# DESIGN AND DEVELOPMENT OF USNIC ACID AND GRAPHENE NANOFORMULATION FOR THE TREATMENT OF WOUND HEALING BY WISTAR ALBINO RATS

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## ABSTRACT

**Objective:** The study aims to investigate the wound healing response of the dug usnic acid and carrier graphene with its nano form.

**Method:** Nano-precipitation method by sonication was adopted to formulate the nanoparticle. SEM test was performed to check the shape and average size of the nanoparticle. FTIR test was performed for the chemical interaction. Ointment was prepared by fusion method and the viscosity test was performed by Brookfield viscometer. Spreadability test was performed by slide method. *In-vivo* wound healing study and topical animal activity was performed to confirm the wound healing effect of the formulated nano-formulation. Excision wound model histology was done to confirm the activity. Statistical analysis was done by Anova.

**Results**: SEM study shows that the particles is in the nano range and possess spherical shape. FTIR study shows no chemical interaction. The result of in-vitro drug release study shows that the nano-formulation posses higher drug release rate as compare to the drug alone. Topical drug administration is more suitable for the treatment of the wound healing, so the nano-formulation 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. *In-vivo* wound healing study was performed on the Wistar albino rats for 16 days. Statistical analysis by Anova gives $p<0.01$. It was found that the usnic acid alone, and the nano form of usnic acid with graphene both possess wound healing activity but the nano-form with graphene possess more significant activity.

**Conclusion:** The present wound healing study revealed that the nano-form of the drug possess higher wound healing activity than the normal formulation of usnic acid.

**Keywords:** Wound-healing, FTIR, Nano-precipitation, Ointment, SEM, Usnic acid.

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## 1. INTRODUCTION

Wounds are the break in the skin continuity takes place by injury or by surgery. The healing process of wound consists of significant challenge. And it also has the potential to lowdown the quality of lifestyle of the peoples **[1].** It was found that the normal healing process of wound is 2-4 weeks. It was observed that healing of wound gets delayed in diabetic conditions. Several kinds of wounds are as follows- cuts, bites, ulcers, stomach ulcers etc. It also depends on several factors such as origin, depth, location, types of damage **[2].**

Wound consists of several issues like delayed wound healing process, reduced synthesis of collagen, extended collagen, delayed neo-vascularization **[3].** The process of wound healing consists of 4 phases which consists hemostasis with creation of blood clots, inflammation with swelling, proliferation and remodeling with the formation of scar tissues **[4].**

Wound healing process normally coexists with a malignant inflammatory response triggered by hypoxia, aging, bacterial colonization **[5]**, increased inflammatory cytokines **[6, 7],** impaired neo-vascularization **[8],** reduced collagen deposition and deposition then morphological changes.

Therefore, wound healing process gets delayed because of pathogenic bacteria, generation of free radicals, impaired collagen deposition and inflammation **[9].**Reactive oxygen species are the key factors of various stages of wound healing and some small changes are essential for the defense against external damage **[10].**

Studies showed that usnic acid possess anti-bacterial activity and graphene also consists of anti-bacterial property. This research presented the formulation of nano-ointment of usnic acid and graphene and also the wound healing activity by Wistar albino rats. 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**.** 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 **[11].**

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 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 **[12].** 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.

## 2. MATERIAL AND METHODS.

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. Chemicals used in this work were of good analytical grade.

### 2.1 Preparation of usnic acid nano-formulation

Usnic acid 1mg/ml was sonicated at pH 5 for 10 minutes 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 particles of usnic acid was remained at the bottom, then it was heated at 40°c in the hot air oven, powder of usnic acid was obtained, then this powder was taken for the characterization studies **[13].**

### 2.2 Characterization of usnic acid nano-formulation

#### 2.2.1 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 20nm.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. **[14]**

#### 2.2.2 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) **[15].**

#### 2.2.3 XRD STUDY

The PAN analytical X'Pert PRO X-ray diffractometer was used to perform X-ray diffraction (XRD) characterization to estimate the physical characteristics of graphene loaded usnic acid. Electromagnetic radiations include X-rays as one of its components. Scattering happens when it interacts with a chemical substance via the electrons of the material's atoms. The phenomenon known as X-ray diffraction is caused by the combination of elastic scattering and destructive interference. This is determined by applying Bragg's law; $2dSin\ \theta = n\lambda$.

