Novel insight into Potential Leishmanicidal Activities of Transdermal Patches of *Nigella Sativa*: Formulation Development, Physical Characterizations and *In vitro In vivo* Assays

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Abstract

Cutaneous Leishmaniasis (CL) is the most common type of Leishmaniasis which annually affects 1.5 million people worldwide. About 90% of cases are reported from countries such as Iran, Afghanistan, Pakistan, Iraq, and Saudi Arabia. The purpose of the present study was to fabricate transdermal patches of *Nigella sativa* (NS), characterize and to check its *in vitro in vivo* anti-*Lieshmanial* activity. Hydroalcohlic extract was analyzed for preliminary phytochemicals. Five formulations of transdermal patches (NS1, NS2, NS3, NS4 and NS5) were prepared by solvent evaporation method. The optimized formulation NS5 was characterized for FTIR, smoothness, brittleness, clarity, thickness, folding endurance, uniformity of weight, percent moisture content, *in-vitro* drug release, release kinetics, *ex vivo* drug permeation and *in-vitro anti-Lieshmanial* activity. *In vivo anti-Lieshmanial* activity was assessed in 30 patients (n = 30) suffering from CL. The FTIR studies showed no incompatibility among the active extract and polymers. *In vitro anti-Lieshmanial* assay was 194.6 ± 1.88 % as compared to standard drug ($p > 0.05$) and *in vivo anti-Lieshmanial* activity was 75 %. The drug release after 24 hours was 87.0 ± 0.94% in NS5 which showed non-Fickian diffusion mechanism while drug permeation across rabbit skin after 24 hours was up to 80.0 ± 0.91%. The results concluded that problems related to the medications parenterally used for *Lieshmanial* treatment can be managed by applying extract of *Nigella sativa* seeds in the form of transdermal patch.

Introduction

Leishmaniasis is a common disease throughout tropical, sub-tropical and temperate regions of the America, Europe, Asia, and Africa, with an estimated 12 million people infected and more than 350 million people at risk.[1] It is vector-borne disease caused by a protozoan endo-parasite species belonging to the genus *Leishmania* that live in the blood and tissues of the host, its mode is through the bite of Phlebotomine sandflies.[2]

Cutaneous *Leishmaniasis* (also known as oriental sore, tropical sore, chiclero ulcer) is the most common form of *Leishmaniasis* affecting humans. There are about twenty species of *Leishmania* that may cause cutaneous *Leishmaniasis*.[3] It is a public health burden in Pakistan. The cases of CL have surged since the end of 2018 in the North West province Khyber Pakhtunkhwa (KPK). According to the health authorities about 28000 cases of CL have been reported since Nov, 2018. These cases were mostly from the newly merged districts of FATA which have borders with Afghanistan. A recent upsurge in the province's southernmost districts, particularly in South Waziristan, has driven people to the neighboring district of Bannu or even to the provincial capital, Peshawar, for treatment.[4]

Not a single ideal and best treatment or vaccination is available for the different clinical forms of *Leishmaniasis*. A number of drugs available for the treatment of *Leishmaniasis* include Pentavalent antimony, Paramomycin, Liposomal amphotericin B, Funconazole and the under-trial drug such as Miltefosine but all these drugs have severe nephrotoxicity due to which its long term use is risky.[5, 6]

For CL there is a need of topical dosage form which can be applied on the skin lesions to avoid the systemic side effects of usual drugs. A new technology developed for release of drug at a controlled rate into systemic circulation through skin is very important for *CL* to avoid the systemic side effects of usual drugs, such innovation includes transdermal patches which can be used for achieving efficient systemic effect by passing hepatic first pass metabolism and increasing the fraction absorbed. The transdermal patches provide continuous drug release through intact skin into the systemic blood stream during a prolong time at a preset rate. This dosage form came into pharmaceutical companies since 1990.[7,8] The examples of marketed transdermal patches in pharmaceuticals are cardiac medicines i.e., nitroglycerine, estrogen patches i.e., hormones, thermal and cold patches, nutrient patches, skin care patches are non-medicated patches.[9] This system provides many clinical advantages as compared to oral and parenteral system. In short transdermal patches are more safe, easy to apply, cheap, painless, and hence leads to positive patient compliance but only some drugs show well delivery across the skin so use of transdermal patches are limited in pharmaceuticals.[10, 11] To avoid all the negative effects of *anti-leishmanial* drug therapy, there is need to evaluate and use herbal extract of medicinal plants in transdermal patches which will be a very good solution of the problem.[12]

Nigella sativa is an annual flowering plant. The black colored seeds are flattened, oblong and angular, funnel shaped, with the length of 0.2 cm and 0.1 cm wide *Nigella sativa L*, which belongs to the *Ranunculaceae* family.[13] These are also used as a carminative and diuretic by oriental people.[14] The seeds are sold in the markets to be used as a condiment and native medicine. Its main chemical constituents are Thymoquinone (TQ), dithymoquinone, fixed oil (32–40 %), saponin (Mohammed & Arias, 2016). The pharmacological activity of *Nigella sativa* is because of quinine constituent, of which TQ is the most part bottomless (Chehl *et al*., 2009), TQ possess anticonvulsant activity antioxidant, anti-parasitic, anti-inflammatory, anti-cancer, antibacterial and antifungal activity.[15]

Based on the above justification, the present study was designed with the aim to formulate and characterize transdermal patches of *Nigella sativa* extract for possible anti Lieshmanial activity *in vitro* and *in vivo.*

Materials And Methods

Methanol, HPMC, Ethyl Cellulose, PVP, PVA (polymers), Propylene glycol, Tween-80, DMSO were purchased from Sigma Aldrich, Germany. Nigella sativa seeds Extract were purchased from local market of D. I. Khan, Pakistan. Benedict's reagent & Wagner's reagent (Iodine Reagent), Chloroform and Distilled Water were obtained from Research Lab, Faculty of Pharmacy, Gomal University, and D.I.Khan, Pakistan. Ether, ethanol, HCL, Sulphuric Acid, Ammonia, Ferric Chloride, Aluminium Chloride, NNN growth medium, fetal bovine calf serum, Gallic acid & Folin and Ciocalteu's reagent, Silica were purchased from Hajwari Chemicals, Lahore, Pakistan.

