DESIGN, OPTIMIZATION AND EVALUATION OF NANO-OINTMENT WITH SMART CARRIER FOR ENHANCED ANTIFUNGAL EFFICACY– A TOOL FOR ANTIMICROBIAL RESISTANCE

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Abstract

Fungal infection of the skin is one of the most common dermatological diseases in the world. ointment formulations are among the most suitable dosage forms for topical use to treat skin infection. Nanotechnology is a promising approach to penetrate the deeper skin layers and enhance permeability of Usnic acid (UA) through the stratum corneum. UA-loaded nanoparticles (UANP) were fabricated using the solvent evaporation method, then Nano conjugation (NC) with 2D structure of the graphene (UGNC) followed by incorporation of UGNC into ointment with water soluble base i.e. PEG400, PEG 4000, SLS, and Glycerin as the ointment forming excipient. The physical properties, in vitro drug release, and antifungal activity of UGNC nanoointment were characterized. UA NPs were spherical in shape with colloidal size <250 nm. The drug release of UGNC ointment was found to be slower than the usnic acid dispersion due to the hindrance caused by the ointment base. Nanoointment of Usnic acid NP's Graphene nano conjugate (UGNC) show augmented effect to kill the pathogens with the triple action of combination therapy with US, NPs and their conjugate with GN. In vivo anti-fungal activity of UGNC nano-ointment on the Wistar albino rats shows significant positive outcome despite hindrance in the in vitro dissolution profile and it is better than the marketed formulation having the steroid.

Keywords: - Graphene (GN), Usnic acid (UA), Usnic acid NP's Graphene nanoconjugate (UGNC) ointment, UA NPs (UANP) ointment, Nanoparticles (NPs).

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1. Introduction

The application of nanotechnology in medicine by using nanoscale materials could be useful to monitor, control, create, and repair biological systems, bringing about a revolutionary advance and progress in the medical sciences [1]. Pharmaceutical scientists have studied nanotechnology for targeted and controlled drug delivery systems more recently [2]. For the effective treatment of infectious diseases, a variety of nanocarrier systems have been developed, including polymeric nanoparticles [3], nanosuspension, nanotubes, nanofibers, and nanosponges (NS) [4-6]. In contrast to commercial hand sanitizers, smart handwash made with silver nanoparticles from Azadirachta indica was found to be efficient against pathogenic bacteria [7]. Chitosan nanoparticle matrix against fungal infections demonstrated promising antifungal efficacy [8]. The permeability and effectiveness of amphotericin B were significantly improved by an olive oil nanoemulsion [9]. B-cyclodextrin-made nanosponges (NS) have been reported to increase the solubility of BCS class II and IV medicines [10]. The delivery of medications, biocatalysts, gases, and the adsorption of harmful substances have all been studied using nanosponges (NS) [11].

Due to their improved penetrability and passive accumulation at the target site, nanocarriers in topical delivery systems are more commonly used [12]. Topical drug delivery systems (TDDS) are primarily designed to deliver a therapeutically effective concentration of medication in the skin or mucosal layers and are easily made into liquid, solid, and semisolid dose forms [13]. Over oral or parenteral dosing forms, topical formulations have some advantages, such as a lower likelihood of inevitable adverse reactions. For the treatment of superficial skin infections and disease conditions, dermatologists favoured topical semisolid formulations [14]. The skin is one of the largest organs, covering around 2 m^2 of the total body surface area and protecting the body from harmful substances. Skin is considered an impermeable membrane that serves as a barrier against xenobiotics and external stimuli. In order to effectively treat cutaneous infections and disease conditions, it is difficult for formulation scientists to develop effective topical dermal dose forms [15]. Globally, there has been an increase in the frequency of superficial fungal infections (SFI) of the skin, hair, and nails. Due to a compromised immune system, the spread of fungal infections can be quick and dangerous [16]. Approximately 20–25% of people worldwide are afflicted by fungi. Dermatophytes and candidiasis are two examples of fungi that can cause an infection, and they are most commonly brought on by humid environments and ambient temperatures between 25 and 28 °C [17].

Usnic acid, one of the most extensively studied lichen secondary metabolites, exhibits a number of biological properties that can be used in medicine i.e. antimicrobial, anticancer and wound healing properties [18-20]. Usnic acid (UA) is a yellowish-coloured crystalline powder. It is a derivative of dibenzofurans, a potent new class of antifungal chemicals. It acts by inhibiting the synthesis of DNA and RNA by pathogens [21]. Graphene materials (GMs) are being investigated for a range of microbiological applications due to their unique physicochemical characteristics, such as high electrical conductivity, a large specific surface area, and solid mechanical strength. It is a 2-D substance made of carbon atoms that are arranged in a honeycomb-like crystal lattice. The antimicrobial activity of graphene is effective against both gram-positive and gram-negative pathogens [22-23].

GMs with antifungal properties can potentially directly kill or starve prokaryotic cells. Membrane tension, oxidative stress, and wrapping isolation are thought to play a role in the bactericidal activity of GMs [24–25]. In cases of membrane stress, GMs eliminate bacteria by puncturing and removing phospholipids from the membrane(s) preserving the integrity of the cell [26]. Oxidative stress includes reactive oxygen species (ROS), which can be created by GMs when there are bacteria present [27]. Proteins, membrane lipids, and nucleic acids in bacteria that are under oxidative stress are all oxidised and destroyed, which results in cell death [28]. Wrapping isolation occurs when fungal cells are wrapped in GM sheets and thus isolate it from it's growth medium [29-30]. Different antifungal medications are marketed in the usual topical dose forms of creams, lotions, and sprays. The challenge with these traditional topical dosing forms is that until infection signs and symptoms are totally gone after many weeks, they need to be used often. There are both local and systemic adverse effects associated with it. As a result, conventional topical dosing forms are frequently ineffective and may be seen negatively by the patient, which can lead to therapy failure [31].

Future treatments should focus on increasing the efficiency of currently available antimicrobials against resistant pathogens. AgNPs recently demonstrated excellent results in numerous trials, efficiently eradicating antibiotic-resistant bacteria

[32–34]. To increase the potency of already available antibiotics and combat antibiotic resistance, AgNPs may be employed in combination with these drugs [35–36]. In current research work, usnic acid and graphene have been used, and both exhibit antifungal properties [37]. Therefore, an attempt has been made to use low-water-soluble drugs as a tool to kill pathogens through the development of novel nanoointments against superbugs.

2. Material and methods

A graphene sample was purchased from the Bengaluru-based GRL (Graphene Research Lab). Himedia, Mumbai, India, has provided a sample of PEG 4000 and PEG 400, while Candida Albicans (MCCB 0290) was provided by the Microbial Culture Collection Bank, SHUATS Prayagraj, India. Usnic acid was obtained from Hubei Honghan Biotech, China. Analytical-grade chemicals were used throughout the study.

