



Formulation, Characterization And In Vitro Antimycotic Activity Of Usnic Acid Loaded Graphene Nano Ointment For Topical Infections

3612

Pradeep Kumar Vishwakarma^{1*}, Revathi A. Gupta²

*Corresponding author: - Pradeep Kumar Vishwakarma

Address: - ^{*1, 2} Institute of Pharmacy, Dr. A.P.J. Abdul Kalam University, Indore 452016, India

E-mail:- pradeepkv@live.com

Abstract

Usnic acid, a secondary metabolite obtained from various species of lichens possess various pharmacological activities including antibacterial and antifungal activity. Due to the poor water solubility of this secondary metabolite its absorption could be limited. This study aims to formulate a nano ointment containing Usnic acid and Graphene and evaluate for antifungal activity. Usnic acid nanoparticles has been prepared by precipitation method and conjugated with Graphene. The prepared formulations were physiochemically evaluated for size, zeta potential, and in vitro release study. The prepared formulations were investigated for antifungal activity against *Candida albicans* (*C. albicans*). The prepared NPs were found in the nano range. Images taken by a Transmission electron microscope showed that all prepared NPs were spherical and smooth. The drug release study showed a prolonged drug release from nanocomposite. In addition, the formulations exhibited a potential antifungal effect against *C. albicans*. From current study, it was concluded that the development of Usnic acid loaded Graphene nano ointment has a great potential for the topical delivery against fungal infections.

Key words: - Antifungal, Graphene, nanoointment, TEM, Usnic acid

Number: 10.14704/nq.2022.20.7.NQ33443

Neuro Quantology 2022; 20(7):3612-3617

Introduction

Fungal infections are caused by microscopic organisms that can invade the epithelial tissues. The kingdom of fungi includes moulds, yeasts, rusts and mushrooms. Like animals, Fungi are heterotrophic, viz. they obtain nutrients from the environment, not from endogenous sources (like plants using photosynthesis) [1]. Most fungi are valuable and having major role in biodegradation; however, a few can cause opportunistic infections if they enter into the skin through wounds, or into the lungs and nasal passages when inhaled [2]. Diseases caused by fungi include superficial infection of the skin by dermatophytes like *Microsporum*, *Trichophyton*, or *Epidermophyton*. Anti-fungal agents work by exploiting the differences between mammalian and fungal cells to kill the Fungal organism without dangerous effects on the host. However. The satisfactory responses of synthetic antifungal drugs are awaited without side effects [3]. Now a day the research is going in a way to use the herbs or some special species like lichens to cure the fungal infections by isolating their chemical constituents and secondary metabolites. The current research

showing that the symbiotic species between algae and fungi that is lichen is a promising genera that contain various chemical constituents that show their antimicrobial properties. In this context the proposed work is concerned with a potent chemical that has been explored for various biological activities isolated from lichen i.e. Usnic acid [4].

Lichens are a world-widespread association of fungal and photosynthetic partners. Usnic acid is secondary lichen metabolite obtained from several lichen genera such as *Usnea* (*Usneaceae*), *Cladonia* (*Cladoniaceae*), *Ramalina* (*Ramalinaceae*), *Lecanora* (*Lecanoraceae*), *Alectoria* (*Alectoriaceae*) and *Parmelia* (*Parmeliaceae*). It has been demonstrated previously to inhibit growth of different bacteria and fungi. Usnic acid shows various activities such as anti-myotic activity, anti-protozoal activity, antioxidant activity, cytotoxic activity and anti-viral activity [5].

Low solubility of Usnic acid in water is the barrier to develop a formulation for oral care, topical ointments, and cosmetic formulations



[6]. Nanonization have been applied to poorly water-soluble compounds in order to increase their dissolution rate and improve bioavailability. Graphene based material are a promising nanomaterial for the development of antibacterial surfaces owing to their biocidal activity [7]. The antimicrobial activity of Graphene has been found to be the synergy of both “physical” and “chemical” effects. When bacteria directly contact with Graphene, intensive physical interactions between Graphene and bacterial cells may cause physical damages on cell membranes, and result in the release of intracellular contents [8]. Since Usnic acid and Graphene having potent antimicrobial property against number of microorganism and poorly water soluble hence an attempt has been made to explore its antimicrobial property as an antifungal through the development of novel nano-ointment.

