International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 12, Issue 5, 2020

Original Article

GRAPHENE CONJUGATED USNIC ACID NANO-FORMULATION FOR THE TREATMENT OF TOPICAL FUNGAL INFECTION

NIMESH KUMAR DUBEY[1*], SHANTI BHUSHAN MISHRA[1], ALOK MUKERJEE[1], AKANKSHA SINGH[1]

[1]Department of Pharmaceutics, United Institute of Pharmacy, Naini, Prayagraj, Uttar Pradesh, India 211001
Email: nimeshdubey0414@gmail.com

Received: 28 Jun 2019, Revised and Accepted: 13 Mar 2020

ABSTRACT

Objective: The study aims to investigate the antifungal response of the dug usnic acid with the carrier graphene.

Methods: Nano-precipitation method by sonication was adopted to formulate the conjugate. SEM test was performed to check the shape and average size of the conjugate. FTIR test was performed for the chemical interaction between the drug and the carrier. Ointment was prepared by the fusion method and the viscosity test was performed by Brookfield viscometer. Spreadability test was performed by slide method. Animal activity was performed to confirm the antifungal effect of the formulated nano-conjugate. Statistical analysis was done by Anova.

Results: SEM study shows that the conjugate is in the nano range and possess a spherical shape. FTIR study shows no interaction between the drug and the carrier. The result of *in vitro* drug release study shows that the conjugate posses a higher drug release rate as compared to the drug alone. Topical drug administration is more suitable for the treatment of the fungal infection, so the nano-conjugate was incorporated into the ointment by geometric mixing. The viscosity and the spreadability test were performed on the different formulations of the ointment and the suitable one was selected for the topical administration. Anti-fungal study had been performed on the Wistar albino rats for 6 d. Skin culture of rats was performed for the formation of the fungal colonies. Statistical analysis by Anova gives $p<0.001$. It was found that the normal form of usnic acid, graphene and the nano form both possess anti-fungal activity as 3/6 and 2/6 experimental animals are cured by normal formulation and nano-formulation.

Conclusion: The present anti-fungal study revealed that the nano-form of the conjugate possess higher anti-fungal activity than the normal formulation of usnic acid with graphene.

Keywords: Anti-fungal, FTIR, Graphene, Nano-precipitation, SEM, Usnic acid

DOI: http://dx.doi.org/10.22159/ijpps.2020v12i5.34724. Journal homepage: https://innovareacademics.in/journals/index.php/ijpps

INTRODUCTION

Microscopic organisms are the responsible ones for the fungal infections, which invades the epithelial tissues. The kingdom of fungus comprises yeast, moulds, rusts and also mushrooms. Animals considered as heterotrophic, because, environment is the nutrient obtaining source of the animals. They are not depended on endogenous sources (such as plants with the photosynthesis). Most of the fungi are considered to be good and they also involves in the process of biodegradation; it is also said that some fungi's are responsible for the infections in case, if fungi's were penetrates into the skin through the wounds, and also by the nasal passage or lungs if they are inhaled [1]. Diseases happens due to fungi, also consists superficially caused skin infection by the dermatophytes in the Microsporum, Trichophyton, and Epidermophyton genera. Anti-fungal agents possess their effect by differentiating between the mammalian cells and the fungal cells to kill the fungal organism without causing hazardous effects on to the host. However the satisfactory responses of synthetic antifungal drugs are awaited without side effects.

Nanotechnologies attracted significant attention in the recent researches. New technologies both in the preparation of the sample and in fabrication of device evoke on development of nano-science. Nanoparticles are employed for the purpose of targeted drug delivery system. It enhances the performance of the drug by increasing their bioavailability. Nanoparticles are of nano-sized colloidal structures which comprises of polymers of having synthetic and semi-synthetic nature [2]. Nanonization process is used for the compounds which are poorly soluble in water in respect to enhance the dissolution rate and increase the bio-availability. Nanoparticles refers as drug delivery systems which has the particle size ranges between 10–1000 nm, it also depends on the preparation method and usage of materials [3].

Ointment considered as the semi-solid preparations which are used for the topical application on to the skin. Basically ointments consist of a medicament which is emulsified or mixed in to the base. They are applied for the emollient effect, protection of the skin. Ointments are also used for the vehicle which is used to administer the drug or the medicament topically.