Amorphous materials have broad and dispersed peaks that correlate to particular crystal lattices existing in them, whereas crystalline materials have narrow and sharp peaks that correspond to specific crystal lattices present in them. Semi-crystalline polymers have both sharp and wide peaks, depending on the amount of crystalline and amorphous components present **[16].**

### 2.2.4 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 (1ml) was taken out at determined period of time up to 500 minutes and the same quantity of fluid was replaced by fresh dissolution medium having phosphate buffer pH 7.4 and analyzed spectrophotomertically at 290 nm **[17].**

### 2.3 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 **[18].**

**Table 1: List of water soluble base formulations.**

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

Formulation no. 3 was selected on the basis of pH, viscosity, spreadability.

### 2.3.1 FORMULATION OF NANO-OINTMENT OF USNIC ACID

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

usnic acid nano-formulation (UAN) had been formulated in a concentration of 0.5% w/w; similarly one more formulation Usnic acid ointment (UAO) in the same concentration has been developed without undergoing Nanonization process.

### 2.3.2 PHYSICOCHEMICAL CHARACTERIZATION

### 2.3.4 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 **[19].** Experiment was performed in triplicates and the mean values were depicted in Table 3.

### 2.3.5 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 3 **[20].**

### 2.3.6 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 7.5cm 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 7.5cm 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 **[21].**Values were depicted in Table 3.

## *2.4 TOPICAL* PHARMACOLOGICAL ACTIVITY

### 2.4.1 Acute skin irritation study

Acute skin irritation study was used to determine any type sensitivity reaction on skin caused by ointment. In this study, the experiment was performed according to the OECD- 402 guidelines. 1st day, dorsal region hairs of the animals were removed and different concentrations (0% w/ w, 5% w/w, 10% w/w, 15% w/w, 20% w/w, 25% w/w, and 30% w/w) of test ointment was regularly applied on dorsal region. The animals were observed till the 16th days for any side effects such as skin reactions itching, inflammation, erythema, swelling, skin redness and irritation **[22].**

### 2.4.2 Animals

Healthy male adult Wistar albino rats, age ranges two and three months, weights 100-150 gm was taken for the wound healing study. The rats were kept in poly-propylene cages and also in prescribed atmosphere which comprises 12 hours light and 12 hour dark cycle at 25±2°c and 30-55% humidity. Rat pellets were given to the rats in their daily diet.

### 2.4.3 Ethical Statement

The Institutional Animal Ethical Committee of (1698/PO/Re/S/13/CPCSEA) Patel College of Pharmacy, Madhyanchal Professional University, Bhopal, M.P., has approved the study.

### *2.4.4 Experimental Design*

Animals were randomly categorized into four groups and every group comprised of six animals. Daily two time application was given to the rats for the interval of seven days. The control group does not receive any treatment. The response of each group was compared to the control group after the period of seven days.

*G1: Control group untreated*

*G2: Usnic acid ointment (UAO-1% w/w)*

*G3: Usnic acid nano-formulation (UAN -1% w/w)*

*G4: Marketed Preparation (POVIDONE-IODINE 1% w/w)*

### 2.4.5 PROCEDURE

### Excision wound model

First of all, Ketamine (40mg/kg) intraperitoneal injection was injected to anaesthetize the animals, the procedure includes, removal of hairs from the back of the rats by using the hair removal cream and round area of 4.40 $cm^2$ was preferred for the application of the prepared formulations. The excision wound was measured on the side of dorsal midline using biopsy punch pliers, 0.2cm deep and 2.4cm thick **[23].** During the entire procedure sterilized and sanitary conditions were maintained. 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 usnic acid with graphene (UAN) and ointment with usnic acid (UAO), Standard marketed preparation (POVIDONE-IODINE 1% w/w) were administered topically. Daily two time application was given to the rats for the interval of sixteen days. The control group does not received any treatment. The response of each group was compared to the control

group after the period of sixteen days. The treatment scores were given to each group as 1 (not treated), 2 (treated with normal formulation), 3 (treated with nano-formulation) and 4 (treated with marketed formulation). Each treated site was wiped properly with 90% ethanol **[24].**