Materials and Method

Graphene was procured as gift sample from Graphene research lab, Bengaluru, Usnic acid was purchased from Hubei Honghan Biotech Co., Ltd. China. Polyethylene glycol (PEG 400 and PEG 4000) were obtained from Himedia, Mumbai, India. Fungal strain, i.e., *Candida albicans* (MCCB 0305) was procured from Microbial Culture Collection Bank, Department of Microbiology & Microbial Technology, Sam Higginbottom University of Agriculture, Technology and Sciences Prayagraj, India. All the reagents and chemicals used in the study were of analytical grade.

Preformulation studies

The preformulation studies for the drug Usnic acid and ointment bases have been performed to assure safety, stability and effectiveness of the formulation. Moreover, the nano- carrier Graphene has been characterized for its genuinity.

Preparation of Usnic acid nanoparticles

Nano-precipitation technique was used to prepare Usnic acid nanoparticles (UANPs) with slight change of a previously reported method [9]. Briefly, 3g of UA was dissolved in 30 ml of ethanol and acetone (1:3) by ultra-sonication at 40 W for 15 s. The resulting 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 polyvinyl

alcohol (PVA) with continuous stirring at 750 rpm. The resulting emulsion obtained was then diluted with 100 ml PVA solution (0.5% w/v in water) in order to minimize coalescence and the mixture was continuously stirred (750 rpm) for 24 h at room temperature to allow evaporation of solvent and nanoparticles formation. The resulting Usnic acid nanoparticle was subsequently cooled down to -40°C and freeze dried.

Conjugation of Usnic acid nanoparticles and Graphene

Usnic acid nanoparticles were loaded onto the Graphene via simple physisorption. Graphene suspension (0.150 mg/ml) was sonicated with Usnic acid (2 mg/ml) at pH 5 for 30 min. and then stirred overnight at room temperature in the dark. Finally the sample was ultracentrifuged at 20000 rpm for 1 h. The prepared nanoconjugate was lyophilized and subjected for the characterization.

Preparation of water-soluble ointment base

The water-soluble ointment bases were prepared by using different grades of Polyethylene glycol (PEG), glycerine, and surfactant and purified water. Briefly, water soluble ointment base was prepared by melting the PEG-4000 on a hot plate/ stirrer (at 70°C) followed by addition of liquid PEG- 400 and glycerin. Sodium lauryl sulphate was mixed to the melted base with continuous stirring. Then the base was cooled with stirring until congealed. Total six formulations of bases with different ratios of PEG 4000 and PEG 400 have been prepared and best one was selected on the basis of their pH, Spreadability and Viscosity.

Formulation of nano-ointment of Graphene-Usnic acid nano-conjugate

The process of geometric dilution has been used for the preparation of nano-ointment. The optimized water-soluble ointment base (PEG 400: PEG 4000 1:1) has been used for nano ointment. The ointment of Graphene- Usnic acid nano-composite (UGNC) had been formulated in a concentration of 0.5% w/w (Fig.1B). Also, the ointment of Usnic acid nanoparticles (UANP) had been formulated in a concentration of 1 % w/w (Fig.1A).

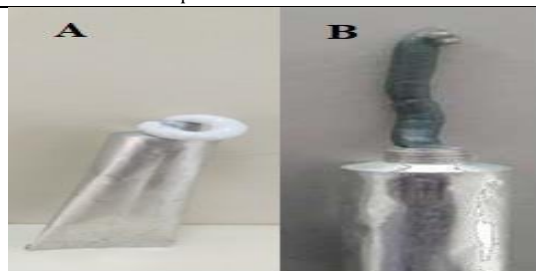


Fig. 1: (A): Ointment containing UANPs. (B): Ointment containing UGNC

Morphological and particle size analysis

The mean particle size of nanoparticles was analyzed by dynamic light scattering, Zetasizer ver. 7.11 Malvern Instrument limited. The size distribution analysis was carried out at a scattering angle of 90° and at a temperature of 25 °C. Furthermore, Morphological analysis was performed by High Resolution Transmission Electron Microscopy (HRTEM).