Now a days 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 i.e. 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. Lichens are considered as a photosynthetic and fungal partners. Usnic acid is considered to be the most common and abundant metabolites of lichens, it is also considered as an antibiotic. It has the ability to inhibit the fungal and bacterial growth [4]. To enhance the bioavailability of usnic acid, nano-conjugate was formulated with the help of graphene used as a carrier in the preparation of nano-conjugate. Graphene having also antibacterial activity, so the conjugate of graphene and usnic acid is developed to accomplished the higher anti-fungal activity.

MATERIALS AND METHODS

Graphene powder was received as gift sample from Nanotech Application Centre, University of Allahabad, Allahabad. Usnic acid was provided by TCI Chemicals (India). Polyethylene glycol (PEG 400 and PEG 4000) were obtained from Merck, India. For the purpose of release study, magnetic stirrer of Remi Pvt. Ltd and the dialysis membrane-70 was gifted by Hi-media Pvt. Ltd. Dermatophyte strain, i.e., *Candida albicans* (MCCB 0290) was procured from Microbial Culture Collection Bank, Department of Microbiology and Microbial Technology, AAIDU, Allahabad. Chemicals used in this work were of good analytical grade.

Preparation of usnic acid and graphene conjugate

Usnic acid was loaded onto the graphene via simple physio-sorption. Graphene 0.145 mg/ml was sonicated with 1 mg/ml usnic acid at pH 5 for 10 min at 20 watt for 3 cycles, then it was stirred overnight at room temperature in the dark by using magnetic stirrer instrument, then it was ultra-centrifuged at 15000 rpm for 1 hour, 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 graphene conjugated usnic acid was obtained, then this nano-conjugate was taken for the characterization studies [5].

Characterization of graphene-usnic acid nano-composite (gun)

Scanning electron microscopy analysis

The SEM imaging of the sample is carried out by type of electron microscope which scans it with a high energy electron beam. In this when electron gets interacted with the atoms of the sample signals is produce which contains information of the sample morphology, its composition and other properties like electrical conductivity. It gives a better resolution than the optical microscope. In the present study, the Carbon coating of the materials was done by using JEOL-JEE-420 vaccum evacuator to make the sample conducting. Coating thickness was 20 nm. SEM images were taken by using EPMA i.e. electron pro-micro analyzer JEOL-J×A 8100. The average size range of the conjugate and its shape was determined [3].

FTIR analysis

FTIR spectroscopy is commanding tool for the identification of functional group present in the compound. It is a helpful tool to identify organic compounds, having polar chemical bonds (such as OH, NH, CH, etc.) with a strong dipoles. It is very useful in the structural analysis of organic compounds, polymers, natural products etc. As every functional group present in a compound has an specific vibration, the IR spectra is seen as their fingerprints. 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 function the samples were mixed with KBr and punched to a tablet applying hydraulic press. The FTIR spectra was recorded at 4000-400 cm-1 using FTIR Spectrometer (PerkinElmer Spectrum Version 10.4.00) [5].

***In vitro* drug release profile**

In vitro drug diffusion study was determined by using the dialysis bag diffusion technique. The diffusion study of usnic acid alone, graphene conjugated usnic acid nano formulation and graphene conjugated usnic acid normal formulation were performed in phosphate buffer (pH 7.4). Total quantity of prepared substance was placed in the cellulose dialysis bag and the bag was tied at both ends. Then the bag was kept in the receptor compartment which consists of 50 ml of phosphate buffer having pH 7.4 at 37 °C under magnetic stirring. An aliquot of receptor media (1 ml) was taken out at determined period of time up to 500 min and the same quantity of fluid was replaced by fresh dissolution medium having phosphate buffer pH 7.4 and analyzed spectrophotomertically at 290 nm [3].

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 [6].

Table 1: List of water soluble base formulations

Formulations	PEG-4000	PEG-400	GLYCERINE	S. L. S
WSB 01	40 gm	60 gm	q. s	q. s.
WSB 02	30 gm	70 gm	q. s.	q. s.
WSB 03	20 gm	80 gm	q. s.	q. s.
WSB 04	50 gm	50 gm	q. s.	q. s.
WSB 05	60 gm	40 gm	q. s.	q. s.
WSB 06	30 gm	60 gm	q. s.	q. s.

Formulation no. 4 was selected on the basis of pH, viscosity, spreadability.

