Development of Usnic Acid Embedded Eudragit Microspheres for Alleviation of Nosocomial Infections

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Abstract: Background: Usnic Acid has been progressively reported in the literature as one of the chiefly significant lichen metabolites exemplified by ample diversity of applications such as antimicrobial, antifungal, antiviral, antiprotozoal agents, etc. Herein, we shed a light on nosocomial infections and formulated topical microspheres loaded with Usnic acid for improved antimicrobial activity. Recent patents and novel researches were referred to pursue the present work.

Methods: Usnic acid embedded Eudragit microspheres were designed applying solvent evaporation method, investigated for topography and drug-polymer compatibility studies. Dialysis bag method was utilized for studying drug release kinetics. In vitro antibacterial assay was carried out against the virulent bacterial strain of Staphylococcus aureus using the disc diffusion method.

Results: Topography studies revealed the formation of regular, micro-sized, smooth surface microspheres. Well defined and specific structural peaks were obtained from FTIR and TGA studies, revealing drug-polymer compatibility. The dissolution profile suggested Korsmeyer Peppas kinetic and Fickian kind of sorption. The pre-eminent activity of formulated microspheres was visualized from the disc diffusion study against Staphylococcus aureus.

Conclusion: The strong biological activity of Usnic acid –loaded Eudragit RS and Eudragit RL microspheres provides a promising application for corresponding material as a bactericidal agent for the alleviation of nosocomial infection. Findings paid attention to the potential of usnic acid microspheres for effective treatment of dermal and nosocomial infections caused by S. aureus.

Keywords: Antimicrobial, usnic acid, biocompatible, nosocomial infection, controlled release, microspheres.