Methods

Extract Preparation

Seeds of *Nigella sativa* were collected from the local market of D.I.Khan KPK Pakistan and relevant permits/permissions/licences were obtained. These were identified by Dr. Mushtaq Ahmad from "Department of Plant Sciences" of Quid I Azam University, Islamabad Pakistan. These seeds were shade dried, crushed in electric grinder and then the seeds powder was macerated in 50 % methanol and distilled water for 5 days with occasional stirring after every 12 h.[12] The macerated seeds granules were filtered through a Muslin cloth for coarse filtration and then filtered through a Whatman # 01 filter paper for clear extract, followed by evaporation at 40ºC in rotary evaporator (Rotavapor®, R-215, Germany). The extract obtained was collected in glass jars and stored in freezer at 0°C.

Phytochemical Screening

Phytochemical screening was performed using standard procedure for alkaloids, saponins, flavonoids, terpenoides (Salkowski test), tannins and reducing sugars.[15–17]

HPLC Analysis

HPLC method was developed for the quantification of extract. Briefly, C18 reversed-phase analytical column (25 x 4.6 mm, 4.6mm particle size) was used. 500 µl of the extract was diluted with mobile phase to get final concentration of 10µl/ml. HPLC conditions of analysis were optimized by varying the mobile phase (Water: Methanol: 2-propanol, 50:45:5% v/v; filtered through a 0.45 mm Millipore filter and de-aerated before use), flow rate (1ml/mint), column temperature 25°C and wave length (245 nm – 270 nm), Retention time was 2 to 4 mints, phosphate buffer solution used with pH 7.4.[18]

Preparation of Transdermal Patches

The transdermal patches were prepared by matrix method. Backing membrane was prepared by using 5 g of PVA (w/v) in a beaker and made quantity sufficient to 100 ml with distilled water as solvent. This beaker was kept on hot plate magnetic stirrer at 80^{o}C until a clear solution is formed. This mixture was then cooled, sonicated for two minutes in a sonicator to remove any air bubbles entrapped. The mixture (15 ml) was poured into each petri dish having a diameter of 70 cm^2, dried in oven at 40^{o}C and stored by raping in aluminium foil for further use.[19] The transdermal patches of crude drug extract of *Nigella sativa* seeds were prepared by using solvent casting method using chloroform as solvent. Different ratios of polymers were evaluated in w/w (**Table I).** First HPMC was taken and mixed with required quantities of PG, PEG 400, Tween 80, DMSO, crude drug extract and chloroform (Q.S). The beaker was kept on magnetic stirrer for 90 min and measured quantity of ethyl cellulose (EC) and PVP were added and beaker was again kept on stirring.[20] This stirred and sonicated solution was poured into the petri dishes having already dried backing membrane and placed at room temperature for drying for 24 hours. Patches were cut into a diameter of 2cm^2 of each patch with help of a sharp blade cutter.

Table I. Composition of Nigella sativa **Transdermal patch**

	Ingredients (w/w), Chloroform (QS to 100mg)					
Formulation	EC : HPMC : PVP (mg)	PG % (mg)	Tween-80 (mg)	DMSO (mg)	PEG (mg)	*Nigella sativa* seed extract (mg).
NS 1	2.1 : 1.7 : 1.2	25 %	8 %	8 %	5 %	25 %
NS 2	3.0 : 0.9 : 1.1	25 %	8 %	8 %	5 %	25 %
NS 3	2.0 : 1.8 : 1.2	25 %	8 %	8 %	5 %	25 %
NS 4	2.5 : 1.5 : 0.5	25 %	8 %	8 %	5 %	25 %
NS 5	1.5 : 2.0 : 2.0	25 %	8 %	8 %	5 %	25%

Physicochemical Evaluation of Transdermal Patches

Smoothness, Brittleness and Clarity

The prepared patches were checked for smoothness and roughness by fingers.

Thickness of the Patch

The prepared drug-loaded patches were selected from all the five formulations and three patches were selected from each formulation and were measured at three different points by using a digital screw gauge. The test was triplicated and results averaged with ± SD.[20]

Folding Endurance

Three patches were selected from each batch and folded repeatedly at the same place till it breaks/cracks. This gave the folding value of a patch. The test was triplicated and the results were averaged with ± SD.

Uniformity of Weight

The prepared drug-loaded patches were weighed using a digital weighing balance. The test was performed to check the uniformity of weight and to check the batch- to- batch variation. The test was triplicated and the results were averaged with ± SD.

Percent Moisture Content

The prepared films were selected from each batch and marked, then weighed individually and kept in a desiccator containing activated silica at room temperature for 24 hours. The films were weighed individually until it showed a constant weight. The percentage of moisture content was calculated as a difference between initial and final weight with respect to final weight. The test was triplicated and the results averaged with ± SD.

Moisture Content (%) = Initial weight of patch - Final weight of patch x 100 **(Eq. 1)**

Final weight of patch

Fourier Transform-infrared Spectroscopy

FTIR spectroscopy was used to detect the vibration characteristics of functional groups in a sample and to investigate and predict physiochemical interaction between different formulation components and therefore it can be applied to the selection of chemically compatible, stable, and therapeutically acceptable ingredients. 10 µl of methanolic extract of *Nigella sativa* seeds, transdermal patch of extract and powder of seed were taken for FTIR analysis. *Nigella sativa* oil with a concentration of 1 mg/ml (methanol as solvent) was taken as standard and both were placed on a diamond window of the spectrophotometer under standard room temperature. A 32 scans with a resolution of 4 cm^{-1} was adopted. The available spectrum region was 4000 to 400 cm^{-1}.[21]

Drug Release Study

Cumulative drug release profiles from matrix patch were examined using the Franz diffusion cell. The receptor compartment was filled with air bubble-free phosphate buffer pH 5.5 in simulation of skin pH using Tuffryn® membrane as barrier. The temperature of the receptor phase was maintained at 32 ± 2°C. The buffer solution was magnetically stirred throughout the study. Two ml aliquot was withdrawn at specific time intervals for 24 h and analyzed by HPLC. Fresh buffer solution was replaced at each interval to maintain the sink condition of receptor compartment. The percentage of drug release was calculated and triplicates were conducted and the results averaged.[21]

Release kinetic

The drug release kinetics were investigated by fitting the drug release data into Korsmeyer pepaas/ and or Weibull kinetic as expressed by equation.[22]

Weibul kinetic: $F = 1\text{-}\exp(-at^b)$ ---- **(Eq. 2)**

Korsmeyer peppas: $M_t / M_\infty = kt^n$ ---------- **(Eq. 3)**

Ex vivo & In vivo **Studies**

Ethical Approval/Informed Consent

This study was approved by the ethical review board of Gomal University, D. I. Khan, Pakistan under reference no. ERB/Gu/1923/2019. All the subjects (Human volunteers) were informed about the study and informed consent was obtained from parents and/or legal guardian of the participants for both study participation and publication of identifying information/images. The animals and human studies were performed in accordance with NIH and Helsinki guidelines respectively. The study is reported in accordance with ARRIVE guidelines.