### 2.4.6 Wound area measurement

Permanent marker and tracing paper were used to measure the wound regions on the 1st, 8th and 16th day. Reduction of wound area in percentage (%) was measured by using the following formula **[25].**

$$\text{Wound contraction as a percentage} = \frac{\text{Healed area of wound}}{\text{Total area of wound}} \times 100$$

Whereas, Healed area of wound = Original area of wound – Current area of wound

### 2.4.7 STATISTICAL ANALYSIS:

The statistical analysis was performed by using GradPad Prism9. The values were depicted as mean ± S.D. for all six Wistar albino rats, the data was analyzed by ANOVA by the Newman-keuls method **[26]. *** $p < 0.05$, **$p < 0.01$ as significant values whereas #$p > 0.05$ non-significant, in comparison to wound control group.

## 3. RESULTS

### 3.1 SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS

Scanning Electron Microscopy is widely used in various fields, such as materials science, biology, geology, nanotechnology, and forensic science, to study the morphology, structure, and composition of a wide range of materials.SEM has been instrumental in advancing our understanding of the microscopic world and has numerous applications, including quality control in manufacturing, semiconductor analysis, biological imaging, and research on advanced materials.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 80-120nm. The shape of the nano-composite is of spherical in nature.

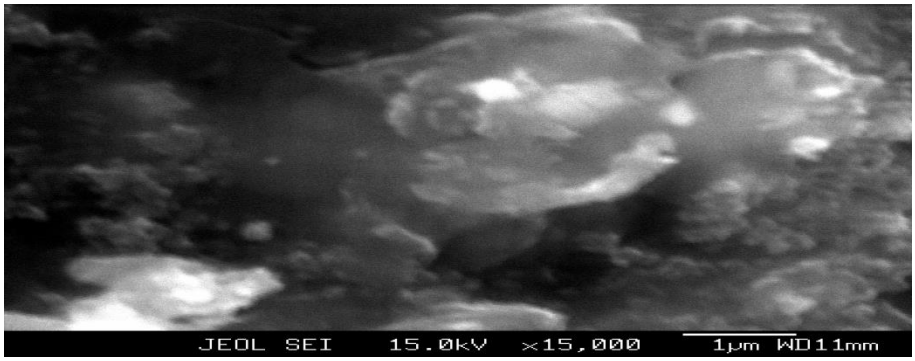



**Fig.1: SEM image of usnic acid and graphene nano-formulation**

## 3.2 FTIR ANALYSIS

FTIR spectra were recorded to assess the compatibility of the pure drug and formulated compound. FTIR spectra of drug, and nano-formulation were examined. All characteristic peaks of usnic acid, and nano-formulation were ascertained in the FTIR test. The results showed that there is no chemical interaction or alteration took place during formulation of nanoparticles. FTIR spectra of usnic acid showed characteristic peaks of C=O stretch at 1631.03$cm^{-1}$, C-O stretch 1000-1200$cm^{-1}$ , C=C aromatic stretch at 1600-1675$cm^{-1}$ were obtained. Usnic acid nano-formulation showed FTIR spectra at C=O stretch at 1631$cm^{-1}$, C-O stretch at 1000-1200 $cm^{-1}$, C-H aromatic stretch at 700-900 $cm^{-1}$.The results of the FTIR test showed that there is no chemical interaction took place during the formulation of nanoparticles and drug was found to be compatible. he measured signal is subjected to a Fourier transform, which converts the time-domain data into a frequency-domain spectrum. This process allows for a more straightforward analysis of the sample's molecular composition. FTIR is a powerful and versatile technique that plays a vital role in the identification and characterization of a wide range of materials and compounds in scientific research, industrial applications, and many other fields. The resulting FTIR spectrum is displayed as a graph that shows the intensity of absorbed infrared radiation as a function of wavenumber or frequency. The spectrum is unique to the molecular composition of the sample and provides valuable information about the functional groups present in the molecule.