1. INTRODUCTION

The co-adjuvant relationship accomplished between fungus and alga arises in the development of lichens, which formulate the use of photosynthesis from algae in the array to supply energy to fungi. Lichens grow up on rocks, tree trunks, and soil and present surprising capacity for water absorption. Their associated natural products are used in aroma, commercial applications such as creams, toothpaste, deodorant, sunscreen and so on. These products make use of imperative properties assigned to usnic acid viz. antiviral (Influenza A) [3, 4], antimicrobial, antifungal [5], anti-inflammatory [6, 7], antiproliferative [8, 9], analgesic, antiprotozoal and antipyretic [10], and in the cure of pulmonary tuberculosis [11]. In addition, health food supplements for weight reduction incorporate usnic acid as an additive. Based on this variety of possibilities, escalating research about usnic acid based innovative materials follows a common trend in the evolution of natural products for biologic applications (such as a bactericidal activity). Attempts to reduce the manifestation of diseases on Earth in human beings has not yet been very successful. The worldwide death toll is 52 million people a year consequent to the estimate by WHO (World Health Organization), and a third of the 17 million people die from infectious disease. Some usually encountered pathogens have been associated with some of the human diseases [12]. The awareness of microbial diseases, accessibility of meliorating diagnostic tools and the discovery of advanced therapeutic agents have aroused an avid deal of scientific developments.
activity in the area of Medical Microbiology. Microbial immunity continues to increase and the prospect for the use of antimicrobial drugs in the future is still not promising. The majority of clinically used antimicrobial drugs are a detriment in terms of toxicity, potency, cost and their frequent use has led to the egress of resistant strains. Thus, there is a cogent need to acquire alternative biodegradable agents, which are free from side effects. This search prompted the exploration of Natural Algal products, that could be exploited as biodegradable, more systemic and non-toxic antimicrobial agents, which would be isolated from side effects and with microbial toxic properties. In recent years, there has been thriving interest in alternative therapies and the use of algal products, for antimicrobial resistance. The algal kingdom is an immense repertoire of phytochemicals and establishes a bright area of current research in physiochemical prevention. It is, therefore, reasonable to predict an immense reservoir of biomolecules of algae, which can be therapeutic agents. Since almost infectious diseases are of microbiological origin with the advent of ever-increasing resistant fungal and bacterial strains, there has been a corresponding rise in the universal need for natural antimicrobial therapeutics. Fungi and bacteria stimulate significant human diseases, particularly in tropical regions and in immune-compromised or immune deficient patients. Despite the creation of potent antibiotic and antifungal agents, resistant or multi-resistant strains are incessantly appearing, imposing the need for an enduring search and evolution of new drugs [13]. We are perpetually in contact with innumerable micro-organisms in the environment. However, we are in even more greater contact with a tremendous number of micro-organisms that exist in our bodies. There are thousands of species of bacteria which stimulate a diversity of diseases in human beings. In recent years, infections caused by bacteria resistant to multiple antibiotics have been of major concern. Nosocomial infections, also known as hospital-acquired infections, are newly acquired infections that are contracted within a hospital environment. Transmission usually occurs via health care workers, patients, hospital equipment or interventional procedures. The most usual sites of infection are the bloodstream, lungs, urinary tract, and surgical wounds. Though any bacteria may induce a nosocomial infection, there is an expanding incidence of multidrug-resistant (MDR) pathogens causing hospital-acquired infections. This can be due to indiscriminate use of antibiotics and lack of hygiene measures, especially among medical staff. Commonly seen multidrug-resistant pathogens include methicillin-resistant Staphylococcus aureus (MRSA), extended-spectrum beta-lactamase-producing bacteria (ESBL), and Vancomycin-Resistant Enterococci (VRE). [14, 15] Lichens are a world-wide widespread consortium of fungal and photosynthetic partners. Usnic Acid is one of the most common and abundant lichen metabolites, well known as an antibiotic, and also endowed with several other interesting properties. Both the (+) and (-) enantiomers of usnic acid are effective against a large variety of Gram-positive (G+) bacterial strains, including strains from clinical isolates, irrespective of their resistant phenotype. The (+) -usnic acid enantiomer appears to be selective against Streptococcus mutans without inducing perturbing side effects on the oral saprophyte flora. On the other hand, the (-) - usnic acid enantiomer is a selective natural herbicide because of its blocking action against a specific key plant enzyme. Other recognized characteristics of usnic acid are ultraviolet absorption and preserving properties [16]. Usnic Acid [2, 6- diacetyl -7, 9-dihydroxy-1, 3- dimethyl -8-9b (2H, 9a/b)H -dibenzo-furandione; C_{18}H_{16}O_{7} is a compound of natural origin resulting from lichen secondary metabolism. It is a yellowish pigment produced by several lichen species. It is a product of the secondary metabolism of the fungal partner and it exists in two enantiomers which differ in the orientation of the methyl group located in position 9b [17]. It is considered as one of the most important biologically active metabolites with important pharmacological properties; antibiotic, antiviral [18], antitumor [19], antioxidant [20], tuberculostatic, anti-fungal [21] anti-inflammatory [22], insecticide [23] and molluscicide.

2. MATERIALS AND METHODS

The usnic acid powder was procured from Triveni Chemicals G.I.D.C., Ahmedabad; Polymers (Eudragit RS100 and Eudragit RL 100) were purchased from Yarrow chem. Products, Mumbai. Polyvinyl alcohol, chloroform, and methanol were purchased from S.D Fine Chemicals Ltd., Mumbai. Dialysis membrane -110 was purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). All other reagents and chemicals used were of analytical reagent grade.

2.1. Preparation of Usnic Acid Microspheres

A solvent evaporation technique was adopted to formulate Usnic acid loaded ERL 100 (Eudragit RL 100) and ERS 100 (Eudragit RS 100) microspheres. In this method, 50 mg Usnic acid and 1 gm ERL 100 and ERS 100 individually, were dissolved completely in 5 ml chloroform as the internal phase using mechanical stirrer at 800 rpm. The solution was then added dropwise with a syringe (24 gauzes hypodermic needle) to a solution of Poly Vinyl Alcohol in water (0.5% w/v) which acts as the external phase. This is the basis of the technology because most of the solvent disappears by evaporation. Solvent removal and hardening of the microspheres were done by continuously stirring the mixture for about 5-6 hours (Table 1). Microspheres were isolated by filtration and washed with n-hexane several times to remove residual PVA and solvent. The produced microspheres were dried at ambient temperature for 24 hours [24].