Preparation of Skin

Healthy male rabbit of family Leporidae and specie *Oryctolagus cuniculus*, weighing 1.5kg, were selected. Hair removing cream was applied on abdominal skin and hairs were removed. Full thickness skin was marked and then rabbit was euthanized and skin was removed with the help of a scissor. The subcutaneous fat present was carefully detached from the skin. The defatted skin was washed with 0.9% w/v NaCl solution; it was wrapped in an aluminum foil and stored in a freezer for further use. The thickness of skin was measured at three different points using a screw gauge and averaged. Prior to use all skin samples were thawed for 3 h at 25 ± 2°C.[23]

Ex-vivo **Drug Permeation**

Franz diffusion cell was used for the diffusion studies by using rabbit skin as a barrier between receiver and donor compartment. The skin was clamped in a way that its epidermis faces upward and dermis was in contact with receiving compartment. The receptor phase of the diffusion cell was filled with air bubble-free USP phosphate buffer saline pH 7.4. Matrix patch was placed on epidermis of the skin and experiment was ran for 24 hr. The temperature of the Franz cell was maintained at 37 ± 1°C. Two ml sample aliquot was withdrawn at specific time intervals of 0, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 16, 20 and 24 hr. Each time, fresh buffer was replaced to maintain the sink condition. The samples were analyzed on HPLC for quantification of extract (Drug) permeated.[23]

In vitro Leishmanicidal **Activity**

Preparation of Lieshmanial **Culture**

Leishmanial major strains of *L. amazonensis* in five test tubes with a maximum capacity of 8 ml and temperature was maintained at 28 ºC having Warren´s medium (Brain-heart infusion plus hemin and folic acid), 10% of inactivated fetal bovine serum, RMPI 1640 solution, penicillin 1001U/ml, streptomycin and gentamycin 100 µg/ml for the time period of 7 days up to the time promastigotes have been formed. This sample was transferred into a sterile culture flask for sub culturing, which have Schneider's insect medium (Sigma Aldrich, USA), pH 4.5, with 20% fetal bovine serum at 32°C. Promastigotes were harvested after 6 days by centrifugation at 2000 RPM for 15 minutes, then pellets were made and washed with phosphate buffer solution having pH 7.2 two times and a haemocytometer (Improved Double Neubauer) was used to calculate the number of promastigotes which was $1x10^{60}$ /ml.[24]

Preparation of Stock Solutions

One milligram of powder and crude extract of *Nigella sativa* seeds and formulation were dissolved in 50 µl of DMSO in separate test tubes and final volumes were made 1ml with same solvent. Then the reference drug Amphotericin B stock solution was made with the DMSO of 0.2 mg/ml. All the stock solutions were kept in tightly closed vials and stored in refrigerator till further use.[25]

Anti-Leishmanial **Assay**

The assay was done by adopting 24 well micro titer plates. 100 µg of *Leishmanial* culture of $1x10^{60}$ promastigotes/ml were transferred to all the wells, only the first well received 180 µg. Then 20 µg of solubilized tested material was taken from the stock solution and added to the first well and mixed well with the micropipette. After the first well mixing sample aliquots of 100 µg was taken from it and shifted to the second well and mixed, from second well shifted to the third well and from third to the fourth well and so on. So the first well received 100 µg/ml of the crude extract of drug while the last well contained only 0.78 µg/ml, for positive and negative control last two wells were used, one contained DMSO and the other have standard drug of Amphotericin B, in both quantities were 0.2 mg/ml, the final volume of DMSO was below 0.5% to avoid its dangerous effect on growth of parasites. Then all the well along with the controls were incubated for 72 hours. After incubation a drop of culture was placed on the slide and the number of promastigotes were counted with a haemocytometer and light microscope. The results were compared with the controls. IC^{50} was calculated and the extract results were compared with the standard drug and percentage inhibition was calculated.[25]

In vivo **Anti-Lieshmanial Studies in Human Being**

For *in vivo* study, 30 insensitive patients were recruited in this study to evaluate the effectiveness of the *N. sativa* loaded formulation. Prior to the formulation application, a dermatologist/physician examined the person to confirm the disease (*Leishmaniasis)*. Volunteers were not informed about the contents of formulation. On the first day, patch test (Burchard test) was performed on the forearms of each volunteer to determine any possible reactions/sensitivity to the formulation. The volunteers were instructed to apply the formulation for 3 weeks. Every individual was instructed to come every week for the skin measurements/lesion observation during study period. All the subjects were informed about the study and informed consent was signed by their families, while the study was approved by institutional ethical committee.

STATISTICAL ANALYSIS

All the results were analyzed by T-test (SPSS, Version 20, IBM software) for determination of mean and standard deviation and level of significance at $P < 0.05$.