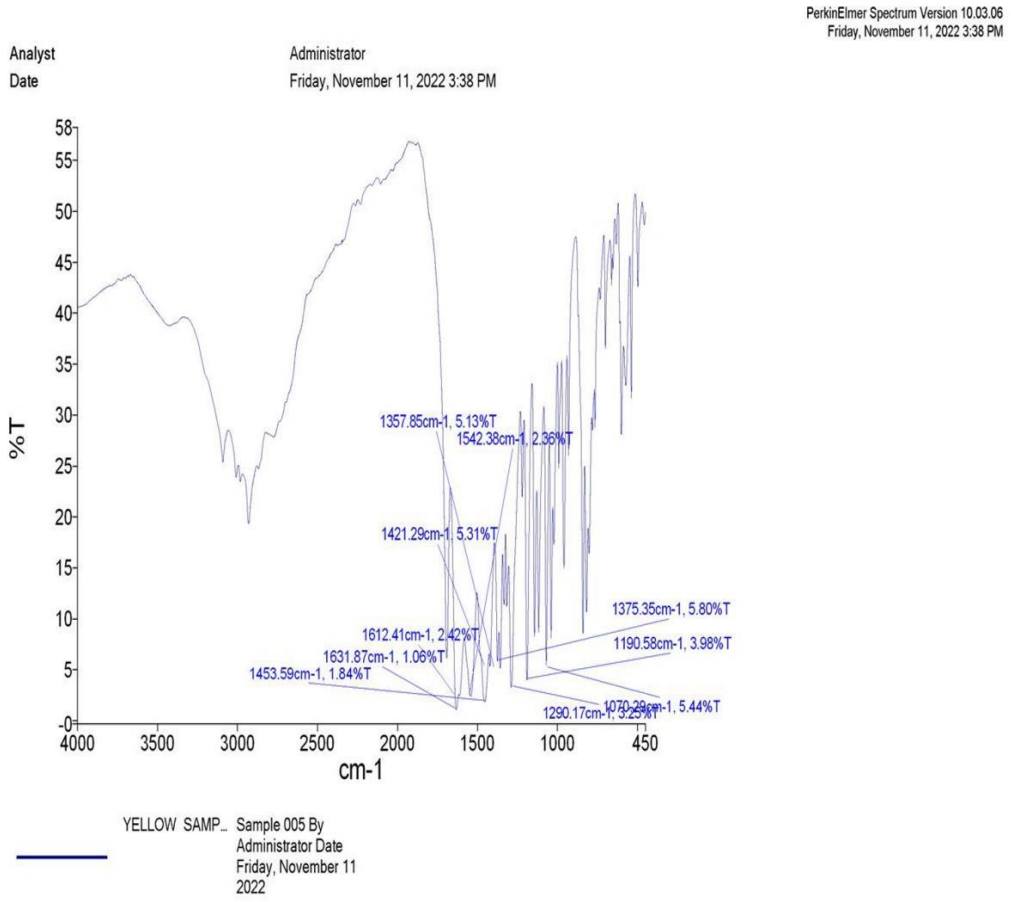



**Fig.2: FTIR spectra of usnic acid**

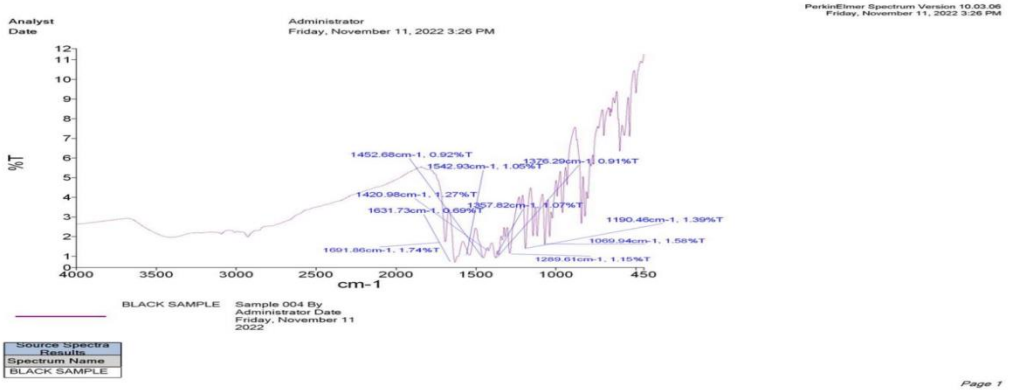



**Fig.3: FTIR spectra of usnic acid and graphene nano-formulation.**

## 3.3 XRD STUDY

The XRD test of the selected formulation depicts that the compound was of crystalline nature.

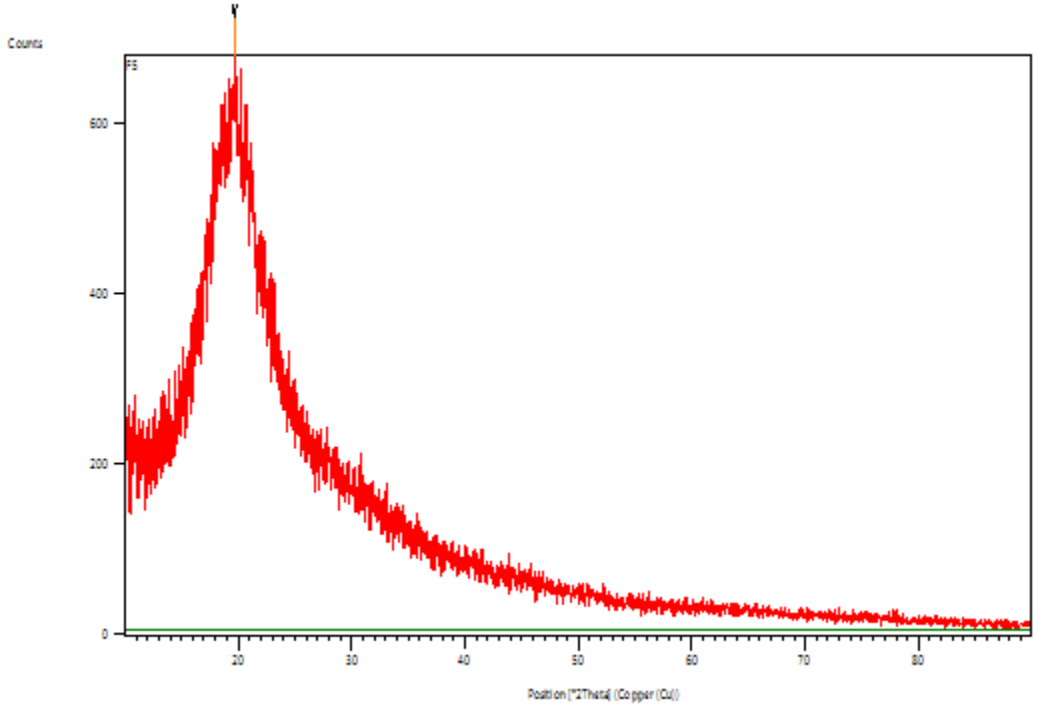



**FIG.4 XRD spectra**

## 3.4 ENTRAPMENT EFFICIENCY

The entrapment efficiency of usnic acid and graphene nano-formulation was determined. The entrapment efficiency of usnic acid and graphene nano-formulation, prepared by nano-precipitation method with sonication was found to be 79.44%. It shows that sonication technique increased the entrapment of drug by decreasing particle size.

