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Research Article

In Vitro and in Vivo Evaluation of Nanoparticles based Ointment with Enhanced Antifungal Efficacy

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Abstract

A fungus infection of the skin is one of the most prevalent dermatological conditions worldwide. One of the most effective dosage forms for treating skin infections topically is an ointment formulation. Nanotechnology is a promising approach for penetrating the deeper skin layers and improving the permeability of Usnic Acid (UA) via the stratum corneum. Using the solvent evaporation method, UA-loaded Nanoparticles (UANP) were manufactured. Consequently, UGNC was incorporated into an ointment base having water-soluble excipients, i.e., PEG400, PEG4000, SLS, and glycerin, which provide sufficient consistency to the ointment. The in vitro and in vivo antifungal activities have been evaluated. The nanoointment of Usnic acid NP's Graphene Nanoconjugate (UGNC) has a greater ability to destroy infections due to the threefold action of combination therapy with US, NPs, and their conjugate with GN. In vitro study results revealed that UANP ointment and UGNC ointment effectively suppressed the growth of C. Albicans when compared. Also Despite hindrances with the in vitro dissolution profile, UGNC nano-ointment's in vivo anti-fungal effectiveness on Wistar albino rats demonstrates a significant positive result and is better than market preparations with steroids.

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

Introduction

The human body's outermost covering, the skin, serves a variety of vital purposes, but its protective function may be the most challenging. The outermost epidermal layer of skin, known as the stratum corneum, serves as the main regulatory barrier to the transcutaneous traffic of water and exogenous materials like bacteria or fungi [1].

When skin is attacked by an external substance like a fungus, the stratum corneum's structure transforms, affecting the permeability of the skin [2]. Fungal infections are becoming more common nowadays, particularly in people with impaired immune systems. The correlation between the causative fungus and diseases like AIDS or the increased use of immunosuppressive medications during this time period is to blame for the surge in the frequency of systemic and cutaneous fungal infections. When stem cell organ transplantation, solid organ transplantation, and neonatology see scientific advancements [3]. The subcutaneous tissue is where topical fungal infections are most common. They may have an invasive nature and have the ability to penetrate the epidermis deeply [4].

Both the location and the presence of particular fungi affect how severe the infection is [5,6]. Multiple studies have revealed that a number of substances from various classes, including polyenes, azoles, echinocandins, nucleoside analogues, and allylamines, can treat fungal infections. The type, location, and susceptibility of the fungal species all affect their efficacy [7,8]. The occurrence and spread of fungal skin infections are greatly influenced by geographic and environmental factors.

Fungal infections are produced by tiny organisms that can infiltrate epithelial tissues. Moulds, yeasts, rusts, and mushrooms are all members of the kingdom of fungi. Animals and fungi both use hetrotrophic improvement, which means that they get their nutrients from their surroundings rather than from within themselves (as plants do with photosynthesis) [9]. However, some fungi can result in infectious diseases if they penetrate the skin through wounds or the lungs and nasal passages when inhaled [10]. The majority of fungi serve a purpose and play a significant part in biodegradation. A superficial skin infection brought on by dermatophytes like Microsporum, Trichophy-

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ton, or Eepidermophyton is one of the diseases brought on by fungi. By taking advantage of the distinctions between mammalian and fungal cells, anti-fungal drugs can destroy the fungus without endangering the host. However, side-effect-free results from synthetic antifungal medications are still required [11].

In the most recent studies, nanotechnologies have received a lot of interest. New technologies employed for manufacturing devices and sample preparations have an impact on the advancement of nanoscience. Nanoparticles are employed for the goal of targeted drug delivery. By increasing their bioavailability, they enhance the drug's performance. Nanoparticles are colloidal structures with nanoscale dimensions formed from synthetic and semi-synthetic polymers [12]. Compounds that are poorly soluble in water undergo exposure to the nanonization process to accelerate rapid dissolution and increase bioavailability. The term "nanoparticles" refers to drug delivery systems with particle sizes between 10 and 1000nm, based on the method of formation and the materials employed [13].