3. Preparation of Usnic acid nanoparticles

(UANPs) Usnic acid nanoparticles has been manufactured through Nanoprecipitation method. 10% w/w solution has been prepared i.e. In 30 ml of ethanol and acetone (1:3) solvent, 3g UA was dissolved by ultra-sonication at 40W. The obtained solution was then injected (1 ml/min) with a micro syringe connected to a thin Teflon tube, into 60 ml distilled water containing 2.5% w/v of (PVA) polyvinyl alcohol with uninterrupted stirring at 750 rpm. The obtained emulsion was then diluted with 100 ml PVA solution (0.5% w/v in distilled water) in order to minimize coalescence and the mixture was continuously stirred at 750 rpm at room temperature to make evaporation of solvent happen and thus nanoparticles formation. The resultant Usnic acid nanoparticles were consequently cooled down to −40˚C and freeze dried [38-39]. Detailed Manufacturing process of preparation of Usnic acid nanoparticles is presented in Figure 1.

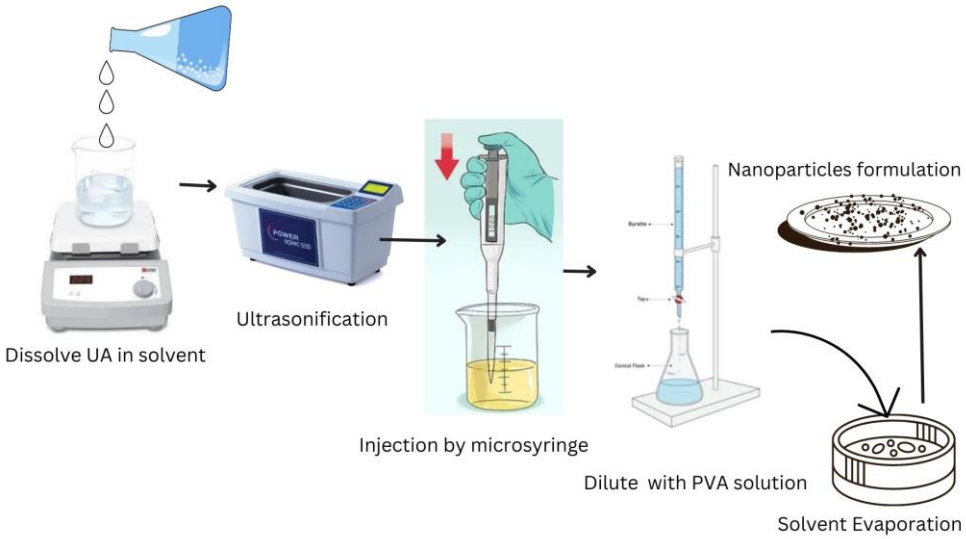


Figure 1 Illustration of manufacturing process of Usnic acid nanoparticles

3.1. Optimization of Usnic Acid Nanoparticles (UANPs)

Formulation variables that were varied for preparing nanoparticles include the concentration of PVA and the Ethanol: Acetone ratio. Process variables include stirring speed. For optimisation, the concentration of PVA was varied (2.0%, 2.5%, 3%, and 3.5%) along with the stirring speed (500 rpm, 750 rpm, 1000 rpm, and 1500 rpm) while keeping the ethanol: acetone ratio constant and also optimised by varying the ethanol: Acetone ratio (1:1, 1:2, 1:3, 1:4) and concentration of PVA (2.0%, 2.5%, 3.0%, 3.5%) while keeping the stirring speed constant (750 rpm). 32 formulations have been prepared and optimised on the basis of Particle size, pdI, and Zeta potential. Plan of optimisation study is presented in figures 2a & 2b with the process variables.

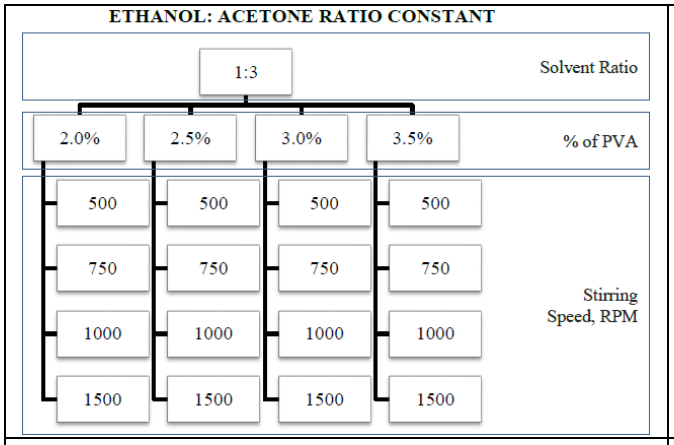


Figure 2a: Usnic acid Nanoparticles Optimization at constant solvent ratio

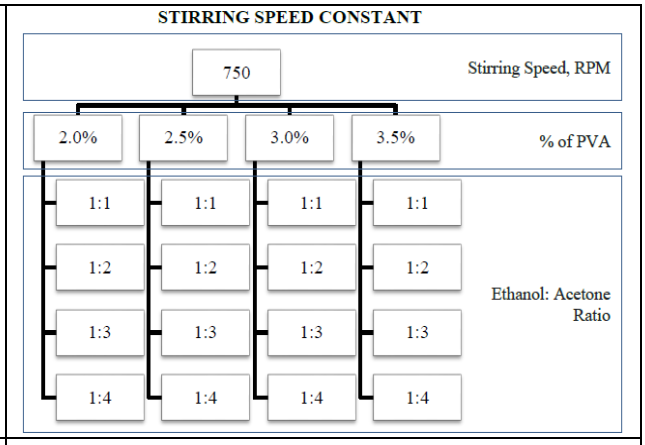


Figure 2b: Usnic acid Nanoparticles Optimization at constant stirring speed

3.2. Conjugation of Usnic acid nanoparticles (UANPs) and Graphene

By adopting physiosorption method, Usnic acid NPs were conjugated on the 2D structure of the Graphene. At pH 5, 0.25% Graphene was sonicated with 0.1% Usnic acid for 10 minutes at 20 watt for 3 cycles, then it was stirred in the dark by using magnetic stirrer instrument, then it was ultra-centrifuged at 15000 rpm, after ultracentrifugation the supernatant was taken out for calculating the entrapment efficiency, the conjugate of Usnic acid and graphene was remained at the bottom, then it was heated at 40°C in the hot air oven, powder of grapheme conjugated Usnic acid was obtained, then this nanoconjugate was taken for the characterization studies [40]. Usnic acid NPs Conjugation on graphene shown in figure 3.