Results And Discussion

Phytochemical Screening

Results of present research comprise a scientific justification and suggested the use of *Nigella sativa* seeds. Qualitative phytochemical evaluation of seeds (*Nigella sativa*) showed the presence of alkaloids, glycosides, terpenoides, tannins, saponins, flavonoids while reducing sugars were not present (Table II). Many studies reported the presence of alkaloidal, saponins, flavonoids, terpenoids, tannins which have *anti-Lieshmanial*, ant parasitic activity, tannins are astringent in nature and resistant to enzymatic attack so good for healing process but reducing sugars are absent in *Nigella sativa* seeds.[25–28]

Table II. Phytochemical Parameters of Extract of *Nigella sativa* Seeds

Screening Result	
Phytochemicals	Nigella sativa **extract**
Alkaloids	+ve
Carbohydrates	-ve
Terpenoids	+ve
Flavonoids	+ve
Tannins	+ve
Saponins	+ve

+; Present, - Absent

Physical Evaluation of Patches

The important factor in stability and activity of transdermal patches depends upon its physicochemical characteristics which are smoothness, clarity, thickness, weight variation, folding endurance, moisture uptake and percent moisture content of the patch. In recent years there has been great advancement in the use of transdermal patches for carrying drugs as a vehicle to the body as the bioavailability of the drugs is high in this dosage form. Smoothness, clarity and brittleness of the patch are very important for its elegant look and for complete contact with the skin. Weight variation test was done to observe that patch weight should not be greater than or lower than a significant level because too heavy patch will detach from the skin after some time.[28] Similar study was done by Namdeo et al, they developed transdermal patches of Quetiapine fumerate for treating psychosis by use of EC and HPMC as polymers, DMSO as penetration enhancer and PEG-400 as plasticizer.[29]

Smoothness, Brittleness and Clarity Analysis

The prepared transdermal patches of *Nigella sativa* seeds extract were smooth, clear and uniform moreover there were no cracks or roughness and brittleness. The results are given in the Table III.

Table III. Physical characteristics of *Nigella sativa* transdermal patches

Formulation Codes	Smoothness	Clarity	Brittleness	Overall Appearance
N 1	+	+	×	Satisfied
N 2	+	+	×	Satisfied
N 3	+	+	×	Satisfied
N4	+	+	×	Satisfied
N5	+	+	×	Satisfied

Thickness of Patch

The thickness of transdermal patches of *Nigella sativa* seeds extract was determined by use of micrometer screw gauge, at different points and the thickness was ranged between 0.201 to 0.250 mm. The results are shown in Table **IV**.

Folding Endurance

From each batch one patch was selected and it was folded at three different places and all the patches of *Nigella sativa* seeds extract have folding capacity of more than 70 times. Results are shown in Table IV.

Table IV. Physicochemical Tests of *Nigella sativa* Transdermal Patches

Formulation Codes	Thickness (mm) ± SD	Weight uniformity (mg) ± SD	%Moisture uptake ± SD	%Moisture content ± SD	Folding endurance ± SD
NS1	0.242 ± 0.22	232.00 ± 1.02	22.00 ± 1.03	3.66 ± 0.500	8.13 ± 1.23
NS2	0.203 ± 0.10	216.00 ± 1.21	16.00 ± 1.11	5.000 ± 1.00	7.33 ± 1.52
NS3	0.234 ± 0.12	212.00 ± 1.10	12.00 ± 1.11	3.333 ± 0.51	7.33 ± 0.52
NS4	0.250 ± 0.32	222.00 ± 1.00	3.33 ± 0.57	81.00 ± 1.01	9.33 ± 0.67
NS5	0.218 ± 0.21	219.00 ± 1.20	29.00 ± 1.00	6.00 ± 1.21	8.66 ± 1.52

Weight Variation Results

All the patches were weighed from five batches by using digital weighing balance and the results were triplicated and weight variation test was satisfactory. No patch showed significant change in weight from that of average of all patches. Results are tabulated in Table IV.

Percentage Moisture Uptake

The percentage of moisture uptake by the transdermal patches at room temperature and relative humidity of 84% for 24 hours of time duration are shown in Table IV. As the formulation contained three different polymers which were HPMC, PVP K30 and EC, so the moisture uptake depends on these polymers, due to more quantity of EC than HPMC and PVP high concentration of moisture uptake was observed in it. The

percentage moisture uptake ranged between 11 to 30%. Those patches which have more quantity of HPMC revealed more moisture uptake due to its hydrophilic nature. The results showed that incorporation of enhancer resulted in increased percent moisture uptake. As a whole the capability of patches to take moisture followed the following sequence; HPMC > HPMC/PVP > PVP > PVP/PVA > PVA. Results tabulated in Table IV.

Fourier Transforms Infrared Spectroscopy

Figures 1a, 1b and 1c exhibit FTIR spectra of pure Nigella sativa seeds extract, transdermal patch of extract and powder of seed respectively at mid infrared region of 4000–400 cm^{-1}. FTIR spectra of all three samples appeared fairly same, however clear examination revealed significant differences in number of peaks. At region 3000–2500 cm^{-1} pure extract have no peak, transdermal patch have two broad peaks and powder have two sharp peaks. These peaks were attributed to O–H stretching vibration. The peaks at region 1700 cm^{-1} showed C = O stretching vibrations. Peaks at 1200–1000 cm^{-1} attributed to C–N stretching, peaks at 1600–1400 cm^{-1} showed C = N vibrations. It is concluded that O–H stretching show alcoholic group, C = N reveals imine group and C = O stretching confirms different groups like aldehydes, ketones, carboxylic acids, ester, amide, anhydride and acid chloride. All the principal peaks were also present in polymer formulation with little changes in frequencies which showed that there was no interaction between polymers and the drug. Results are shown in Fig. 1. The broader peaks at 3200cm^{-1} in Fig. 3 (crushed powder from seeds) shows the presence of alcohols and phenols in the seed powder. While the intensity of same peak was much higher in case of crude extract and extract loaded transdermal patches possibly because of strong O-H stretching of hydroxyl group from methanol or water. Multiple peaks from 600 to 1600 in Fig. 4.3 confirmed the presence of various active phyto-constituents ranging from alkenes (1600) to aromatics (1400) to alkyl halides (600–700). Strong carbonyl stretching at 1650 further confirms the presence of wide variety of organic compounds such as 1550 to 1650 resulted from C = N which shows an imine group. In case of crushed seeds powder the C = O stretching confirms many different groups like aldehydes, ketones, carboxylic acids, ester, amide, anhydride and acid chloride.[30]