Nanocarriers are becoming increasingly popular in topical delivery systems because of their enhanced penetrability and passive accumulation at the target site [14]. Topical Drug Delivery Systems (TDDS) are frequently readily developed in liquid, solid, and semisolid dosage forms and are primarily intended to deliver a therapeutically effective concentration of medication in the skin or mucosal layers [15]. Topical formulations offer certain benefits over oral or parenteral dosage form alternatives, such as a lesser probability of unavoidable adverse reactions. Dermatologists favour topical semisolid formulations for the treatment of skin diseases and superficial infections [16]. Usnic acid, one of the most extensively studied secondary metabolites of lichens, possesses a range of physiological characteristics that can be utilised in medicine, including antibacterial, anticancer, and wound-healing effects [18-20]. Usnic Acid (UA) is a yellowish crystalline powder. It is a product of the potent new class of antifungal substances identified as dibenzofurans. It works by preventing infections from synthesising DNA and RNA [17]. Graphene Materials (GMs) are under investigation for a variety of microbiological applications due to their unique physicochemical properties, such as high electrical conductivity, a large specific surface area, and high mechanical strength. It is a 2-D material made up of carbon atoms structured in a crystal lattice that resembles a honeycomb. Both gram-positive and gram-negative bacteria are susceptible to graphene's antimicrobial properties [18,19].

GMs with antifungal characteristics have the ability to directly kill or starve prokaryotic cells. It is believed that membrane tension, oxidative stress, and wrapping isolation lead to the GMs' capacity to eradicate bacteria [20,21]. When there is membrane stress, GMs kill bacteria by penetrating the membrane(s) and extracting the phospholipids while maintaining the integrity of the cell [22]. Reactive Oxygen Species (ROS), which can be produced by GMs when there are bacteria present, are a part of oxidative stress [23]. Whenever bacteria are exposed to oxidative stress, their proteins, membrane lipids, and nucleic acids are all oxidised and damaged, which leads to cell death [24]. By wrapping fungal cells with GM sheets, it is possible to isolate them from their growing medium [25,26]. Usnic acid and graphene have both been used in recent studies and both have antifungal properties [27]. In order to combat superbugs, new nanoointments have been developed that use low-water-soluble drugs as a weapon to destroy pathogens.

Material and Methods

A graphene sample was purchased from the Bengaluru-based GRL (Graphene Research Lab). PEG 4000 and PEG 400 samples were provided by Himedia in Mumbai, India, while Candida Albicans (MCCB 0290) came from the Microbial Culture Collection Bank in SHUATS Prayagraj, India. Chinese company Hubei Honghan Biotech provided the usnic acid. All compounds used in the study were of analytical grade.

Formulation of Nanoparticle and Its Nanoconjugate

(UANPs) Usnic Acid Nanoparticles has been manufactured through Nanoprecipitation method obtained NPs were cooled down to −40°C and freeze dried. For nanoconjugate, physiosorption method was employed to conjugate NPs on the 2D structure of the Graphene. Obtained nanoconjugate was dried at 40°C in the hot air oven.

Usnic acid NPs Conjugation on graphene shown in Figure 1.

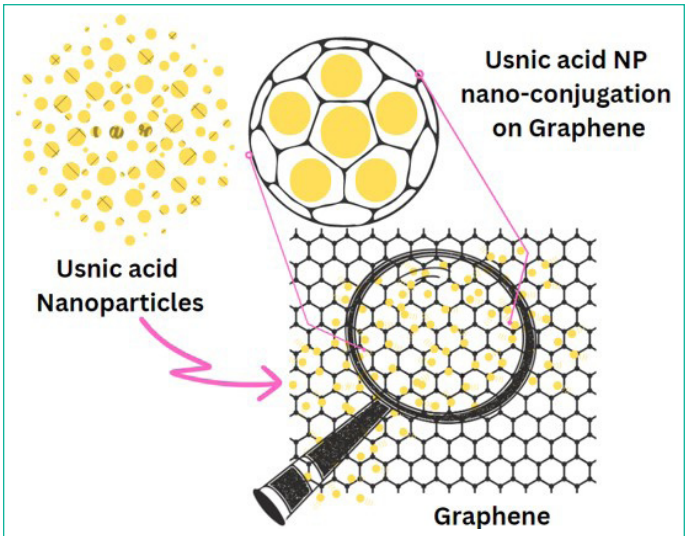

Figure 1: Conjugation of Usnic Acid nanoparticles (UANPs) and Graphene.