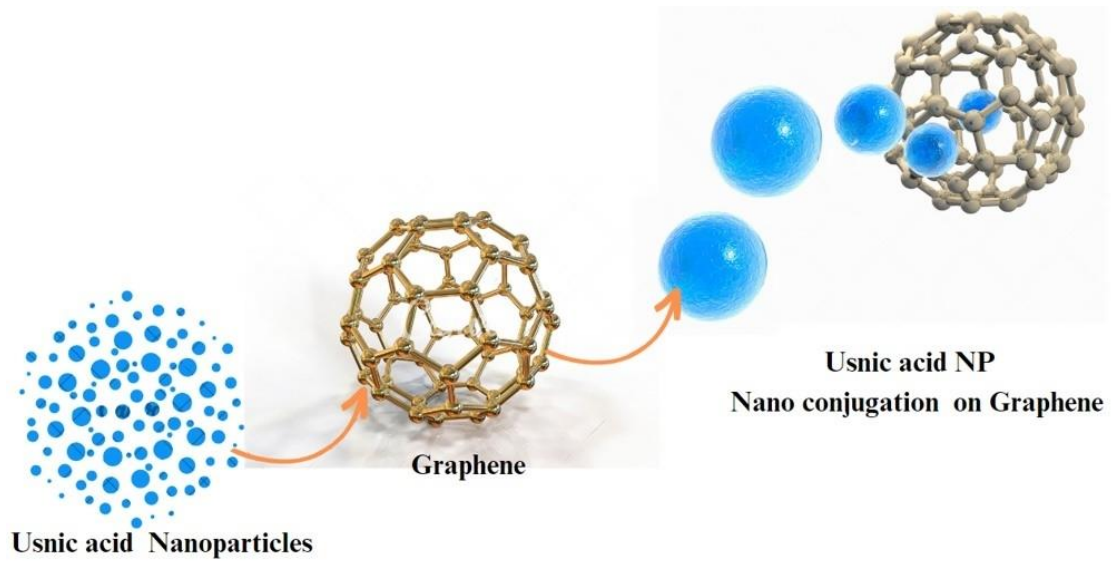


Figure 3 Conjugation of Usnic acid nanoparticles (UANPs) and Graphene

3.3. Preparation of water-soluble ointment base

Ointment base was prepared by selecting water soluble ingredients like PEG derivative and glycerin, which facilitate to make water soluble base for the ointment where different grades of PEG, glycerin, surfactant and Purified water has been used. In order to make water soluble ointment base, first in suitable container PEG-4000 was melted at 70°C temperature followed by PEG-400 and glycerin was added subsequently under stirring. Once the molten base is ready then Sodium lauryl sulphate (surfactant) was added under continuous stirring. once it is uniformly distributed then base was cooled down until congealed, this activity is done under continuous stirring. With different ratio of PEG 4000 and PEG 400, six formulations have been prepared and among all best one has been selected based on the pH, Spreadability and Viscosity [41].

Manufacturing process of making ointment base is presented diagrammatically in Figure 4.

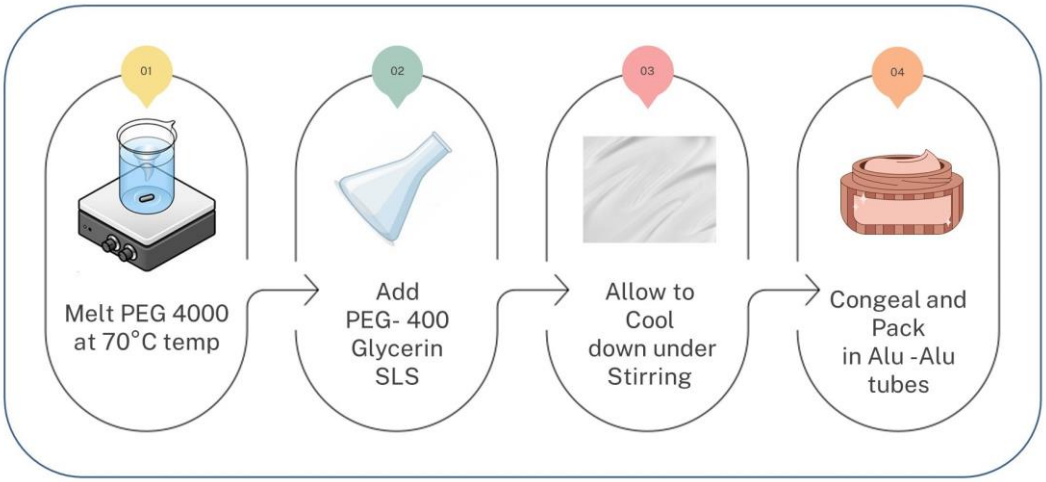


Figure 4 Preparation of water-soluble ointment base

3.4. Formulation of nanoointment of Graphene-Usnic acid nanoconjugate

The geometric dilution method was used to prepare the nanoointment of the graphene-Usnic acid nano-conjugate, in which Usnic acid NPs were gradually added to the ointment base while homogenization was taking place. Once the total amount of nanoparticles had been mixed into the base of the ointment, homogenization was carried out to ensure that the NPs were uniformly distributed and the ointment had a homogeneous consistency. After preparation, the ALU-ALU tube was filled with nanoointment and tightly sealed. Figures 5a and 5b, referring to the ointments with usnic acid nanoparticles (UANP) and usnic acid-graphene nanoconjugate (UGNC), respectively. Usnic acid nanoointment (UANP) and graphene-usnic acid nanocomposite (UGNC) were created with a 0.5% w/w concentration. An overview of the entire manufacturing process is presented in figure 6.

Figure 5 Description of nanoointment (5a. UANP & 5b UGNC)

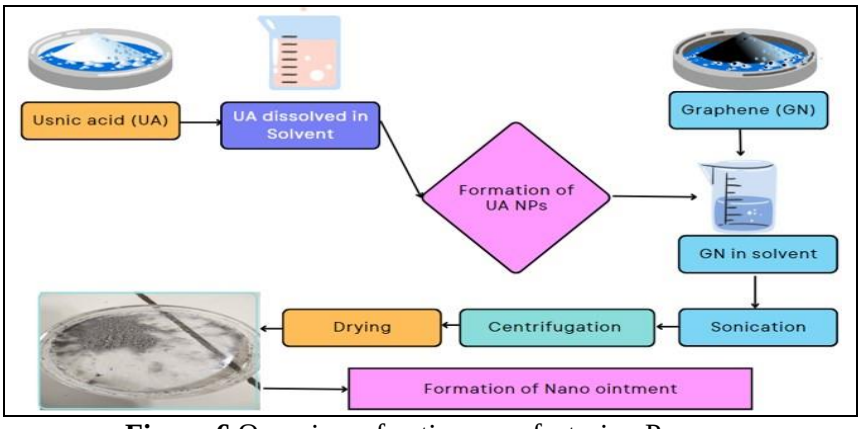


Figure 6 Overview of entire manufacturing Process

3.5. Determination of Usnic acid by UV Spectroscopy

UV spectroscopy has been used to determine the usnic acid content. Methanol and water were mixed in a 50:50 ratio to create the standard solution, to which was then added usnic acid to dissolve it. Two solutions were prepared. One solution was blank, and another contained usnic acid. Between 200nm - 800nm of absorption spectra were recorded against a blank, and a calibration curve was constructed using the data. With a wavelength of 290.0 nm, different absorption spectra were produced at dilutions of 2-10 µg/ml.