Formulation of nano-ointment of graphene-usnic acid nano-composite (GUN)

The process of geometric dilution has been used for the preparation of nano-ointment. The selected water soluble ointment base has been used for nano ointment. The nano ointment of graphene-usnic acid nano-composite (GUN) had been formulated in a concentration of 0.5% w/w; similarly one more formulation Graphene-Usnic acid composite (GUC) in the same concentration has been developed without undergoing Nanonization process.

Physicochemical characterization

pH

The pH of the formulated products were determined by the usage of Digital pH meter (361, Systronics). The electrode which was connected to the pH meter was cleaned with distilled water and made it dry with the help of tissue paper, then immerse the electrode and temperature probe in a beaker containing ointment formulations. After that wait for few minutes then note the readings of pH of samples which were displayed on pH meter [7, 10]. Experiment was performed in triplicates and the mean values were depicted in table 2.

Determination of viscosity

Measurement of viscosity is defined as the process of fluid resistance. Viscosity determines the process of internal friction of a fluid which is a moving state. Units of viscosity are Poise, Centipoises, Pascal-second. Viscosity test of ointment was performed by the help of Brook-field Viscometer (model no. DV-E viscometer, Helipath-spindle S-61). The test was done in a triplicate manner and then the mean values of each prepared formulation were depicted in table 2 [12].

Spreadability

Spreadability of the semi-solid preparations, is the ability of a preparation to evenly spread on to the skin. It plays an important role in the administration of a dose of a preparation on to the skin. Spreadability is measured in respect of times in seconds. 2 sets of glass slides of standard dimension of 7.5 cm were taken. The prepared ointment was kept over on the first slide and the second slide was kept on top of the ointment, in the manner that the ointment was sand-witched in between both slides. 100g weight of ointment was kept on the upper slide in such a manner that the ointment gets compressed properly to form a thin layer. Then the previous weight of 100g was removed from the upper slide and 20g of new weight was tied to the upper slide and the slides were tilted in such a manner that only the upper slide to slips off by the applied force on to it. The time interval in which the upper slide travels the distance of 7.5 cm and separated away from the lower slide was measured. The test was performed in triplicate manner and the mean value was considered. In the spreadability study lower the time interval of separation of the slides better will be the spreadability. Formula for the spreadability study is as follows:

$$S = M \times L/T$$

Where, S denotes the spreadability, M denotes the weight tied on to the upper slide, L denotes the length of glass slides and T denotes the time taken by the slides to get separated [13]. Values were depicted in table 2.

***In vivo* pharmacological activity**

Anti-fungal activity

Microbial strain

Candida albicans (MCCB 0290) was obtained from Microbial Culture Collection Bank, Microbiology department, Allahabad Agriculture Institute of Deemed University, Allahabad.

Animals

Healthy male adult Wistar albino rats, age ranges two and three months, weights 100-150 gm was taken for the anti-fungal study. The rats were kept in poly-propylene cages and also in prescribed atmosphere which comprises 12 h light and 12 hour dark at 25±2 °c and 30-55% humidity. Rat pellets were given to the rats in their daily diet. The Institutional Animal Ethical Committee, UIP, Allahabad, India (UIP/IAEC/March-2019/03) has approved the study.

Procedure

Candida albicans (MCCB 0290) was preferred to induce the mycosis in Wistar albino rats for the *in vivo* study of the formulated nano-formulation. The procedure includes, the removal of hairs from the back of the rats by using the hair removal cream and an area of 2 × 2 cm^2 was preferred for the application of the prepared formulations. On the next day the skin was slightly abraded with the help of sandpaper and then the inoculum of *Candida albicans* were applied on to the skin of the rats by using a cotton swab. The animals were separated into four different groups, which comprises one control group, 6 rats were taken in every group. The prepared products, i.e. nano ointment of graphene conjugated usnic acid (GUN) and Ointment with graphene and usnic acid (GUC), Standard marketed preparation (SURFAZ-SN 0.5% w/w) were administered topically. Daily one time application was given to the rats for the interval of six days. The control group does not received any treatment. The response of each group was compared to the control group after the period of six days. The treatment scores were given to each group as 1 (not treated), 2 (50% treated), 3 (75% treated) and 4 (100% treated). Culture study was performed to check the effect of the given treatment. Each treated site was wiped properly with 70% ethanol. The skin from each treated site was excised, minced, by the help of scissors, and then it was homogenized in 4 ml of saline by using the tissue homogenizer. Then, small portion of homogenate was streaked on the solidified Sabouraud dextrose agar medium. All plates were incubated at 25 °C for 5 d in the BOD incubator (Indosati Scientific, Ambala). The numbers of colony forming units in the agar plates were counted by using colony counter and the number of colony forming units per infected site was determined. If more than one fungal colony was found in the plates then it was termed as fungal positive [9].