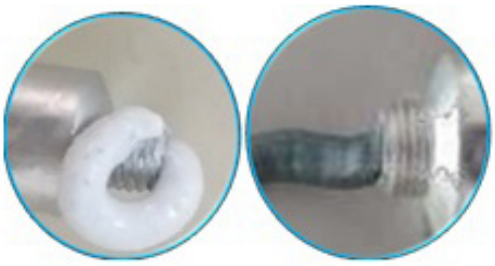

Figure 2: Description of nano ointment (2a. Usnic Acid Nanoparticles (UANP) & 2b Usnic Acid Graphene Nanoconjugate (UGNC).

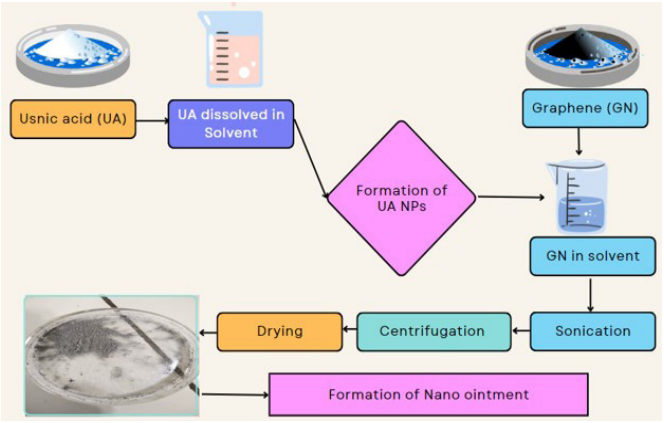

Figure 3: Overview of entire manufacturing Process.

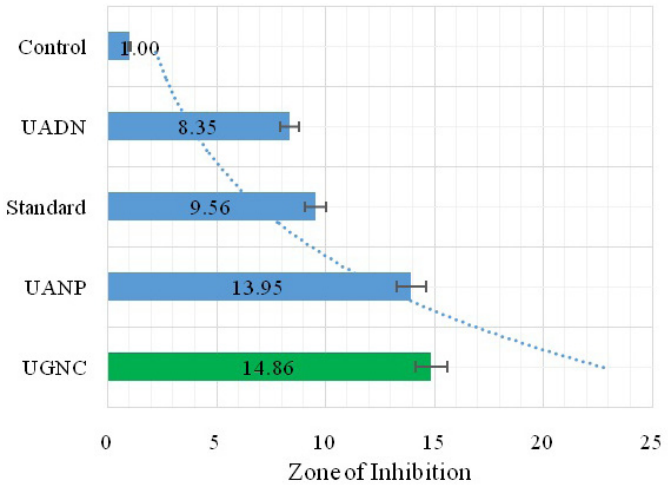

Figure 4: In-Vitro antifungal activity.

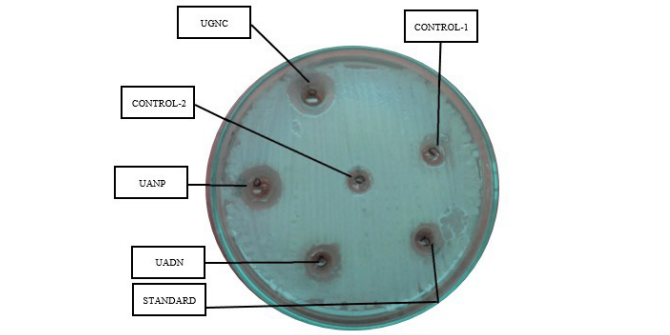

Figure 5: In-Vitro antifungal activity on Agar plate.

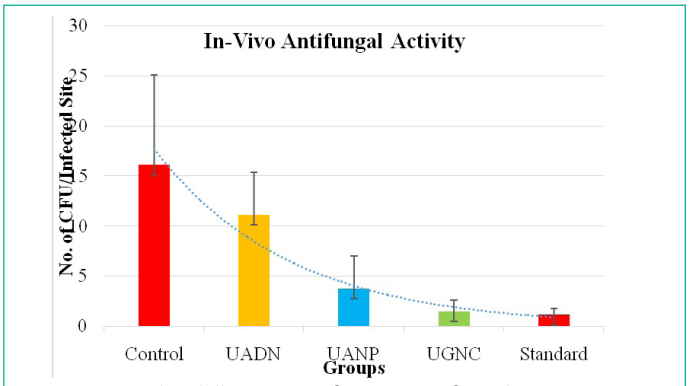

Figure 6: Graphical illustration of In vivo antifungal activity.

Table 1: Table In-Vitro antifungal activity.