FTIR Analysis

In this study, the FTIR analysis was performed to identify the potential interaction between the drug and the carrier. FTIR analysis was performed on Usnic acid, Graphene and on Usnic acid- Graphene nano-conjugate. For this, the samples were mixed with KBr and punched to a tablet applying hydraulic press. The FTIR spectra was recorded at 4,000 cm⁻¹ to 450 cm⁻¹ using FTIR Spectrometer (PerkinElmer Spectrum Version 10.4.00) [10].

In vitro drug release Study

The in vitro drug release study of Graphene-Usnic acid nano-composite (UGNC) containing ointment, Usnic acid nanoparticles (UANP) containing ointment and plain Usnic acid dispersion (2 % w/v) were performed in triplicate using diffusion cells, whereby a dialysis membrane with a molecular weight cut-off (MWCO) of 12,000- 14,000 Da, separated the acceptor compartment from the donor compartment, consisting of drug equivalent to 10 mg. The release studies were conducted in phosphate buffer at pH 7.4. Sink conditions and constant temperature (37 ± 0.5 °C) were maintained throughout the study. Samples were quantitatively analyzed spectrophotometrically using Systronics 10 UV- Vis spectrophotometer at 290 nm. All measurements were performed in triplicate and the SD was calculated.

Physicochemical Characterization

The Physicochemical characterizations viz. determination of pH, determination of viscosity, and determination of spreadability of the Nano-ointment were performed as per the earlier reported methods [11].

In vitro antifungal studies

Candida albicans strains (MCCB 0305) isolates were procured from Microbial Culture Collection Bank, Department of Microbiology and Microbial Technology, Sam Higginbottom University of Agriculture Technology and Science, Prayagraj Uttar Pradesh. All isolates were kept on Sabouraud Dextrose Agar (SDA) media.

The cup-plate method was used to evaluate the antifungal activity of different formulations. Briefly, SDA media was prepared by comprising of 3 g dextrose, 2 g agar, and 1 g mycological peptone. Prepared SDA (5g) was dissolved in 100 mL of distilled water with continuous heating and agitation followed by autoclaving at 121 °C for 15 min.

Three sterile 93 mm glass petri dishes were taken and the media was aseptically poured into the dishes. The plates were kept for solidification. As the agar medium solidified, a sterile stainless-steel borer was used to pierce the surface of the agar plate to make small wells of 6mm in all three petri dishes. The *C. albicans* were inoculated by streaking of the surface of the agar medium. The Usnic acid dispersion (200 µl) was taken and placed in the first dish. In the second dish, 100 mg of UGNC ointment was placed, while holes of the third dish were subjected to 100 mg of UANP ointment. After subjecting the three samples, the petri dishes were kept and left for at least 30 min and were incubated for 24 h at a temperature of 25°C. After 24 hours, the most uniform outside diameter of the zone of inhibition in millimeters was recorded with a standard ruler. The diameter of the zones, including the diameter of the well, has been recorded. Each assay was performed in triplicate [12].

Statistical analysis

Data were expressed as Mean ± S.D. Results were analyzed statistically using one-way ANOVA test followed by Student Newman Keuls test. *p < 0.05 considered significant.

Results and Discussion
Preformulation studies

Pre-formulation is a process to check the authenticity of a drug by determining their properties i.e., the physical and chemical properties, which are important factors in the development of a good quality drug i.e., the drug possess good stability, high efficacy and prolonged shelf life. Pre-formulation study merely confirms that there are no significant barriers to the development of compound. Table 1 shows the preformulation studies of prepared ointment base and Usnic acid which was performed to assure safety, stability and effectiveness of the formulation.