Statistical analysis

The statistical analysis was performed by using GradPad Prism 5.01. The values were depicted as mean±SD for all six Wistar albino rats, the data was analyzed by ANOVA by the Newman-keuls method.

RESULTS

Scanning electron microscopy (sem) analysis

SEM 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 SEM. The average size of the prepared nano-composite was found to be in the range of 90-125 nm. The shape of the nano-composite is of spherical in nature.

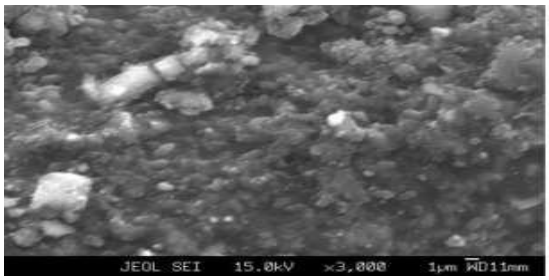

Fig. 1: SEM image of graphene-usnic acid

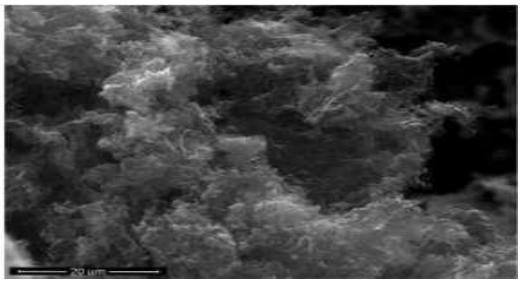

Fig. 2: SEM image of graphene nano-sheets

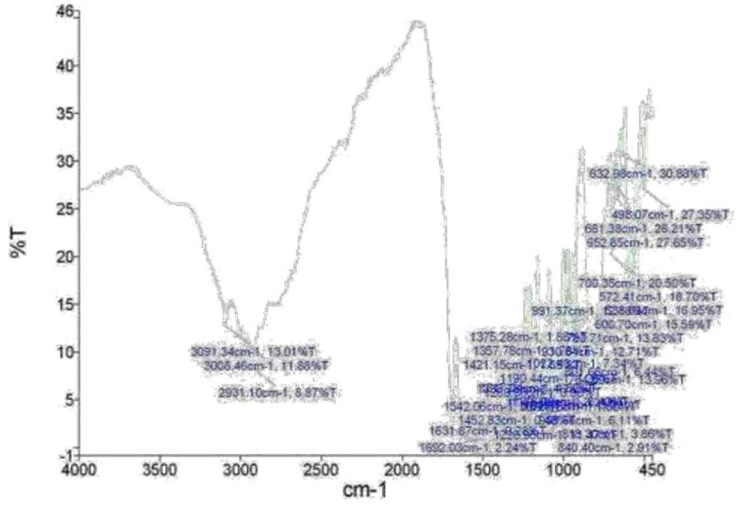

Fig. 3: FTIR spectra of usnic acid

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 2931.10 cm^{-1}, C=O stretch at 1692.03 cm^{-1}, C-O stretch 1000-1200 cm^{-1}, O-H aromatic stretch at 3091 cm^{-1}, C-H aromatic stretch at 700-900 cm^{-1}, C=C aromatic stretch at 1600-1675 cm^{-1} were obtained. FTIR spectra of graphene showed characteristic peak at 1600 cm^{-1} was obtained. Graphene usnic acid conjugate showed FTIR spectra at C-H stretch at 2929 cm^{-1}, C=O stretch at 1631 cm^{-1}, C-O stretch at 1000-1200 cm^{-1}, C-H aromatic stretch at 700-900 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.