In vitro **Release Study**

In vitro release study was performed by Franz diffusion cell using three transdermal patches to confirm the type of release. Significant differences were observed in the release of *Nigella sativa* patches containing hydroxyl-propyl-methyl-cellulose (HPMC), Polyvinyl-pyrolidone (PVP) and Ethyl cellulose (EC). During this process the patches were swelled forming a gel layer on the exposed patch surfaces. The loosely bound polymer molecules in these patches were readily eroded, allowing the easy release of *nigella sativa* extract. It was found that the crude extract released from the patches varied with respect to the proportion of polymers. After 24 hours the release was found to be 55.3%, 68.5% and 87.0% in the formulation NS1, NS3, NS5 respectively. Amongst all formulations, formulation NS5 showed significantly good release pattern as compared to others. Results are given in Fig. 2. Little difference was observed in the release of *Nigella sativa* seed extract patches containing different proportions of hydroxyl-propyl-

methyl-cellulose (HPMC), ethyl cellulose (EC) and polyvinyl-pyrolidone (PVP) due to the binding capacity of these polymers. It was revealed that ethyl cellulose has the highest binding force with the drug *Nigella sativa* seed extract. Amongst all formulations the formulation NS4 showed the good release pattern as compared to others because of high concentration of ethyl cellulose. Same studies and findings were revealed by Mutalik and Udupa (2004) and the drugs released from the patches were calculated by korsmeyer pappas kinetics which varied with respect to the proportion of polymers. All formulations shown good release pattern as compared to others.[31]

Evaluation of Drug Release Mechanism by Various Kinetic Models

Various kinetic models can be employed to investigate drug release mechanism of the formulations using *in vitro* release data, but here only Korsmyers Peppas model was used. The *in vitro* release data was fitted to this model to find that either the release follows fickian or non fickian diffusion. The mechanism by which the drug is released from the patch can be determined by the value of n. If the value of n equals 0.5 then the diffusion is fickian diffusion but if it lies between 0.5 and 1 then called anomalous diffusion and if the value of n is equal to 1 than it shows Case-II transport and if it's greater than 1 then Super case-II transport mechanism. Hence, to confirm the exact mechanism of drug permeation from these patches, the data were fitted to the Korsmeyer-Peppas model. In the present study, the coefficient of determination (R2 = 0.992 to 0.995) was found to be much closer to 1 and the release exponent 'n' value varied between 0.426 to 0.771, which explained that drug released from the patch occurred by Non-fickian type of diffusion. Overall results of kinetic modeling suggest that diffusion is dominant mechanism for drug release following Non-Fickian type of diffusion.

Release data were analyzed by Korsmeyer-Peppas model.

$M_t / M_\infty = kt^n$

Where Mt / Mα is the fraction of drug released at time 't' and 'k' is the rate constant and a function of the physical properties of the drug delivery system and 'n' is the release exponent The values for the release exponent 'n' are listed in Table V. The slope of Korsmeyer-Peppas plot were found to be 0.5688, 0.5292, 0.5580 in the formulation NS1, NS3 and NS5 respectively which confirms that the drug release is mediated solely by fickian diffusion mechanism. As the drug release depends on hydrophobicity of the polymers and it decreases with increase in hydrophobic increase. Hence, to confirm the exact mechanism of drug permeation from these patches, the data was fitted to the Korsmeyer-Peppas model and the results for n meant that the mechanism for drug release follows Anomalous non fickian diffusion. Fickian diffusion is derived from ficks law of diffusion which explains that the polymer relaxation time (t_r) is much higher than the solvent diffusion time (t_d) but when this polymer relaxation time (t_r) is almost equal to solvent diffusion time (t_d) than its called anomalous non fickian diffusion to simplify the complex release process from the transdermal patches there is need of mathematical modeling which give details

about release mechanisms of a specific material system, similar study was done by Mutalik and Udupa (2004) and they confirmed that the drug release is mediated solely by diffusion mechanism.[31]

Table V. Korsmeyer-Peppas values for drug release from NS transdermal patches

Formulation	K ± SD	r^2	N	
NS1	0.0190 ± 0.04259	0.9492 ± 0.0600	0.5688 ± 0.0231	Anomalous non fickian diffusion
NS3	0.0130 ± 0.03525	0.8863 ± 0.0279	0.5292 ± 0.0011	Anomalous non fickian diffusion
NS5	0.0190 ± 0.04448	0.9441 ± 0.0632	0.5580 ± 0.0001	Anomalous non fickian diffusion

Ex vivo **Drug Permeation**

As samples were taken at regular time intervals and evaluated by HPLC. In this work, it was observed that as the concentration of polymers varied in the formulations (NS1, NS3, and NS5). The mean accumulative amounts of percent crude extract permeated also showed variation in drug permeation. Hence formulation NS5 has very good permeation pattern up to 80% across the skin, also the percent drug permeated was increased with time (Fig. 3). The percent drug permeated was 40% at six hours, 60% at 12 hours and 80% at 24 hours, which shows that the drug permeated well with the passage of time due to DMSO. Similar study was done by Nicoli *et al*., (2006), they made oxybutynin bio-adhesive films by use of PVA (30% solution), Plastoid E35H® 27, Sorbitol 70% and evaluated its *in-vitro* permeation on rabbit skin, with a good permeation.[32]

In vitro Anti-Leishmanial **Assay**

In vitro assay of *Leishmanial* parasite was done for crude extract of *Nigella sativa* seed, grinded powder of *Nigella sativa* seed and transdermal patch of *Nigella sativa* seed extract. The IC^{50} values of standard drug amphotericin-B, crude powder, crude extract and extract loaded transdermal patch were found to be 20.0ug/ml, 18.50ug/ml, 45.20ug/ml and 38.92ug/ml respectively. The percentage inhibition of amphotericin-B was supposed to be 100 ± 0.02% and then percentage inhibition of crude powder, crude extract and formulation were calculated from it, which were found to be 92.5 ± 0.21%, 226.0 ± 0.43% and 194.6 ± 0.21% respectively. Assay result is given in Table VI. There was significant increase in *in vitro anti-leishmanial* activity of the *Nigella sativa* seeds crude extract and its loaded transdermal patch as compared to standard drug amphotericin-B. Similar research study of *anti-Leishmanial* activity was done by Hossein *et al* in 2014 who concluded that *Nigella sativa* extracts exhibited an effective *Leishmanicidal* activity against *L. tropica* on *in-vitro* model, using a clinical setting further works are required to evaluate the exact effect of these extracts on *Leishmania* species.[35]