3.6. Morphological and particle size analysis

Zetasizer by Malvern employed to determine the particle size of the Usnic acid NPs by dynamic light scattering technique. The SD was calculated after three measurements (n = 3). Whereas morphology was carried out using TEM.

3.7. Physicochemical Characterization

The nanoointment formulations UANP and UGNC undergone physical chemical characterizations, such as pH, viscosity, spreadability, etc. [42].

3.8. In-vitro release study

The dialysis bag diffusion technique was used to conduct in vitro dissolution studies in phosphate buffer pH 7.4. Selected nanoointment was placed in a cellulose dialysis bag, which was then knotted at both ends. The receptor compartment contained 50 ml of phosphate buffer with a pH of 7.4 and was kept at 37 °C under magnetic stirring. During the in vitro dissolution, the sink condition was maintained. After each time interval, 1 ml of the dissolving medium was taken out and replaced with fresh media. After additional dilution and filtering via a 0.45 micron PVDF filter, the obtained aliquot was tested for absorbance at 290 nm. In vitro dissolution was carried out of both UANP and UGNC along with drug dispersion to understand the drug release profile against the drug in the dosage form, percentage drug released at different time points was calculated. All readings were carried out in triplicate and SD was calculated.

3.9. In vivo antifungal studies

Experimental animals:

To test the antifungal activity, Wister albino male rats between the weights of 100 and 150 g and the age of 2 and 3 months were used. The authority to carry out this study has been given by the AEC (Animal Ethical Committee, UIP/IAEC/Sept-2020/07), UIP, Prayagraj, India, an organisation that was granted government approval.

Experimental Design

To conduct this study experiment were divided into five groups of animals and in each one group comprises of 6 rats. First group is control which didn't receive the treatment, second group got treatment of Usnic acid API dispersion, third group is standard which got treatment of Standard marketed formulation, fourth group got treatment of Usnic acid Nanoparticles ointment, fifth group got treatment of Usnic acid Graphene nanoconjugate ointment. In all the groups formulation were administered topically. The response of each group was compared to the control group after the period of six days.

Groups	Treatment	Study
Group I	Control	Normal control rats, which didn't received treatment
Group II	UADN	Group of 6 Rats got treatment of Usnic acid API dispersion
Group III	UANP	Group of 6 Rats got treatment of Usnic acid Nanoparticles ointment
Group IV	UGNC	Group of 6 Rats got treatment of Usnic acid Graphene nanoconjugates ointment
Group V	Standard	Group of 6 Rats got treatment of Standard marketed formulation

Antifungal activity:

First, a hair removal treatment was used to remove hair from backside of the rats. The prepared formulations were then applied topically to 2 cm x 2 cm square area. Before the study began, a selected part of the skin was lightly abraded with sandpaper. After that, a cotton swab was used to apply a fungal strain inoculum. Individual formulations were applied. After six days, responses from each group to that formulation were noted and compared to the control group.

Culture study:

A study was conducted to evaluate the effects of each group. Rats were sacrificed, and tissue from the treated site was excised and minced. The obtained tissue portion was homogenised in a homogenizer with 4 ml of 0.9% saline. Following the streaking of the obtained homogenate onto the

nutrient solidified Sabouraud dextrose agar plates, the treated agar plates were incubated for 5 days at 25°C in an incubator. The number of colonies that had formed after 5 days was recorded using a colony counter, and the number of colonies per infected location was assessed.

4. Results

4.1. Characterization of Nano formulations

TEM analysis and zeta potential measurement

The best tool for characterising NPs is transmission electron microscopy (TEM), which allows for direct analysis of NP size distribution and shape. The size of the produced NPs was measured at a magnification of 20–50 nm, demonstrating their size <250nm and spherical shape. Both the UANP and UGNC formulation's shapes are shown in Figures 7a and 7b.

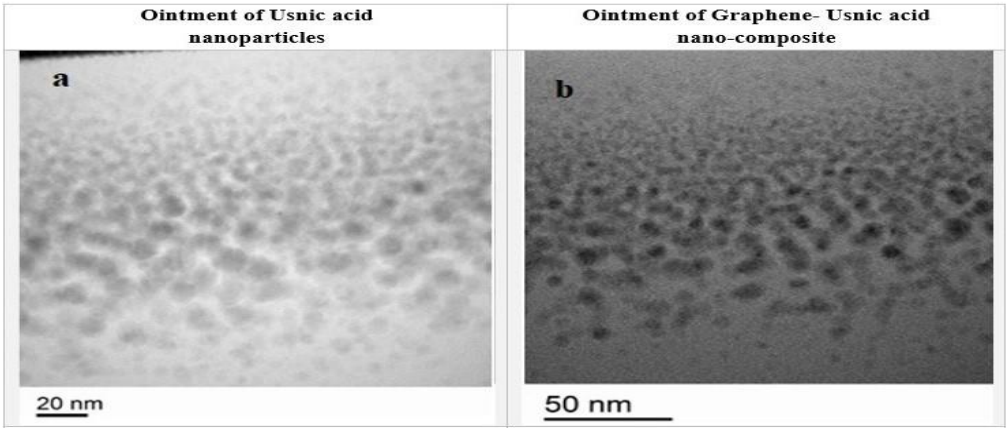


Figure 7 TEM image of Usnic acid nanoparticles (7a UANP & 7b UGNC)

4.2. Content determination of Usnic acid by UV-Spectroscopy

UV-Spectroscopy was employed to evaluate the nanoointment's usnic acid content. Through the application of the regression equation, the assay for usnic acid was determined to be y = 0.0857x - 0.0097 of the standard curve.

4.3. Optimization of Usnic Acid Nanoparticles (UANPs)

Results of the response variables i.e. particle size, pdI, and zeta potential are presented in table 1 & 2 and figure 8 & 9.