2.2. Characterization

2.2.1. Fourier Transform Infrared (FTIR) Analysis

Identification and chemical interactions were studied by FTIR spectroscopy. Usnic acid, polymers (ERL100/ERS100), and formulated microspheres were crushed with potassium bromide to get pellets by applying a pressure of 600 kg/cm². Spectral scans were taken in the range between 4000- 1500 cm⁻¹ on a Nicolet, FTIR 8400S, Shimadzu, Japan instrument [25].

2.2.2. Thermal Gravimetric Analysis (TGA)

TGA recorded a decrease in the weight of the sample as a function of the temperature. Usnic Acid, polymers (ERL 100 /ERS 100), and formulated microspheres were placed in aluminum pans and heated in nitrogen atmosphere at
10°C/min utilizing Perkin –Elmer TGA-7. The heating range of TGA-7 was 20–400°C [26].

### 2.2.3. Particle Size Determination and Field Emission Scanning Electron Microscope (FESEM)

The particle size of the microspheres was determined by using optical microscopy method, (Olympus, Dewinter, New Delhi, India). A small number of dry microspheres were suspended in distilled water. A small drop of suspension was placed on a clean glass slide. The slide containing suspended microspheres was mounted on the stage of the microscope and 300 particles were measured using an ocular micrometer [27]. Surface topography and morphology examination of the dried microspheres was carried out using a scanning electron microscope equipped with a secondary electron detector at an accelerating voltage of 10 KV. The dried samples were dispersed in water and were dropped onto metal stubs and allowed to dry in air under ambient conditions. Each sample was coated with gold to a thickness of about 30 nm in a vacuum evaporator [28].

### 2.2.4. Determination Of Drug Content

The various batches of the microspheres were subjected to drug content analysis. Accurately weighed microsphere samples were mechanically powdered. The powdered microspheres (5 mg) were dissolved in adequate quantity (10 ml) of phosphate buffer pH 6.8 and then filtered. The UV absorbance of the filtrate was measured using a UV spectrophotometer (Shimadzu, pharma-Spec1700) at 291 nm.

### 2.2.4.1. In Vitro Drug Diffusion

Accurately weighed microspheres equivalent to 10 mg usnic acid drug were added in the dialysis bag containing 5 ml methanolic phosphate buffer pH 6.8. Tied dialysis bag was kept in a beaker containing methanolic phosphate buffer pH 6.8 and allowed for diffusion for 8 hours. 5 ml aliquots were withdrawn and replaced with the same medium to maintain the sink condition at predetermined 30 minutes time interval [29]. Each aliquot was analyzed through UV-visible spectrophotometer at 291 nm and drug release profile was studied.

### 2.2.4.2. In Vitro Antibacterial Study

In vitro antibacterial activity was carried out by the disc diffusion method. 250 ml of PDA (Potato dextrose agar) media, was inoculated with 0.5 ml of a suspension of the Staphylococcus aureus, mixed well with a sterile loop and allowed to set in Petri plate to solidify. The stock solution of formulations B1 and B2 was each equivalent to 10 mg pure drug usnic acid prepared in DMSO (Dimethyl sulfoxide). 100 µl of this was impregnated over 10 mm paper disks on the PDA plate of S. aureus and allowed for incubation at 30°C for 48 hrs. The zone of inhibition was recorded and compared with the pure drug for antibacterial activity [30].