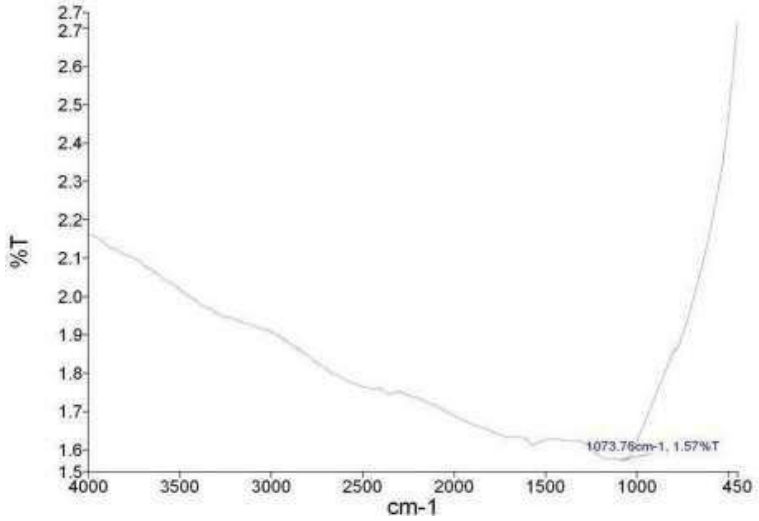

Fig. 4: FTIR spectra of grapheme

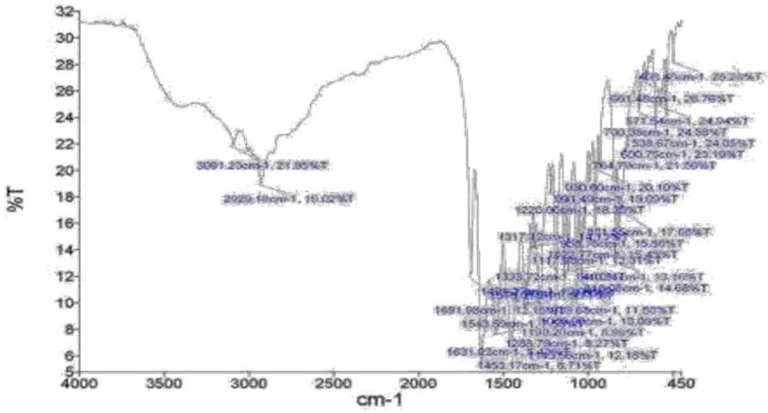

Fig. 5: FTIR spectra of usnic acid and graphene nano conjugate

Entrapment efficiency

The entrapment efficiency of graphene usnic acid nano-conjugate was determined. The entrapment efficiency of graphene usnic acid nano-conjugate, prepared by nano-precipitation method with sonication was found to be 79.33%. It shows that sonication technique increased the entrapment of drug with polymer by decreasing particle size and increasing the surface area between drug and carrier.

***In vitro* drug release analysis**

It was observed that the release rate of usnic acid and graphene-usnic acid conjugate (GUC) is lesser in comparison to graphene conjugated usnic acid nano-conjugate (GUN), it was concluded that the nano-conjugate of graphene conjugated usnic acid facilitates more drug release because of its size and presence of graphene as compare to usnic acid alone and GUC.

Table 2: Drug release kinetics of usnic acid, graphene and conjugate of usnic acid and graphene

S. No.	Data	Usnic acid	Graphene	Conjugate
1	Standard Deviation	18.389	21.47	26.89
2	Relative Standard Deviation	0.58	0.54	0.52
3	Average Release Rate	31.38	39.56	50.74
4	R^2(Regression)	0.977	0.948	0.941

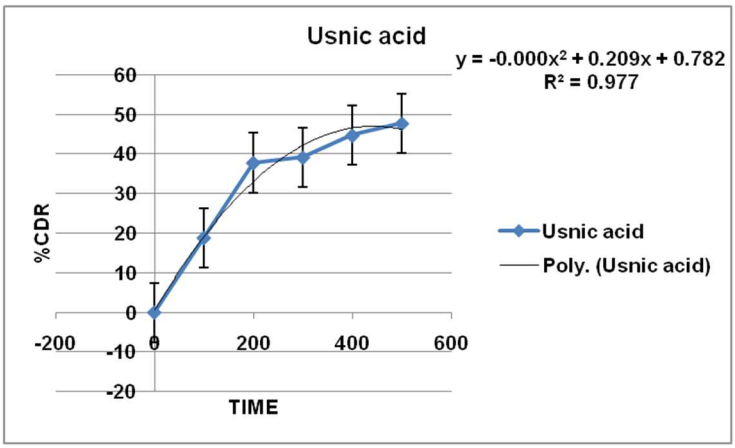

Fig. 6: *In vitro* drug release of usnic acid data points represents mean±SEM (n=3)