Table VI. *In-vitro anti-Leishmanial* Assay results

Samples	IC_{50}(ug/ml)	%Age Inhibition	Comments
Amphotericin-B	20.00 ± 0.57	100 ± 0.02	LAA
Transdermal patch PL	0.00 ± 0.00	00 ± 0.00	NAA
Crude powder	18.50 ± 0.23	92.5 ± 0.21	LAA
Crude extract	45.20 ± 0.52	226.0 ± 0.43	SAA
NS transdermal patch	38.92 ± 0.76	194.6 ± 0.21	SAA

SAA: Significant anti-Leishmanial activity, LAA: Low anti-Leishmanial activity, NNA: No anti-Leishmanial activity

In vivo Anti-Lieshmanial **Studies in Human Volunteer**

In this study the effectiveness of the *NS* extract containing transdermal patch was evaluated in human volunteer. Patients (n = 30) were included in the study according to inclusions criteria for antileishmanial treatment. The mean number of lesion per patient was 1.2 and mean lesion size of the patients was 25.3 mm. Every patient was treated for 2 to 3 weeks. The cure rate in the patients included in the study was 73%. Measurements and calculations for all lesions and indurations were recorded separately and presented in Table VII. Figures 4 show the pre-treatment and post-treatment photographs of the patients. NS formulations showed good results during therapy period with percentage cure rate of 73% while failure rate was 27 % which may be due to non-compliance to treatment, involvement of other species of *leishmania* or secondary infections to which therapy may not be effective. During the study, *L. major* did not produce visceral infection in the patients so they seemed to be suitable model/volunteers for the present study. The fact that there was no other treatment applied by the patients indicates that the healing, to any extent, was due to topical treatment alone using NS formulation. These finding are in agreement to the results of the study conducted by Parvaneh *et al* who formulated topical ointment containing crude extracts of *Peganum harmala*, however they used animal model rather than human volunteers.[36]

Table VII. *In-vivo study* on NS extract containing transdermal patches in patients with cutaneous *Leishmaniasis.*

No of patients	**30**
Mean no of lesions per patients	1.2
Mean lesion size SD (mm)	25.3 ± 0.1
No. of cured patients	22
No. of patients for whom therapy failed	08
Cure rate in percentage	73 %

Conclusion

The finding of this work revealed that the problems of drugs parenterally used for *Lieshmanial* treatment can be managed by applying extract of *Nigella sativa* seeds in the form of transdermal patch. *Nigella sativa* seeds extract contained thymoquinone which have potentiating effect for anti-parasitic activity. As an extension of this work pharmacokinetic studies, *in-vivo* studies on higher animals and controlled clinical studies on human beings can be carried out in future.

Declarations

Consent for publication

Not applicable

Availability of data and materials

It can be obtained from the corresponding author on request.

Competing interest

All the authors declare that they have no competing interest

Funding

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Author's contributions

BAK designed and supervised the study plan. YA performed all the experiments. THK helped in writing of the manuscript. MQ edited the manuscript and did the formal analysis. SMA and MKK helped in the formulation development and statistical analysis. All authors have read and approved the manuscript.

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Figures

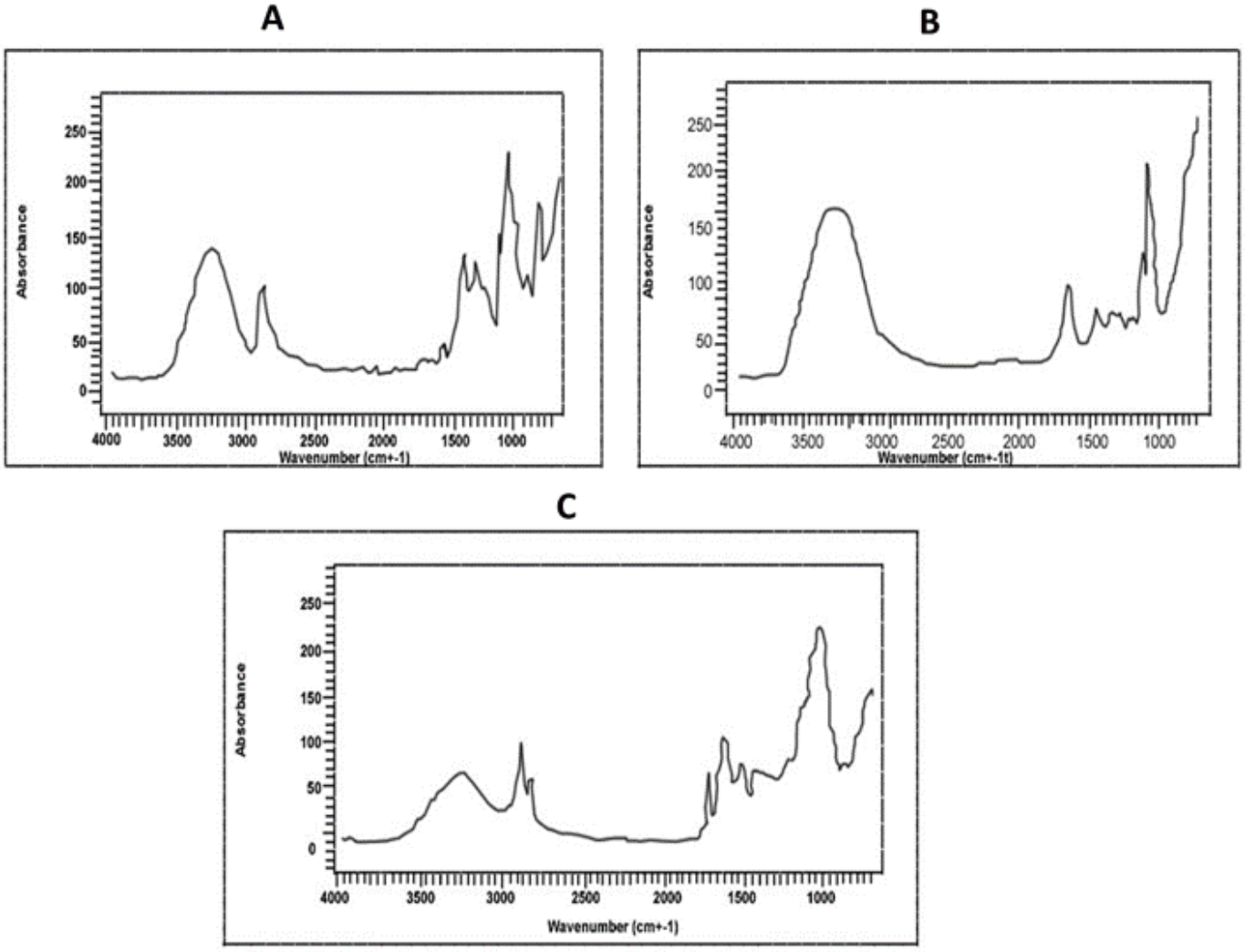

Figure 1

FTIR of Nigella sativa seeds extract 1b) FTIR of transdermal patch of Nigella sativa seeds extract 1c) FTIR of crushed powder of NS seeds