Table 1: Pre-formulation studies of ointment base and Usnic acid

S.No	Preformulation parameters	Ointment base	Usnic acid
1.	Physical appearance	Off-white semisolid	White amorphous powder
2.	Odour	Mild Aromatic	Odourless
3.	Taste	Characteristic	Bitter
4.	Melting point	52.6 °C	221°C
5.	Solubility in water	Soluble	Insoluble
6.	Spreadability	Moderate to easy	--
7.	Washability	Washable	--
8.	Occlusiveness	No	--
9.	Chemical test for identification	Passed	Passed

Conjugation of Usnic acid to Graphene nanosheets

The rationale behind selecting pH 5 for loading of Usnic acid on to Graphene oxide was considering that at this low pH hydrogen bonding between the (-OH) and (-COOH) groups of Graphene oxide and the (-OH) groups of Usnic acid can occur..On the other hand under basic conditions, (-COOH) of Graphene oxide exists as (-COO⁻) and cannot form hydrogen bonds with any group of Usnic acid. Therefore, at pH 5 comparatively strongest hydrogen bonding interaction is expected between Graphene oxide and Usnic acid.

Characterization of Nano formulations TEM analysis and zeta potential measurement

Transmission electron microscopy (TEM) is considered to be the most popular technique in characterizing nanomaterials in electron microscopy. TEM is a surface imaging method in which the incident electron beam scan across the sample surface and interact with the sample to generate the signals. The size distribution and shape of nano-material can be directly obtained from TEM. The TEM sample must have 100 nm thickness, so electrons can pass through the specimen, depending on sample density and

electron acceleration voltage [13]. The shape of the nanoparticles is of spherical in nature.

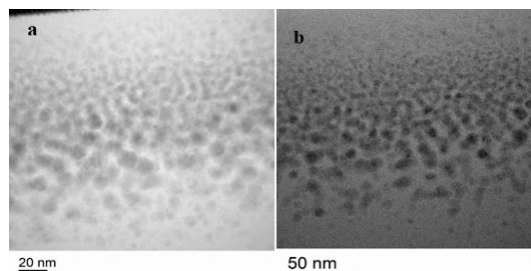


Fig. 2: TEM image of a) UANPs and b) UGNC

The mean size of the prepared nano- conjugate and polydispersity index was found to be 100.46±5.23 nm and 0.27 respectively. The shape of the nano-composite is of spherical in nature. Zeta potential of prepared nanoparticles was -12.7±6.46 mV as shown in figure 3.

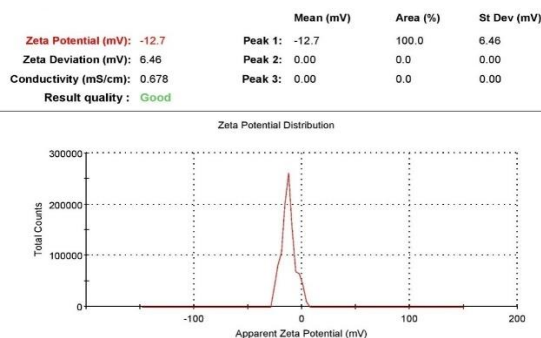


Fig. 3: Zeta potential of UGNC

FTIR Analysis

FTIR spectra were recorded to assess the compatibility of the pure drug and formulated compound. FTIR spectra of drug, Graphene and conjugate were examined. All characteristic peaks of Usnic acid, Graphene and conjugate were ascertained in the FTIR test of nano-conjugate. The results showed that there is no chemical interaction or alteration took place during formulation of nano-conjugate. FTIR spectra of Usnic acid showed characteristic peaks of C-H stretch at 2931.10cm⁻¹, C=O stretch at 1692.03cm⁻¹, C-O stretch 1000-1200cm⁻¹, O-H aromatic stretch at 3091cm⁻¹, C-H aromatic stretch at 700-900cm⁻¹, C=C aromatic stretch at 1600-1675cm⁻¹ were obtained. FTIR spectra of Graphene showed characteristic peak at 1600cm⁻¹ was obtained. Graphene Usnic acid conjugate showed FTIR spectra at C-H stretch at 2929cm⁻¹, C=O stretch



at 1631cm^{-1} , C-O stretch at $1000\text{-}1200\text{ cm}^{-1}$, C-H aromatic stretch at $700\text{-}900\text{ cm}^{-1}$, O-H aromatic stretch at 3091 cm^{-1} . The results of the FTIR test showed that there is no chemical interaction took place during the formulation of nano-conjugate and drug was found to be compatible with Graphene.