Table 1 Optimization of Usnic acid Nanoparticles Prepared by Varying PVA Concentration and Stirring Speed

ETHANOL: ACETONE RATIO CONSTANT						
S. No.	**Formulations**	**PVA (%)**	**Stirring Speed (rpm)**	**Particle Size (nm)**	**PdI**	**Zeta Potential (-mv)**
1	A1	2.0	500	498.9	0.268	17.6
2	A2	2.0	750	310.3	0.169	25.8
3	A3	2.0	1000	435.8	0.215	17.7
4	A4	2.0	1500	427.7	0.264	13.7
5	B1	2.5	500	479.3	0.298	14.3
6	B2	2.5	750	284.9	0.158	28.8
7	B3	2.5	1000	467.7	0.231	29.6
8	B4	2.5	1500	415.3	0.276	15.9
9	C1	3.0	500	500.8	0.326	11.6
10	C2	3.0	750	320.3	0.121	32.9
11	C3	3.0	1000	430.7	0.295	23.7
12	C4	3.0	1500	468.5	0.322	17.8
13	D1	3.5	500	515.7	0.335	15.8
14	D2	3.5	750	363.6	0.184	29.6
15	D3	3.5	1000	498.7	0.345	14.8
16	D4	3.5	1500	515.8	0.363	13.7

Figure 8: Optimization of Usnic acid Nanoparticles by varying PVA Concentration and Stirring Speed

Figure 8a Relation between PVA% and stirring speed to Particle Size (nm)

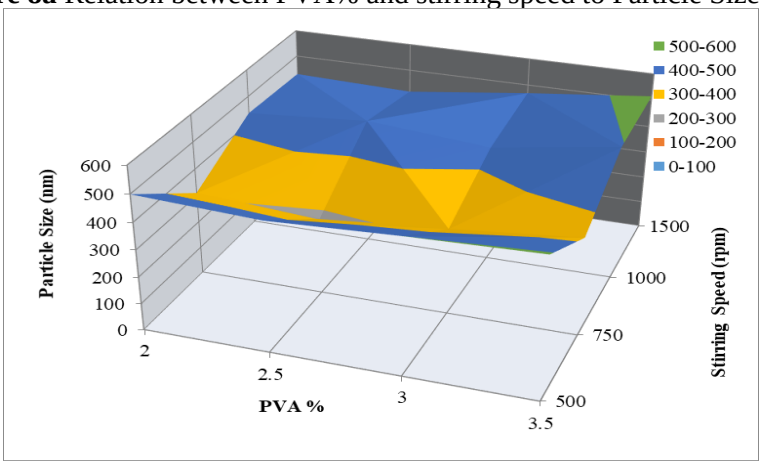


Figure 8b Relation between PVA% and stirring speed to Polydispersity index

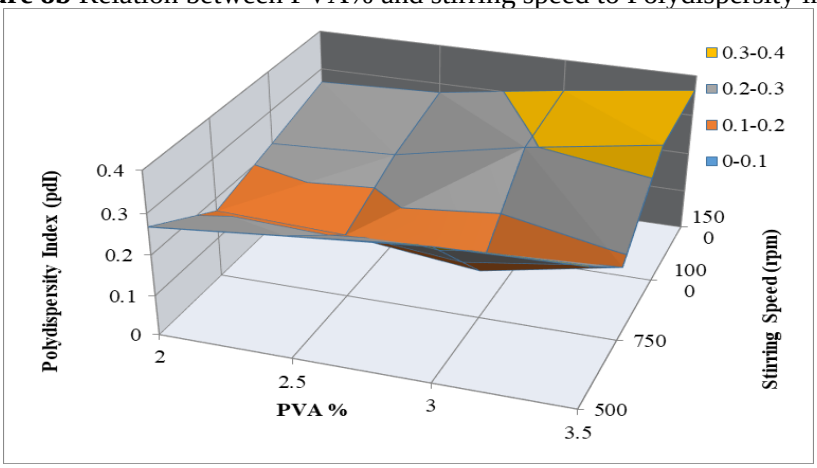


Figure 8c Relation between PVA% and stirring speed to Zeta potential (mv)

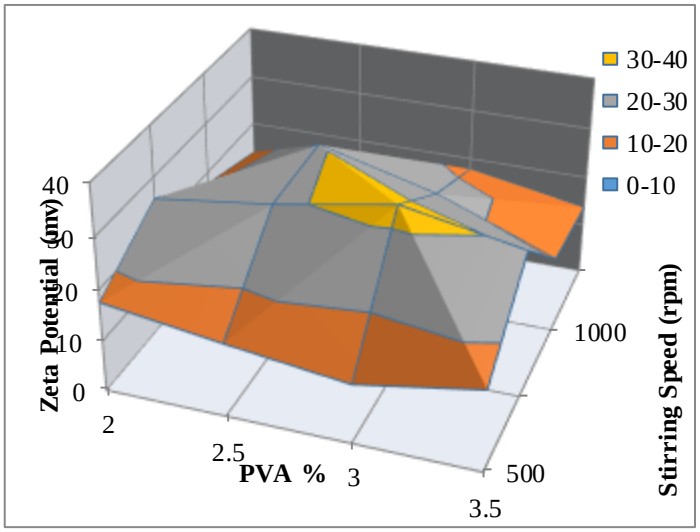


Figure 8d: Overall relation between PVA% and stirring speed to Zeta potential & pdi

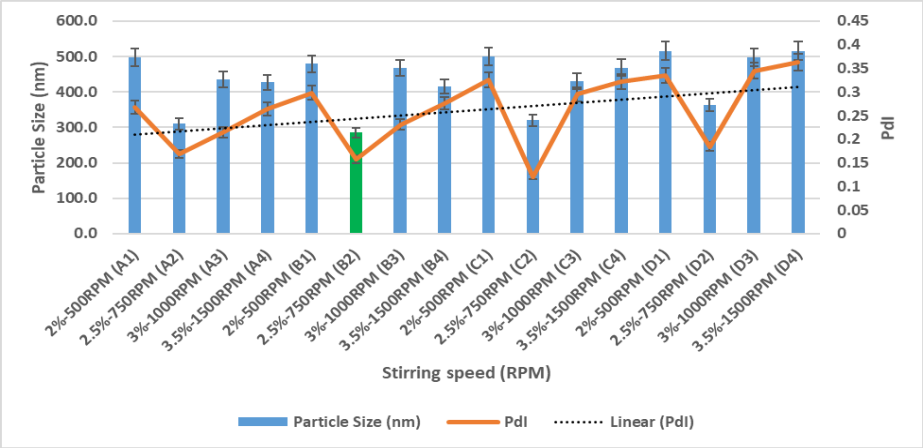


Table 2 Usnic acid Nanoparticles Prepared by varying PVA Concentration and Ethanol: Acetone ratio (v/v)

STIRRING SPEED CONSTANT (750 RPM)						
S. No.	**Formulations**	**PVA (%)**	**Ethanol: Acetone (v/v)**	**Particle Size (nm)**	**PdI**	**Zeta Potential (-mv)**
17	E1	2.0	1:1	305.9	0.062	18.5
18	E2	2.0	1:2	350.7	0.168	20.9
19	E3	2.0	1:3	248.6	0.282	23.7
20	E4	2.0	1:4	350.3	0.243	21.4
21	F1	2.5	1:1	320.9	0.213	28.8
22	F2	2.5	1:2	266.9	0.238	30.2
23	**F3**	**2.5**	**1:3**	**236.2**	**0.207**	**31.2**
24	F4	2.5	1:4	315.8	0.219	28.9
25	G1	3.0	1:1	330.8	0.233	22.6
26	G2	3.0	1:2	368.4	0.220	22.9
27	G3	3.0	1:3	286.5	0.245	18.5
28	G4	3.0	1:4	302.2	0.248	20.9
29	H1	3.5	1:1	334.6	0.211	17.8
30	H2	3.5	1:2	316.6	0.242	16.7
31	H3	3.5	1:3	325.4	0.235	15.9
32	H4	3.5	1:4	293.2	0.230	14.9