### 3. RESULTS

#### 3.1. Characterization

##### 3.1.1. Fourier Transform Infrared (FTIR) Analysis

FTIR spectra of Usnic Acid showed a characteristic peak of N-H stretching at 3415.51 cm⁻¹, C-H stretching at 2931.20 cm⁻¹, and C=C stretching at 1633.25 cm⁻¹. FTIR spectra of ERL 100 and ERS 100 exhibited the O-H stretching of hydrate band at 3429.99 cm⁻¹, C=O stretching at saturated aldehyde at 1735.65 cm⁻¹, N-R stretching of quaternary amine salt at 1384.86 cm⁻¹ and C-CO-C stretching of a strong band of ester at 1735.65 cm⁻¹. FTIR spectra of usnic acid showed a characteristic band of the conjugated ketone cyclic group at 1645 cm⁻¹. The peak at 3446 cm⁻¹, 3303 cm⁻¹, 3124 cm⁻¹ was due to stretching of O-H bond in intramolecular hydrogen bond. Alkane groups (-CH3) demonstrated peaks at 2921 cm⁻¹ and 2854 cm⁻¹ due to C-H bond stretching. Likewise, -C=C group depicts the peak at 1645 cm⁻¹ which indicates the presence of aryl group. The conjugation, electron donation of the constituent rings and possible hydrogen bonds imparted to the small wavelength of the aromatic methyl ketone at 1632 cm⁻¹. It was possible to specify the conjugated cyclic ketone group to the 1694 cm⁻¹ band. In addition, the IR spectrum of usnic acid comprised a band of hydroxyl phenolic groups at 3150 cm⁻¹. It was also possible to attribute the anti-symmetric and symmetric stretching n(C-O-C) aryl alkyl ether bands at approximately 1277 and 1070 cm⁻¹ respectively. FTIR of formulated microspheres revealed that no chemical interaction or changes took place during the formulation of microspheres and the drug was found to be stable (Fig. 1).

#### 3.1.2. Thermal Gravimetric Analysis (TGA)

TGA recorded a decrease in weight of the sample as a function of the temperature range 20°C- 400°C. TGA indicated
Fig. (1). Complied FTIR spectra of Usnic Acid, ERL/ERS 100 drug-loaded microspheres.

Fig. (2). Thermal gravimetric analysis of Usnic acid, ERL/ERS 100 loaded microspheres.
the dehydration and decomposition of each Usnic Acid, ERS 100 RS & ERL 100 microspheres. The TGA curve indicated a slight decrease in the weight of the Usnic acid, showing a fast weight loss step, which occurred between 250°C and 350°C, whereas it showed a rather slow weight loss over the range of 350°C-500°C. ERL 100 microspheres of the drug showed a fast weight loss step, which occurred between 100°C and 200°C, whereas it showed stability at 420°C, and a rather weight loss over the range of 430°C-800°C. ERS 100 microspheres of the drug depicted a fast weight loss step, which occurred between 230°C and 350°C, whereas it showed a rather slow weight loss over the range of 350°C-800°C. TGA of formulated microspheres described stability for up to 800°C indicating its better thermal behavior Fig. (2).

3.1.3. Particle Size Analysis and Scanning Electron Microscopy (SEM) Analysis

The particle size was determined using both simple and optical microscope (Olympus Dewinter, New Delhi, India). At least 20 particles of microspheres were counted for precise size distribution. Microspheres were found to be almost spherical, smooth and of regular surface. Microspheres B2 exhibited regular, smooth and spherical surface, which was due to high permeability property of B2 microspheres as they have more quaternary ammonium groups which make the polymer permeable as compared to B1 microspheres. The particle size distribution of microspheres formulation of batches B1 and B2 showed an average particle size of 80 µm, 25 µm respectively. Fig. (3a, b). The morphology of the microspheres prepared was investigated by Scanning electron microscopy. Microspheres of both the batches exhibited spherical porous surface. It can be seen that microspheres are almost spherical with a smooth surface and no drug crystals were found on the microsphere surface which might be attributed to the uniform removal of the solvent by evaporation to produce even polymer distribution. The SEM images of the microspheres are shown in Fig. (3c, d).