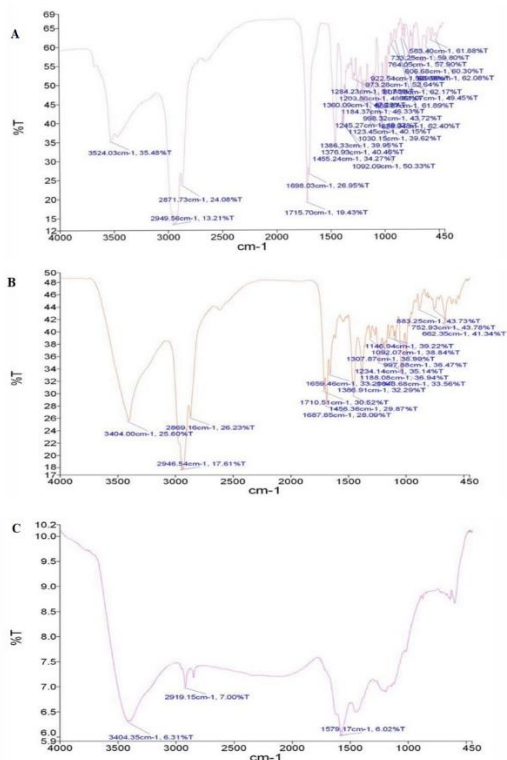


Fig. 4: FTIR spectra of A: Usnic acid, B: Graphene, C: UGNC

Physicochemical parameters

The Physicochemical characterizations viz. pH, viscosity and spreadability of the both Nano-ointments were performed as per the reported method. The results obtained shows that all the formulations exhibit good spreadability and acceptable rheological properties.

Table 2: The parameters investigated during physicochemical characterization of Nano- ointments

Formulations	pH	Viscosity (cps) Mean ± SD	Spread ability (sec)
UANPs	6.28	15.82 ± 1.32	21
UGNC	6.21	17.01 ± 1.78	24

In vitro release study

The in vitro release study provides prognostic potential to imitate critical features of in vivo conditions. The cumulative percent drug release after 480 min for UANP ointment and UGNC ointment was found to be 97.92% and 66.32 %, respectively (Fig. 5). However, plain Usnic acid

dispersion was found to release more than 99% drug within 360 minutes of study. The drug release from UANP ointment was found slower than the Usnic acid dispersion due to hindrance caused by the ointment base. The release of Usnic acid by the UGNC ointment was found still slower as a result of controlled release of Usnic acid by the Graphene- Usnic acid nano-composite accompanied with the hindrance caused by the ointment base.

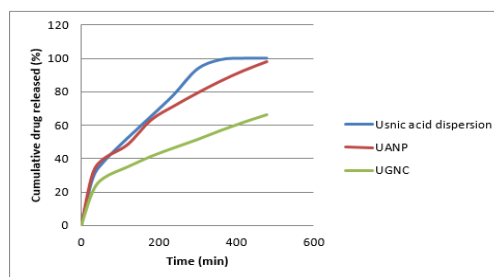


Fig. 5: Cumulative drug released (%) from Usnic acid dispersion, UANP ointment and UGNC ointment

in-vitro antifungal activity

The agar diffusion method was used for the microbiological study (Fig.6). The mean diameters of zone of inhibition against *C. albicans* were: Usnic acid dispersion, 8.35 ± 2.76 mm; UANP ointment 13.95 ± 1.54 mm; and UGNC ointment, 14.86 ± 1.46 mm. Results showed that UANP ointment and UGNC ointment significantly inhibit the growth of *C. albicans* when compared with Usnic acid dispersion despite of having four and eight times lower concentration, respectively ($p < 0.05$). This is due to the nano particulate structure of Usnic acid present in both the UANP ointment and UGNC ointment 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 having greater ability to inhibit the growth of *C. albicans*, when compared to simple UANP ointment. This was due to the fact that UGNC ointment caused more inhibition (as greater zone of inhibition) despite of having two-fold less 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 with higher penetration ability of Usnic acid leading to higher antifungal activity.