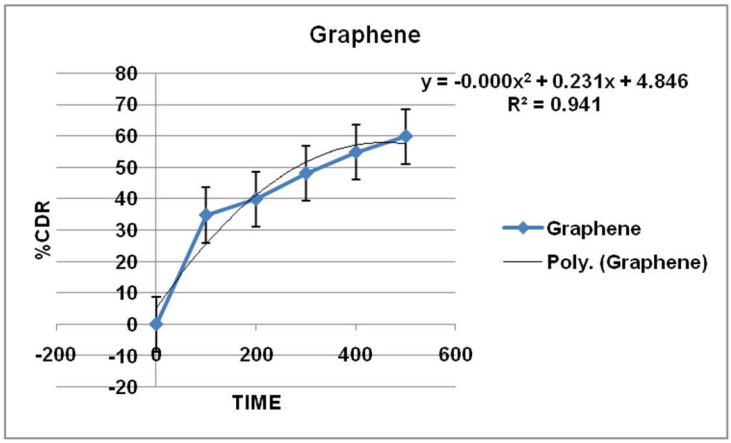

Fig. 7: *In vitro* drug release of graphene acid data points represents mean±SEM (n=3)

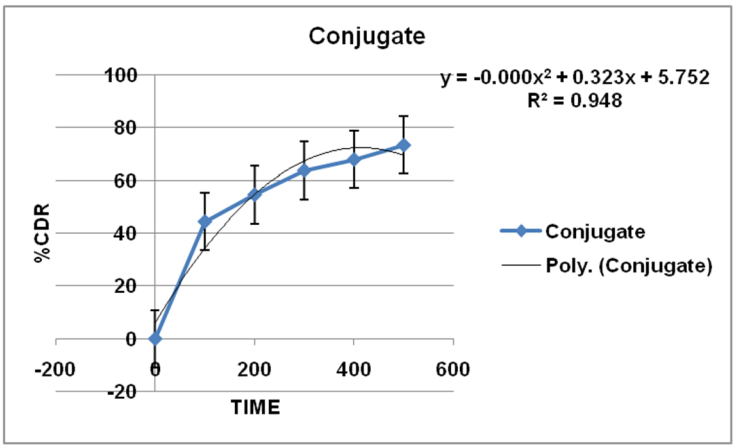

Fig. 8: *In vitro* drug release of conjugate data points represents mean±SEM (n=3)

Physico-chemical characterizations of prepared and selected formulation

Table 3: Physico-chemical characterizations

Formulations	pH	Viscosity(cps)	Spreadability (gm. cm/sec)
Selected Base	6.3±0.2	25.12±0.14	29.73±0.52
GUC	6.2±0.2	25.22±0.16	28.21±0.17
GUN	6.3±0.2	25.12±0.14	30.01±12

Each value represents the mean±SD (n=3)

Pharmacological activity

***In vivo* antifungal activity**

The *in vivo* efficacy of graphene conjugates usnic acid nano-ointment was assessed in male albino rat model (Wistar; 100–150 g). Isolate of *candida albicans* was used for production of cutaneous candidiasis in albino rats. Table 3 depicts the efficacy of graphene conjugated usnic acid nano-ointment formulation against cutaneous candidiasis in rats as compared to that of standard preparation and normal graphene conjugated usnic acid formulation. It was observed that graphene conjugated usnic acid (GUC) ointment showed moderate control over the fungal infection as three animals out of six were positive in culture test whereas graphene conjugated usnic acid nano-ointment (GUN) depicts greater efficiency in the treatment of candidiasis, as only two animal out of six exhibited a positive culture test. Fast recovery from fungal infection was found in the case of standard marketed preparation (Surfaz SN) as there is only one animal exhibited trivial CFU on infected area.

Table 4: Colony forming unit of *Candida albicans* in skin of rats after treatment with different formulations

Treatment	Number of animals with positive culture/ Total number of animals	mean±SD CFU/ Infected sites
Control (Group I)	6/6	15±13.01
GUC (Group 2)	3/6	3.5±2.7*
GUN (Group 3)	2/6	1.33±1.3*
Standard (Group 4)	1/6	1.0±1.0*

The value represents the means±SD for 6 rats per group. P<0.001 was considered as significant compared to control group.