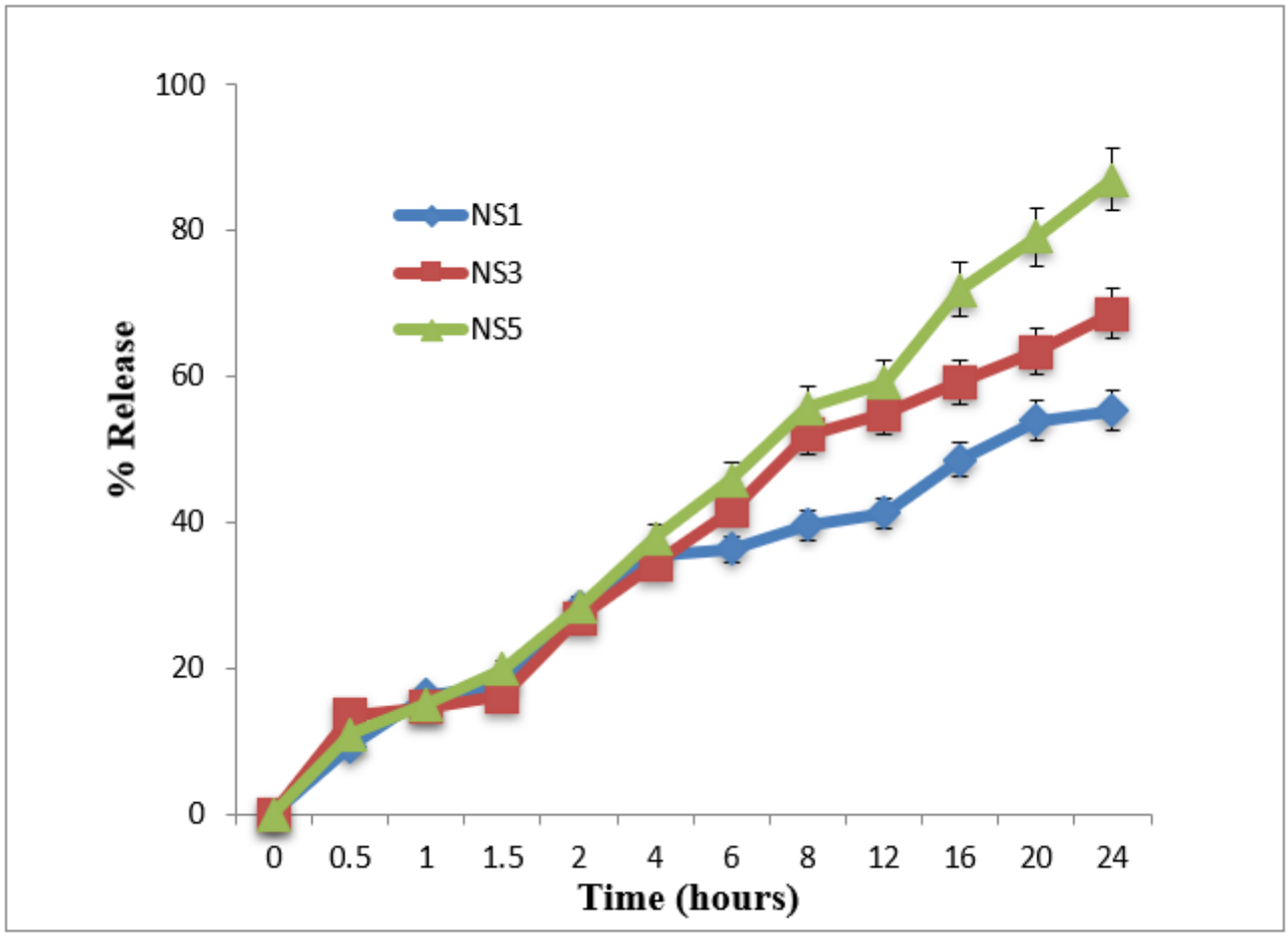

Figure 2

Percentage release of Nigella sativa seeds extract from three formulations of transdermal patches at different time intervals.