Figure 9: Optimization of Usnic acid Nanoformulation by varying PVA Concentration and ethanol: acetone ratio

Figure 9a Relation between PVA% and Ethanol :Acetone Ratio to Particle Size (nm)

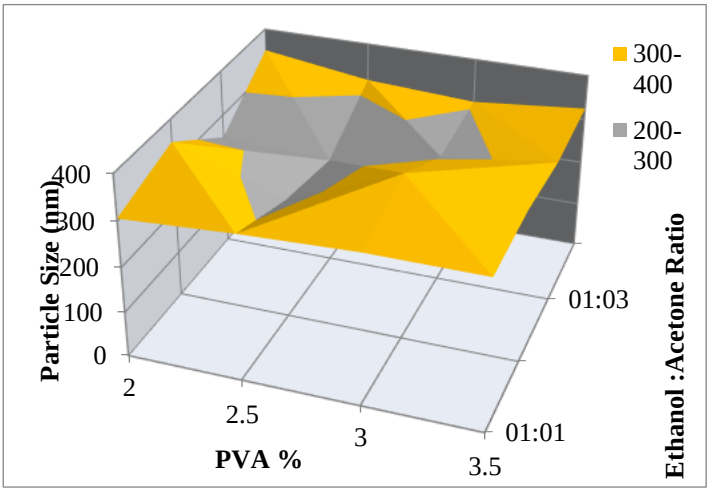


Figure 9b Relation between PVA% and Ethanol :Acetone Ratio to Polydispersity Index (pdI)

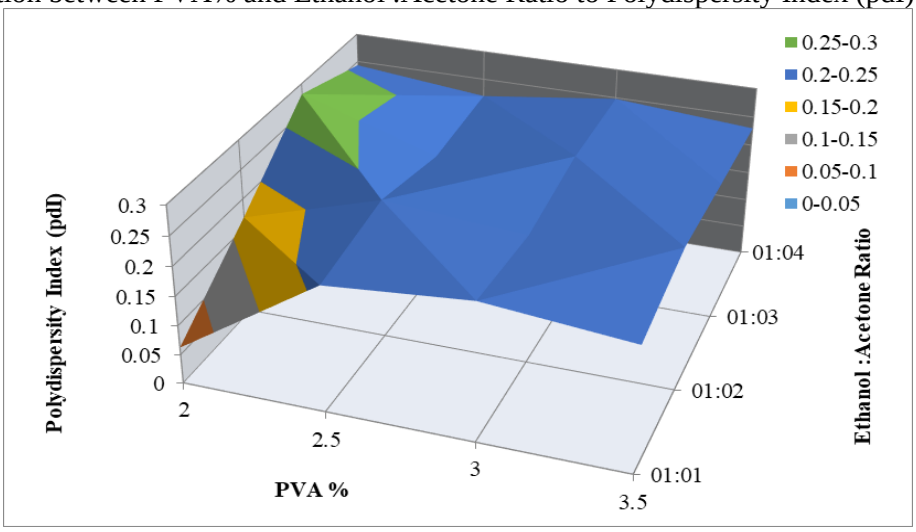


Figure 9c Relation between PVA% and Ethanol :Acetone Ratio to Zeta potential (mv)

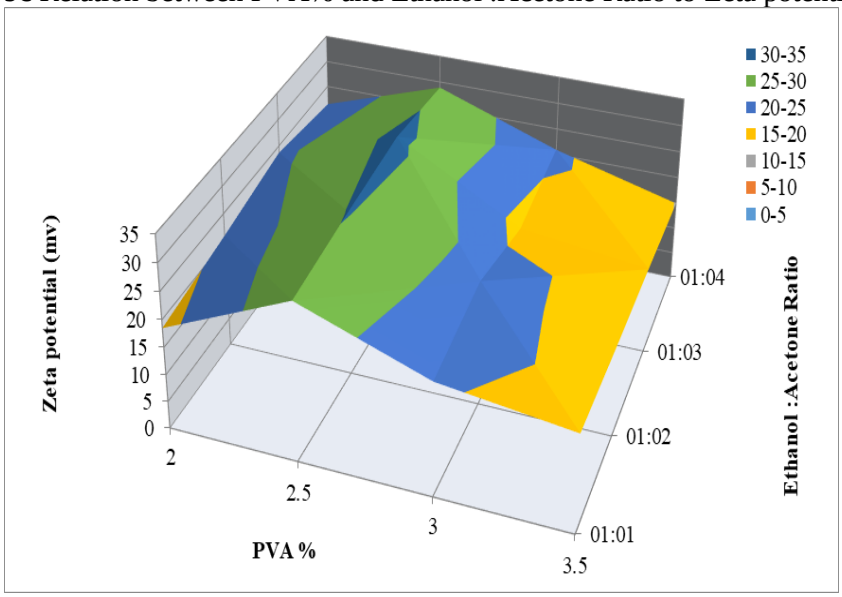
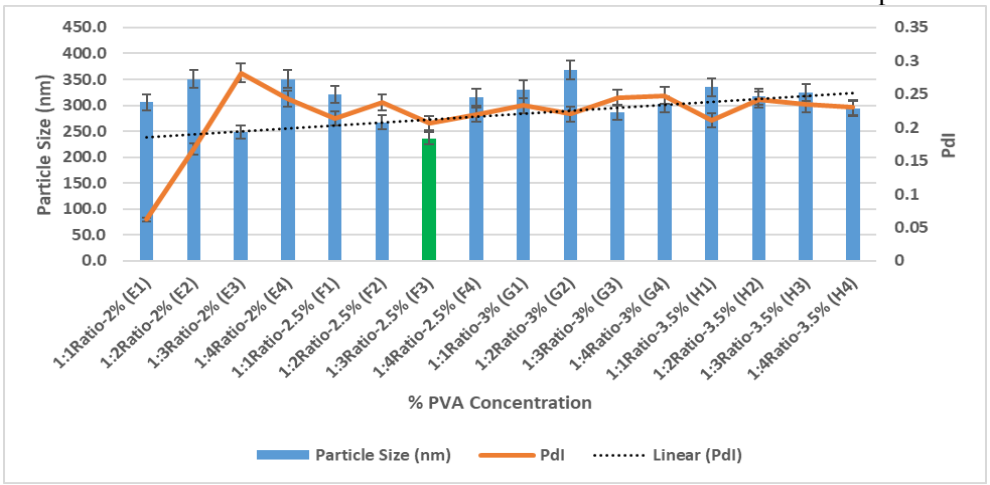