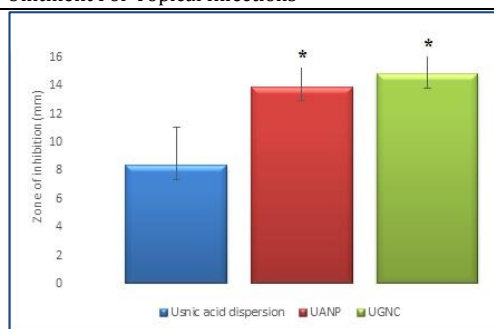


Fig. 6: In vitro antifungal activity of Usnic acid dispersion, UANP ointment and UGNC ointment against *Candida albicans*. Study performed in triplicate (n=3), data represents as mean \pm S.D., $p < 0.05$ considered statistically significant.

Conclusions

In this study, usnic acid graphene nano ointment was prepared with water soluble base. Moreover, their effects were also investigated based on physicochemical properties. The prepared usnic acid-graphene nanoparticles were ranged from 50 nm to 115 nm. The FTIR data shows that there is no chemical interaction between the drug and carrier. The aqueous base was prepared by fusion method and the selection of base is based on its pH, viscosity and spreadability. The formulation of nano-ointment of the nano-composite with aqueous base was done by geometric method. In vitro drug release interpreted controlled liberation of Usnic acid from UGNC ointment in dissolution media. Findings from antifungal activity reported the superior antifungal activity of UGNC ointment as compared to UANP ointment and recommended potential antimycotic preparation for an efficient regimen against *C. albicans* infections. Therefore, we suggested that ointment containing UGNC is promising for the bioavailability improvement of effective topical delivery. It can be concluded that the development of nano formulations has great potential for topical delivery against fungal infection.

References

- Uma A, Angela Mercy A, Karal Marx K. *Chemotherapy and Aquatic Therapeutics*, Abingdon: Oxon, CRC Press; 2020.
- Myers RS. *Immunizing and Antimicrobial Agents*, 4th ed, Livingstone: London; 2006.
- Abd Rashed A, Rathi DG, Ahmad Nasir NAH, et al. Antifungal Properties of Essential Oils and Their Compounds for Application in Skin Fungal Infections: Conventional and Nonconventional Approaches. *Molecules*. 2021 Feb 19; 26(4):1093.

Ranković, B, Kosanić, M. Lichens as a Potential Source of Bioactive Secondary Metabolites. In: Ranković, B, editors *Lichen Secondary Metabolites*. Cham: Springer; 2019.

Alahmadi AA. Usnic acid biological activity: history, evaluation and usage. *Int J Basic Clin Pharmacol*. 2017; 6(12):2752-59.

Francolini I, Norris P, PiozziA, et al. Usnic acid, a natural antimicrobial agent able to inhibit bacterial biofilm formation on polymer surfaces. *Antimicrob Agents Chemother*, 2004; 48(11):4360-65.

Kumar P, Huo P, Zhang R, et al. Antibacterial Properties of Graphene-Based Nanomaterials. *Nanomaterials (Basel)* 2019; 9(5):737.

Zhang X, Kong H, Yang G, et al. Graphene-Based Functional Hybrid Membranes for Antimicrobial Applications: A Review. *Appl Sci*. 2022; 12, 4834.

Mishra SB, Pandey H, Pandey AC. Nanosuspension of *Phyllanthus amarus* extract for improving oral bioavailability and prevention of paracetamol induced hepatotoxicity in Sprague-Dawley rats. *Adv Nat Sci: Nanosci Nanotechnol*. 2013; 4, 035007

Pandey S, Misra SK, Sharma N. Synthesis and characterization of graphene-usnic acid conjugate microspheres and its antibacterial activity against *Staphylococcus aureus*. *Int J Pharm Sci & Res*. 2019; 10(2): 939-46

Maru AD, Lahoti SR. Formulation and evaluation of ointment containing Sunflower wax. *Asian J Pharm Clin Res*. 2019; 12(8): 115-20

Asmerom D, Kalay TH, Tafere GG. Antibacterial and antifungal activities of the leaf exudate of *Aloe megalacantha* Baker. *Int J Microbiol*. 2020; 2020, 1-6.

Williams DB, Carter CB. *Transmission electron microscopy: A textbook for materials science*. New York: Plenum Press; 1996.