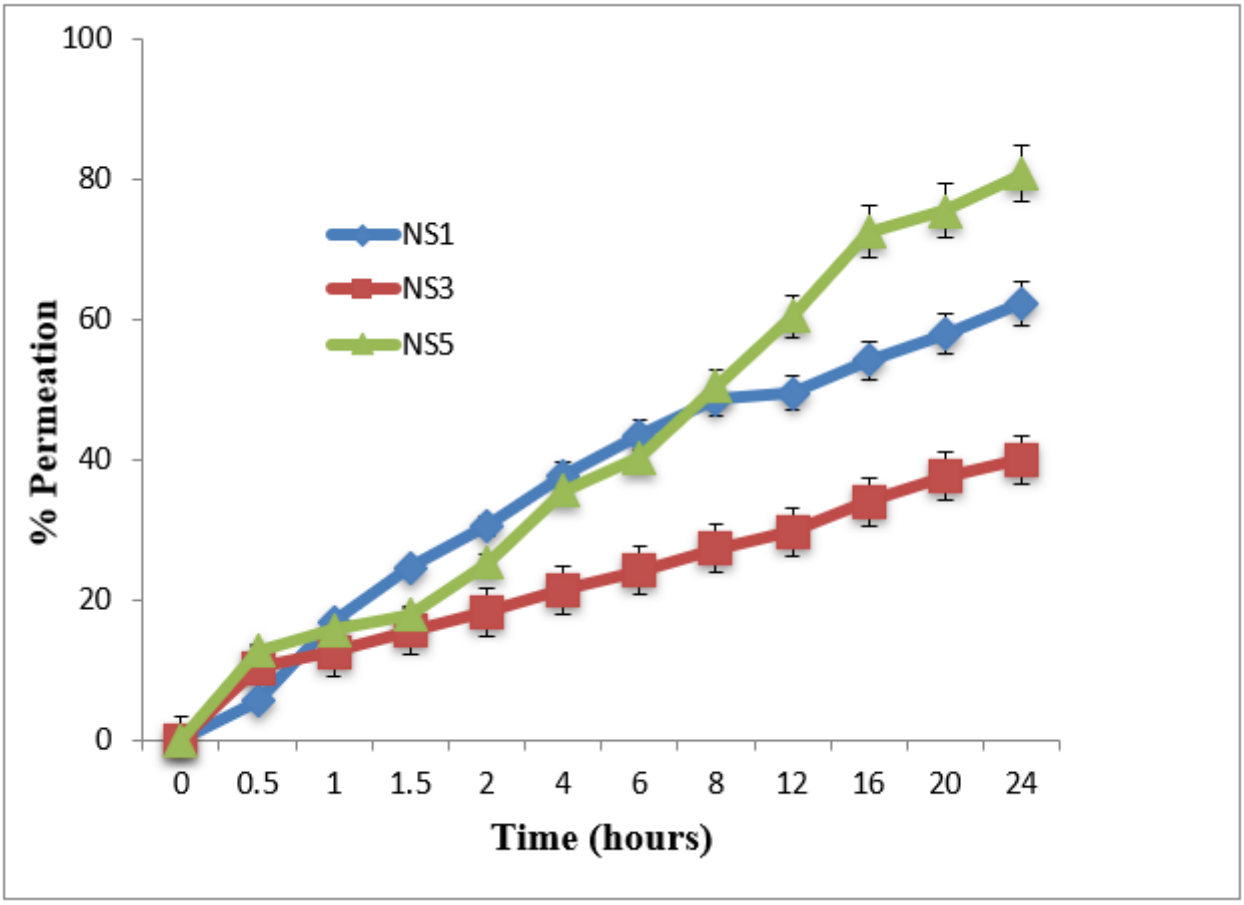

Figure 3

Percent permeation of Nigella sativa seeds extract from three formulations of transdermal patches at different time intervals

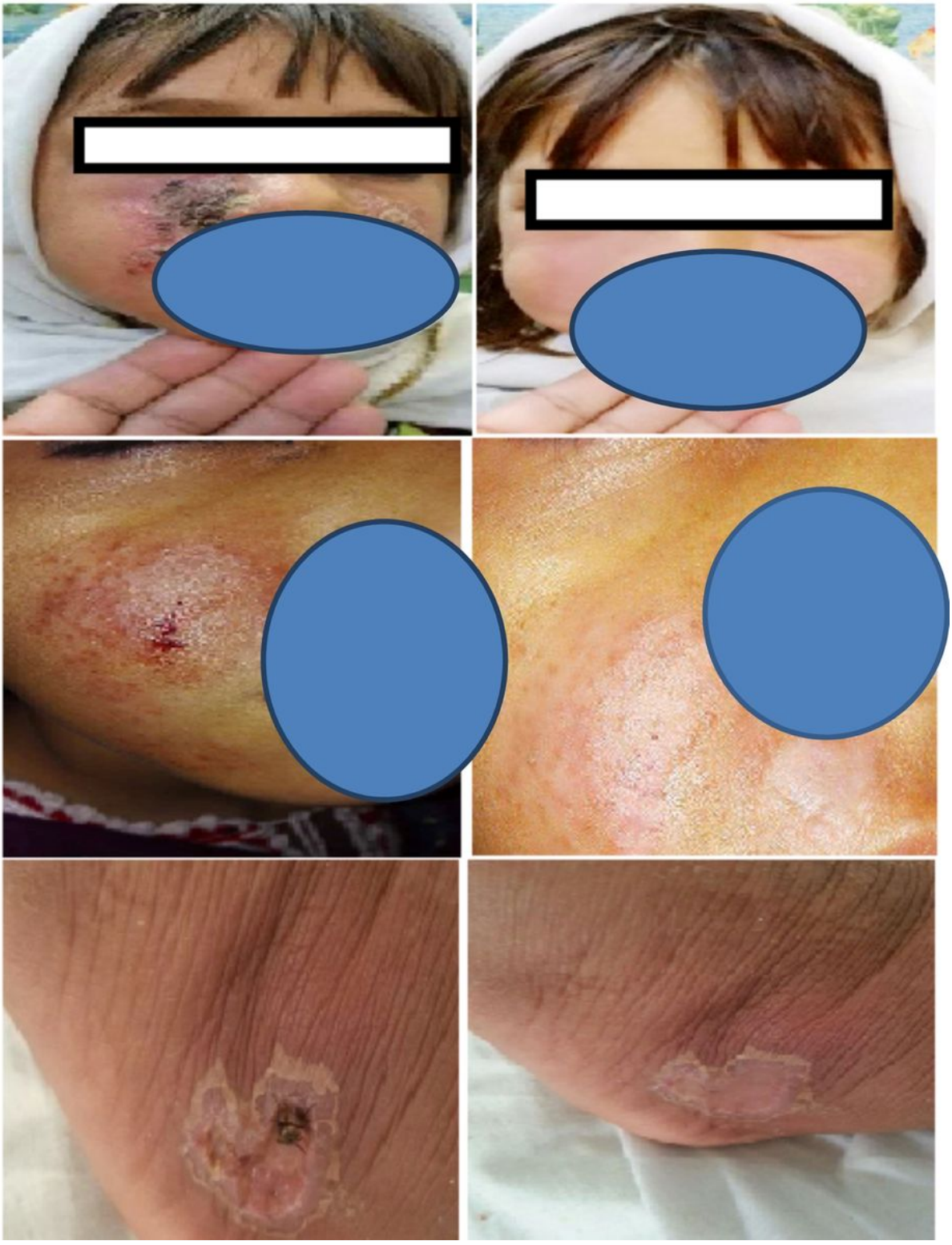

Figure 4

Pre-treatment and Post treatments photographs of Patients treated with NS formulation for 2-3 weeks