## 3.5 IN VITRO DRUG RELEASE ANALYSIS

It was observed that the release rate of usnic acid is lesser in comparison to usnic acid and graphene nano-formulation (UAN), it was concluded that the nano-formulation of usnic acid and graphene facilitates more drug release because of its size.

**Table 2. Drug Release kinetics of usnic acid, graphene and usnic acid.**

| S.NO. | DATA | USNIC ACID | NANOFORMULATION |
|---|---|---|---|
| 1 | Standard Deviation | 18.389 | 26.79 |
| 2 | Relative Standard Deviation | 0.58 | 0.51 |

| | | | |
|---|---|---|---|
| 3 | Average Release Rate | 31.38 | 50.64 |
| 4 | $R^2$ (Regression) | 0.977 | 0.951 |

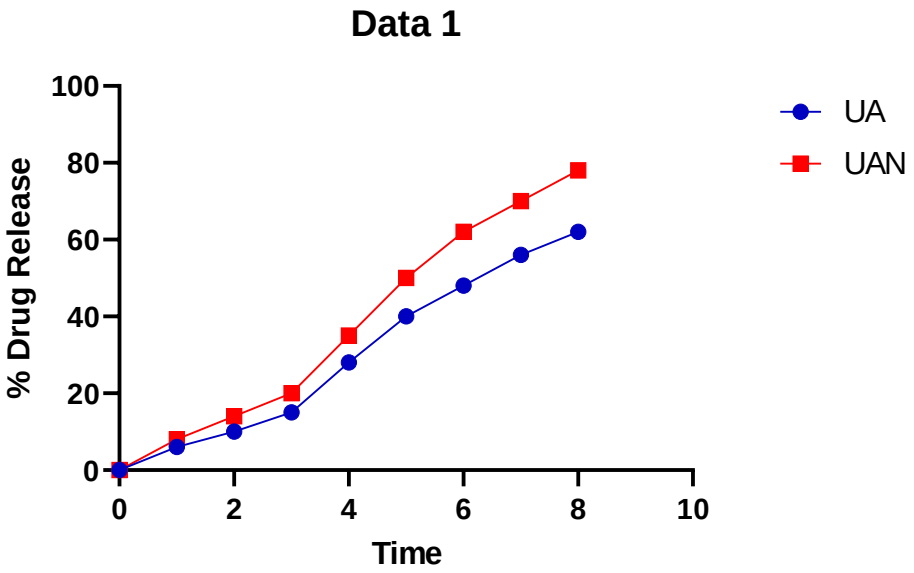



**Fig.5 Percentage drug release of UAN and UAO**

## 3.6 PHYSICO-CHEMICAL CHARACTERIZATIONS OF PREPARED AND SELECTED FORMULATION.

**Table 3: Physico-chemical characterizations.**

| Formulations | pH | Viscosity(cps) | Spreadability(gm.cm/sec) |
|---|---|---|---|
| Selected Base | 6.4±0.2 | 24.12±0.14 | 28.73±0.52 |
| UAO | 6.3±0.2 | 24.22±0.16 | 27.21±0.17 |
| UAN | 6.4±0.2 | 24.12±0.14 | 30.11±12 |

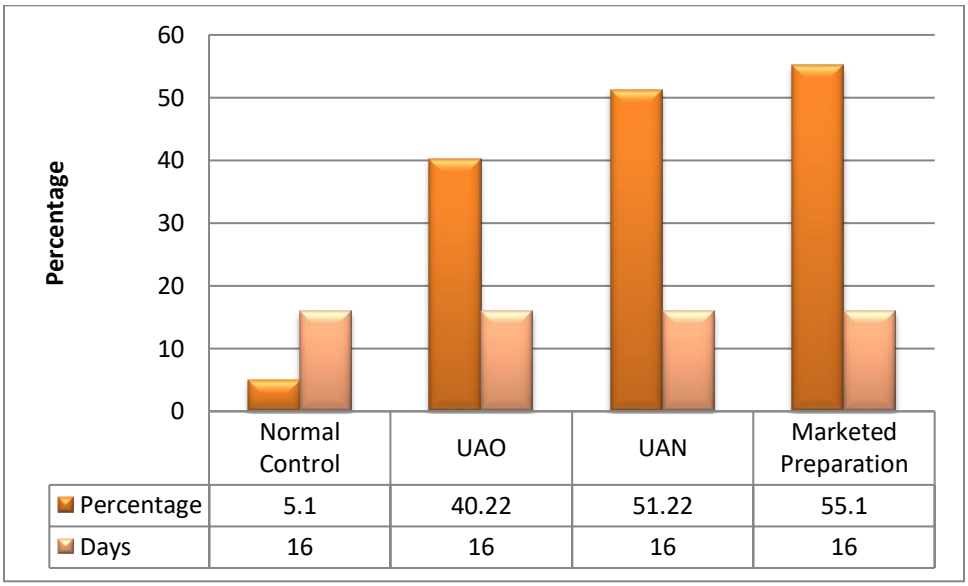
Each value represents the mean ±SD (n=3)