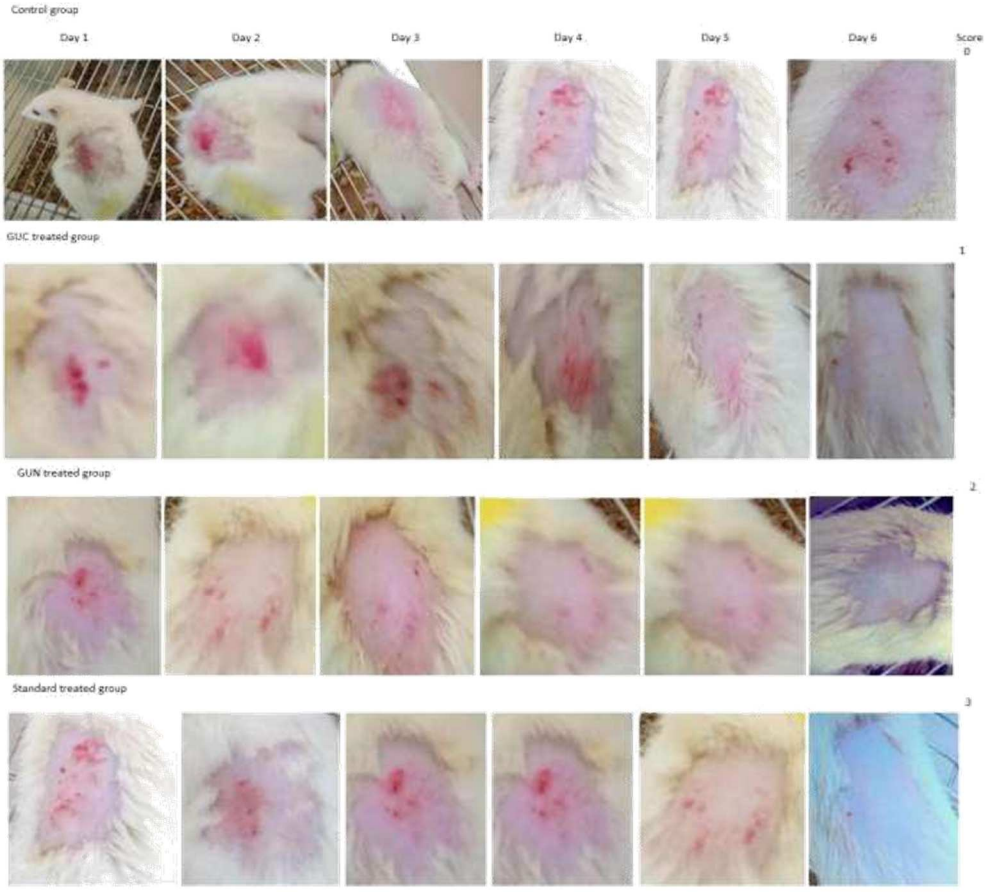

Fig. 9: Anti-fungal effect of GUC and GUN on experimental animals

DISCUSSION

In the present work the drug usnic acid which is a dibenzo-furan derivative a common lichen metabolite reported for their many pharmacological activities was conjugated with versatile carrier graphene which is already reported for its anti-microbial property. This nano-conjugate was prepared by nano-precipitation method and characterized by SEM which confirms that the average particle size is 90-125 nm and also particles are in spherical in nature as spherical particles having better cellular entry in biomedical activities. The FTIR data shows no chemical interaction between the drug and the carrier as the obtained FTIR spectra of usnic acid showed characteristic peaks of C-H stretch at 2931.10 cm^{-1}, C=O stretch at 1692.03 cm^{-1}, C-O stretch 1000-1200 cm^{-1}, O-H aromatic stretch at 3091 cm^{-1}, C-H aromatic stretch at 700-900 cm^{-1}, C=C aromatic stretch at 1600-1675 cm^{-1} were obtained. FTIR spectra of graphene showed characteristic peak at 1600 cm^{-1} was obtained. Graphene usnic acid conjugate showed FTIR spectra at C-H stretch at 2929 cm^{-1}, C=O stretch at 1631 cm^{-1}, C-O stretch at 1000-1200 cm^{-1}, C-H aromatic stretch at 700-900 cm^{-1}, O-H aromatic stretch at 3091 cm^{-1}. The *In vitro* drug release study shows that the nano-form of the drug possesses greater release rate than the normal forms of the drug (normal form of usnic acid gives 40%-50% release of drug and graphene gives 50%-60% whereas the nano-conjugate of graphene