Figure 9d Overall relation between PVA% and Ethanol :Acetone Ratio to Zeta potential&pdi



Observation: PVA at different concentrations, including 2%, 2.5%, 3.0%, and 3.5%, has been used as a stabiliser and polymer for preparing nanoparticles. Different stirring speeds of 500

rpm, 750 rpm, 1000 rpm, and 1500 rpm were tried for the formulation of NPs. Figure 8 & 9 shows that on increasing the concentration of PVA from 2.0% to 2.5% and stirring speed from 500 rpm to 750 rpm, the particle size of nanoparticles (Figure 8a) and pdI (Figure 8b) was decreasing, but the zeta potential was increasing (Figure 8c) when compared with an increased particle size on increasing the concentration of PVA above 2.5% and stirring speed above 750 rpm. Various combinations of ethanol, acetone (1:1, 1:2, 1:3, 1:4) as solvent, and PVA (2.0%, 2.5%, 3.0%, and 3.5%) have been tried for the preparation of usnic acid nanoparticles. Ethanol and acetone are both less toxic and also produce suitable nanoparticles on precipitation. From the studies, the effect on particle size has been observed at different solvent ratios, and it was found that at 2.5% PVA and an ethanol: acetone (1:3) ratio, optimum and uniformly sized nanoparticles were formulated, as shown in Figure 9. On decreasing the solvent ratio and at lower concentrations of PVA, the particle size and pdI increased, but the zeta potential decreased. On increasing the solvent ratio and at intermediate levels of PVA, the particle size (Figure 9a) and pdI (Figure 9b) decreased, but the zeta potential (Figure 9c) increased.

4.4. Physicochemical parameters

Characterizations of physicochemical parameters like Viscosity, pH and Spreadability of both nanoointment i.e. UANP and UGNC was carried out. pH of final formulation of both nan-ointment i.e. UANP and UGNC was near to the skin pH i.e. 6-7, viscosity lies between 15-17 cps and Spreadability 20-21. Shows that both nanoointment having pH which is near to skin so its nonirritant.; Viscosity and Spreadability parameters are encouraging shows the formulation exhibit good Spreadability which facilitate to meet the patient compliance. Results of physicochemical parameters are presented in Table 3 and figure 10.

Table 3: Physicochemical results of Nanoointments

Formulations type	pH	Viscosity (Cps) Mean ± SD	Spreadability
UANPs	6.28	15.82 ± 1.32	21
UGNC	6.21	17.01 ± 1.78	24

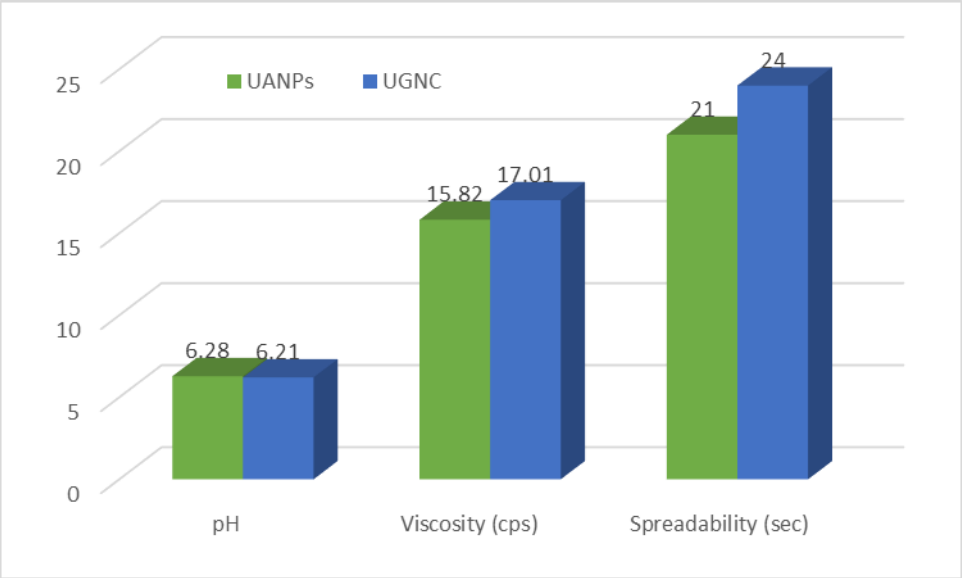


Figure 10 Physicochemical characterization of Nanoointment

4.5. In vitro release analysis

In vitro drug release was found to be 97.92% and 66.32% for UANP and UGNC respectively in 480 minutes. Release profile of UGNC was found to be on lower side in comparison to UANP shows that this could be because of the hindrance caused by the ointment base and Graphene matrix. However plain Usnic acid dispersion has shown 99% drug release in 360 minutes itself. Graphical representation of comparative dissolution profile is presented in figure 11.

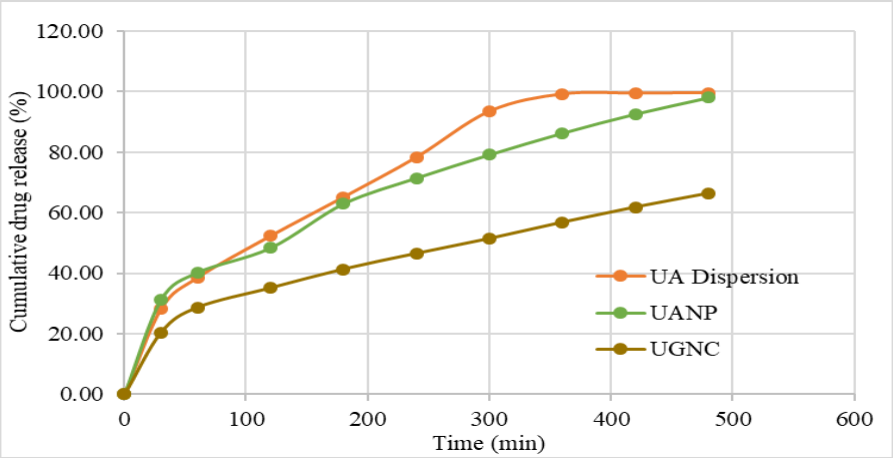


Figure 11 Comparative In-vitro release profile

4.6. In-vivo antifungal activity

In vivo antifungal activity was carried out on fungal strain of Candida albicans (MCCB 0290). Outcome of in vivo antifungal activity is presented in table 4 and graphically represented in figure 12. In vivo results shows that

UADN shown poor control over the fungal infection, as five animals out of six were positive in the culture test, whereas UANP showed moderate control over the fungal infection, as three animals out of six were positive in the culture test, whereas a synergistic effect was observed in UGNC, where out of six animals, only two were positive in the culture test. However, in the standard market formulation, only one animal out of six was positive in the culture test. The antifungal activity of the UGNC and marketed formulations is quite close despite having steroid in the marketed formulation, which could have a similar antifungal effect. This study clearly demonstrates that without having any steroid, i.e., Beclomethasone dipropionate 0.025 % in the UGNC formulation, the closer pharmacological effect proves that this formulation has more pharmacological potential than that of the marketed formulation.