## 3.7 PHARMACOLOGICAL ACTIVITY

## IN-VIVO WOUND HEALING ACTIVITY

## 3.8 EFFECT ON WOUND CONTRACTION

The results shows that nano-formulation of Usnic acid with graphene of 1%w/w significantly promotes wound reduction. The percentage of wound reduction in normal control group (16$^{th}$ day) was found to be 5.10%. However in the treated group of usnic acid nano-formulation the wound reduction was found to be 40.22%. The nano-formulation of usnic acid and graphene exhibited 51.22% wound contraction. Maximum wound contraction percentage was obtained in the group of marked preparation of povidone-iodine i.e. 55.10%.




## 4. 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. This nano-formulation was prepared by nano-precipitation method and characterized by SEM which confirms that the average particle size is 80-120nm and also particles are in spherical in nature as spherical particles having better cellular entry in biomedical activities. The FTIR data shows no chemical interactions, as the obtained 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$^{-1}$ , C-H aromatic stretch at 700-900cm$^{-1}$, C=C aromatic stretch at 1600-1675cm$^{-1}$ were obtained. Usnic acid nano-formulation showed FTIR spectra at C=O stretch at 1631cm$^{-1}$, C-O stretch at 1000-1200 cm$^{-1}$, C-H aromatic stretch at 700-900 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 whereas the nano-formulation of usnic acid gives 70%-80% release of drug). The nano-ointment 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 24.12±14cps gives spreadability 30.11gm.cm/sec) the obtain data was compatible. The data of pH was also very compatible to the skin. Then finally the prepared nano-ointment was subjected for wound healing activity by using wound excision model. The *topical*

wound healing activity shows significant ($p<0.01$) wound healing activity that corresponds similar to activity of standard marketed preparation. The obtained data was compatible.

## 5. CONCLUSION

The present study clearly indicates that the nano form of usnic acid and graphene shows greater wound healing response as compare to the nano-form of usnic acid alone. The study illustrated that the secondary metabolite of lichen i.e. usnic acid possess wound healing activity and with graphene which considered as having anti-bacterial property, the prepared formulation exhibits good results. The exact mechanism of wound healing is still unknown. Further research can determine the exact wound healing mechanism and we are also working in that direction. is essential to note that while these findings are promising, further research is required to determine the specific mechanisms of action and potential side effects of plant-based treatments for wound healing. Additionally, the effectiveness of plant extracts may vary depending on the plant species, extraction methods, and the type and severity of the wound.

Overall, the studies on wound healing activity in rats suggest that plant-based treatments hold potential as natural and cost-effective alternatives for promoting wound healing. However, more extensive clinical trials and research are necessary to establish their safety and efficacy in human wound healing applications.

## AUTHORS CONTRIBUTIONS

Nimesh Kumar Dubey had performed and designed the study as well as written the manuscript. Dr. Ritesh Jain and Dr. Neeraj Sharma helped in conceptualization and supervision of study. Dr. Dharmendra Singh Rajput helped in conceptualization.

## CONFLICT OF INTEREST

There is no conflict of interest

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