Treatment	Zone of Inhibition	S.D.
UGNC	14.86	1.46
UANP	13.95	1.54
STANDARD	9.56	1.32
UADN	8.35	2.76
CONTROL	1.00	0.00

Table 2: 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

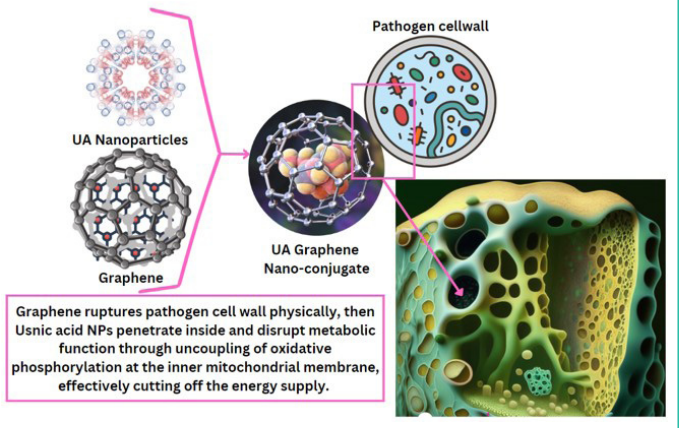

Figure 7:

Preparation of Ointment Base and Nanoointment of Graphene-Usnic Acid Nanoconjugate

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. Among all best one has been selected based on the pH, Spreadability and Viscosity. And for preparation of nanoointment of Graphene-Usnic acid nanoconjugate, geometric dilution method was used, in which Usnic acid NPs were gradually added to the ointment base till uniformly homogenised then filled into Alu-Alu tube. Figures 2a and 2b, referring to the ointments with usnic acid nanoparticles (UANP) and Usnic Acid-Graphene Nanoconjugate (UGNC), respectively. An overview of the entire manufacturing process is presented in Figure 3.

In Vitro Antifungal Activity

Fungicidal activity test: A series of test tubes containing 10ml of SG broth medium incorporating from 0 to 25µg of nano-conjugate per ml were prepared, inoculated with 0.1ml of a standard spore suspension of Candida albicans (MCCB 0290), and incubated at 25°C for 10 days. Samples (5ml) were taken after 10 days of exposure to the drug, washed twice with large volumes of sterile saline to remove unabsorbed nano-conjugate, and re-suspended in 0.5ml of sterile saline; 0.2ml samples of this suspension were spread over each of the SG agar slants, and the survival or death of the spores was determined by the presence or absence of colonies that developed within 15 days of incubation.

In Vivo Antifungal Studies

To test the antifungal activity, Wister albino male rats between the weights of 100 and 150g 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. 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.

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.

Results

In Vitro Antifungal Activity

The in vitro anti-fungal activity of the optimised formulation of nano-ointment was investigated against Candida albicans (MCCB 0290). The zones of inhibition were obtained. The diameter of the zone of inhibition was measured by an antibiotic zone finder. Readings were taken in triplicate. Results of in vitro anti-fungal activity are presented in Table 1 and graphically represented in Figures 4 and 5.

Observation: The agar diffusion method was used for the microbiological study (Figure 5). The mean diameters of the zone of inhibition against C. albicans were: standard marketed formulation, 9.56±1.32mm, usnic acid dispersion, 8.35±2.76mm; UANP ointment, 13.95±1.54mm; and UGNC ointment, 14.86±1.46mm.

Results showed that UANP ointment and UGNC ointment significantly inhibited the growth of C. albicans when compared with Usnic acid dispersion despite having four and eight times lower concentrations, respectively ($p<0.05$). This is due to the nanoparticulate structure of usnic acid present in both the UANP and UGNC ointments, resulting in greater penetration into the agar medium facilitating higher antifungal effect. Whereas the inhibition zones of UANP ointment and UGNC ointment were found identical ($p>0.05$). However, the UGNC ointment was found to have a greater ability to inhibit the growth of C. albicans when compared to the simple UANP ointment. This was due to the fact that UGNC ointment caused more inhibition (a greater zone of inhibition) despite having a two-fold lower concentration than UANP ointment. This was due to the conjugation of the drug usnic acid and graphene in UGNC ointment, which facilitated the controlled release accompanied by a higher penetration ability of usnic acid, leading to higher antifungal activity.

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 2 and graphically represented in figure 6. 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.

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 <250nm, 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.

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.

Author Statements

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.

Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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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Availability of Data and Materials

Respective data shall be provided through cloud link based on the reviewer requirement.

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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