Table 4: Results of in vivo antifungal activity of various treatment type

Group Number	Treatment type	No. of animals with positive culture	Total animals	Infected sites/ Mean CFU	S.D.
Group I	Control	6	6	16.10	9.01
Group II	UADN	5	6	11.13	4.22
Group III	UANP	3	6	3.78	3.20
Group IV	UGNC	2	6	1.46	1.16
Group V	Standard	1	6	1.12	0.65

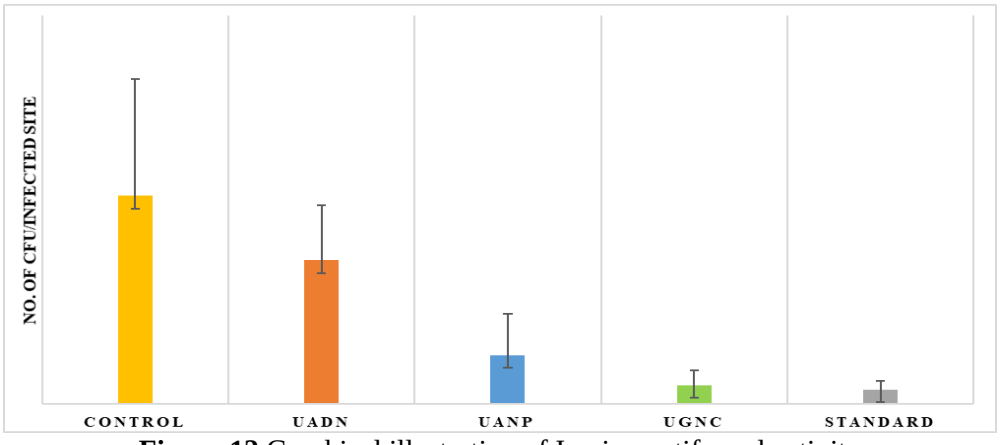
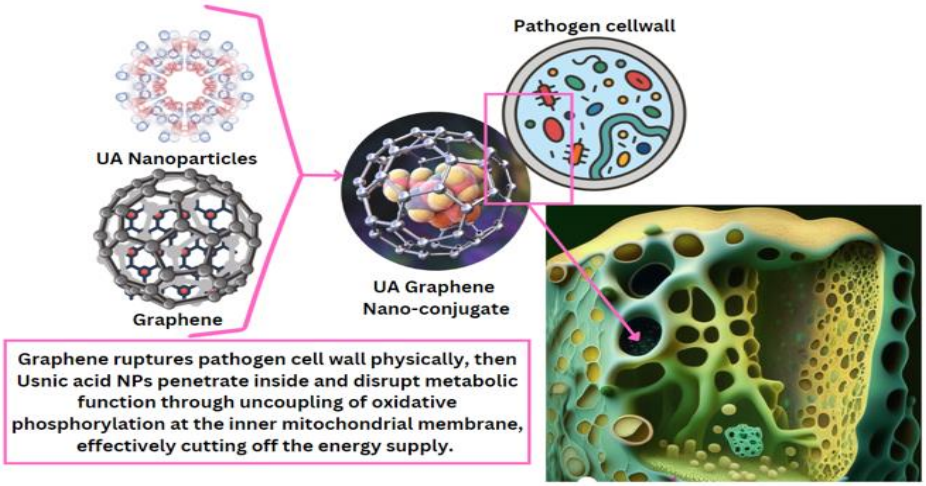


Figure 12 Graphical illustration of In vivo antifungal activity

5. Discussion

In the present study, a novel tool has been developed to eradicate microorganisms that are resistant to antimicrobial resistance (AMR) and are applied topically. This tool offers new research in the field of AMRs, which the WHO considers to be a worldwide threat. Two new raw materials were selected to demonstrate this idea: one was usnic acid, a lichen derivative with demonstrated antimicrobial activity, and the other was graphene, which serves as a carrier and also has antimicrobial properties. As a dosage form, NDDS, or nanoparticles, were chosen by dispersing into the water-soluble ointment base, which facilitates the drug's delivery to the site of infection.



Usnic acid nanoparticles were produced, conjugated with graphene, and then dispersed into the base of the ointment. The base of the ointment was made water-soluble to allow for greater penetration into the afflicted area. The final formulation's physicochemical results showed that the nanoointment has a balanced pH and good rheological properties, which makes the formulation patient-centric to ensure patient compliance through skin application.

The particle size of the UANP and UGNC was reported < 250 nm, which would help to directly attack the pathogens and rupture the cell wall directly as this would be in the ointment base, so escaping of the pathogen from the site would be vanished. In fact, the same phenomena have been reflected in in-vitro dissolution and in vivo activity, where in the case of the UGNC formulation, in vitro dissolution was found to be on the slower side among all the formulations, but it's in vivo activity was found to be encouraging. This could be the impact of the combination therapy that resulted in the synergistic effect.

On the other hand, the antifungal activity of the UGNC is better than marketed formulations despite the presence of steroids in the marketed formulation, which could boost the antifungal effect. This study clearly demonstrates that without having any steroid, i.e., Beclomethasone dipropionate 0.025 % in the UGNC formulation, the closure pharmacological effect proves that this formulation has more pharmacological potential than that of the marketed formulation. This could be because of the graphene, as graphene first ruptures the fungal cell wall and creates a channel to penetrate drug inside the fungal cell, which further augments the eradicating process of pathogen by stopping the energy supply to the fungal cell wall through mitochondria.

6. Conclusion

Present research concludes that usnic acid-graphene nanoconjugate (UGNC) has proven antimicrobial results against superbugs. This formulation is patient-friendly and easy to apply. The combination effect of drug and carrier has been proven and is a great tool to eradicate microbial infections as well as superbugs.

Declarations

Ethical Approval

Authors have followed all applicable international, national and/or institutional guidelines for the care and use of animals. The animal studies were accomplished according to CCSEA guidelines. The animal studies were approved (Approval no. UIP/IAEC/Sept-2020/07) by IAEC of United Institute of Pharmacy, Prayagraj, India.

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Authors' contributions
PKV has worked on the all-experimental work and written the manuscript. RAG and SBM have supervised and reviewed all experimental work and statistical analysis. GJ and KhV have prepared figures and graphics. All authors reviewed the manuscript.

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Consent for publication
The consent of all the authors has taken to publish the research in this journal.

Highlights:
Concept of this fundamental research breach the pathogen cell walls and then delivery of the antimicrobial drugs to their cell and led to cell death.

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