and usnic acid gives 70%-80% release of drug) the obtained data was compatible with [5,8]. The nano-ointment of this conjugate was prepared with water soluble base by geometric mixing method. The various evaluation studies of the prepared ointment like pH, viscosity and spreadability gives proper data of the standard ointment. The obtained data also gives the justification of the evaluation tests like the data of viscosity and spreadability, lower the viscosity higher will be the spreadability and the obtained data exactly indicates this concept of standard ointment (viscosity 25.12±14cps gives spreadability 30 gm. cm/sec) the obtain data was compatible with [7]. The data of pH was also very compatible to the skin. Then finally the prepared nano-ointment was subjected for anti-fungal activity by using fungal strain *Candida albicans. The in vivo* anti-fungal activity shows significant ($p<0.001$) anti-fungal activity that corresponds similar to activity of standard marketed preparation. The obtained data was compatible with [9]. This significant anti-fungal property shows synergistic response of both the drug and carrier as reported for their anti-microbial property.

CONCLUSION

The present study clearly indicates that the nano form of usnic acid with graphene shows greater antifungal response as compare to the normal form of usnic acid with graphene. This provides an indication that if the fungal infection is penetrated into the deeper layers of skin the nano form of the prepared formulation treats it better with respect to the normal form of the usnic acid and graphene. It also indicates that the nano form of the prepared formulation penetrates through the barrier layers of the skin like stratum corneum and treats the deeper infection of the skin whereas the normal form was not penetrated through the barrier layers of skin and it remains on the top surface of the skin only. This study shows that how effectively the nano-formulations works in the present scenario and also into the upcoming years the nano-technology is going to play a vital role in the efficiency of the drug formulation.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All authors contributed equally.

CONFLICT OF INTERESTS

There is no conflict of interest.

REFERENCES

1. Mayer M. Usnic acid a non-genotoxic compound with anti-cancer properties. Anti-Cancer Drugs 2005;16:805-9.
2. Vyas, Khar. "Targeted and controlled drug delivery" 1st edition. CBS Publishers and Distributors Pvt. Ltd. 2013. p. 331-86.
3. Pandian Sundara M, Karthikeyini Chitra S, Nagarjan M. Fabrication, characterization and pharmacological activity of usnic acid loaded nanoparticles. Int J Pharm Sci Res 2017;8:4758-66.
4. Krzeminska Guzow. Antibacterial activity of lichen secondary metabolic usnic acid is primary caused by inhibition of RNA and DNA. FEMS Microbiol Lett 2014;353:57-62.
5. Pandey. Synthesis and characterization of graphene-usnic acid conjugate microspheres and its antibacterial activity against *staphylococcus aureus.* Int J Pharm Sci Res 2019;10:939-46.
6. Gupta SK. Introduction to pharmaceutics II" fourth edition. CBS Publishers and Distributors Pvt. Ltd; 2011. p. 184-215.
7. Nayak SH, Nakhat PD, Yeole PG. Development and evaluation of cosmeceutical hair styling gels of ketoconazole. Indian J Pharm Sci 2005;52:231-33.
8. Jain S, Padsalg BD, Patel AK, Moale V. Formulation development and evaluation of fluconazole gel in various polymer base. Asian J Pharm 2007;1:63-8.
9. Pershing LK, Corlett LJ, Jorgensen C. *In vivo* pharmacokinet Bics and pharmacodynamics of topical ketoconazole and miconazole in human stratum corneum Antimicrob. Agents Chemother 1994;38:90–5.
10. Queiroz MBR, Marcelino NB, Ribeiro MV, Espindola LS, Cunha F, Silva MV. Development of gel with *Matricaria recutita* L. extract for topical application and evaluation of physical-chemical stability and toxicity. Lat Am J Pharm 2009;28:574-9.
11. Bhattacharya Sankha, Prajapati G Bhupendra. Formulation and optimization of celecoxib nanoemulgel. Asian J Pharm Clin Res 2018;11:353-65.
12. S Princely, MD Dhanaraju. Design, formulation and characterization of liposomal encapsulated gel for transdermal delivery of fluconazole. Asian J Pharm Clin Res 2018;11:417-24.
13. Nihal Badduri, Gupta Vishal N, Gowda DV, M Manohar. Formulation and development of anti acne formulation of *Spirulina* Extract. Int J Appl Pharm 2018;10:229-33.