: Percentage (%) of cells migrated towards the wound and were involved in wound closure

Formulation Code	Time		
	0 hrs (mm)	24 hrs (mm)	48 hrs (mm)
Control	00 %	42 %	44 %
Standard Cipladine (5 µg/ml)	00 %	78 %	81 %
Nanoemulgel (100µL)	00 %	34 %	37 %



Control Standard Nanoemulgel (N-RW)

Figure 9: Percentage (%) of cells migrated towards the wound and involved in wound closure

Microscopically, images representing the *In-vitro* wound healing nature of nanoemulgel: L929 cells were incubated in the presence or absence of nanoemulgel and the standard drug Cipladine, and images were captured at different time intervals up to 48 hrs. According to the images, the sample of nanoemulgel formulation shows moderate activity.

CONCLUSION:

In the present work, a nanoemulgel of TFG *(oil)*, and AML *(extract)* was formulated by the ultrasonication method and evaluated. Finally, In comparison to Standard gel, it can be concluded that designed nanoemulgel formulations loaded with TFG (oil) and AML (extract) may be generated with the right size, maximum drug content, and improved transdermal flow. Also, it concluded that the herbal nanoemulgel shows moderate activity for wound healing at a medium amount of herbal drug usage, so it needs to increase concentration of drug to identify an appropriate dose of the TFG oil and AML extract for maximum healing of the wound.

ACKNOWLEDGEMENT:

We are thankful to Truelixir Biotech Pvt. Ltd., Dewas, Madhya Pradesh for providing gift sample of TFG seed oil. We are also thankful to the principal, Dr. V. V. P.Fs, College of Pharmacy, Vilad Ghat, Ahmednagar, Maharashtra, India, for providing laboratory facilities for research work.

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