3.1.4. Drug Content Studies

Drug content was determined through UV spectrophotometer and the sequence of drug present in both the batches were 58.66 (B2), 50.25 (B1) mg drug/100 mg of microspheres. UV-vis spectrum of usnic acid is shown, in which it is possible to identify a characteristic absorption band centered at 291 nm, the typical signature of material (Fig. 4).

3.1.4.1. In Vitro Drug Release Study by Dialysis Bag Method

It was observed that the release rate of the drug from Eudragit RL 100 microspheres was a little higher than that of Eudragit RS 100 microspheres because Eudragit RL 100 contains a higher amount of quaternary ammonium groups, which renders it more permeable and accelerates the drug release. The release rate of Eudragit RS 100 microspheres exhibited a lag time at the initial release and the best release...
was observed with formulation B2. In vitro diffusion study was carried out through dialysis bag method and various models applied. The highest regression coefficient was found in Peppas model with n-value less than 0.5 predicting Fickian diffusion of drug release from formulated microspheres [B2] (Fig. 5), Table 2.

3.1.5. Drug Release Kinetics of Drug Loaded Topical Microspheres

3.1.5.1. In Vitro Antibacterial Study

Antibacterial activity of formulated microspheres was carried out by the disc diffusion method. The purpose of the study was to evaluate and compare the potential of usnic acid loaded microspheres along with polymer Eudragit RS 100 [B1] and Eudragit RL 100 [B2] loaded usnic acid microspheres. The in-vitro antibacterial study revealed that usnic acid microspheres along with formulations B1 and B2 possess antibacterial activity against S. aureus and are able to inhibit the growth of bacteria. In vitro disc diffusion analysis was done for the estimation of zone diameters (which is measured with help of the caliper scale) of formulated microspheres (B1 and B2), which demonstrated that S. aureus is highly susceptible to bioactive usnic acid (Fig. 6). Although there could be seen a pronounced clear zone of inhibition surrounding the pure drug, it must be its great susceptibility towards this virulent bacterial strain which must be attributed to free diffusion of usnic acid into culture media.

4. DISCUSSION

Usnic acid loaded Eudragit RL 100 and Eudragit RS 100 microspheres were explicated by a solvent evaporation technique. The formulations evinced controlled release of drug through the skin, signifying risky potential of the delivery system as equated with the drug of usnic acid. The formulation revealed enhanced retention of the drug in the skin, indicating improved potential of the delivery system. On the grounds of efficacy and improved patient compliance due to reduced frequency of application, microsphere-based formulations will have a significantly better role in the systemic treatment of anti-bacterial infection. Formulated microspheres were found to have a spherical, smooth and porous surface, facilitating high payload and meliorate drug release. FTIR spectra of microspheres revealed deep-rooted presence of the drug and polymer by predicting the wave number of their respective functional groups. FTIR spectra were recorded to assess the compatibility of the drug and excipients. FTIR spectra of the drug, Eudragit RS 100 and Eudragit RL 100 prepared microspheres were examined. The results showed that no chemical interaction or alteration took place during formulation of microspheres and the drug was found to be enduring. TGA of formulated microspheres showed stability for up to 800 °C signifying its better thermal behavior. The formulated B1 microspheres showed a better thermal stability as compared to drug and B2 microspheres because of the presence of quaternary ammonium compounds. The TGA curve indicated a slight decrease in weight of the Usnic acid, showed a fast weight loss step, which occurred between 250°C and 350°C, whereas it showed a rather slow weight loss over the range of 350°C-500°C. ERL 100 microspheres of the drug showed a fast weight loss step, which occurred between 100°C and 200°C, whereas it showed stability at 420°C. ERS 100 comprising microspheres depicted a fast weight loss step, which occurred between 230°C and 350°C, whereas it showed a rather slow weight loss over the range of 350°C-800°C. The particle size distribution of microspheres formulation of batches B1 and B2 showed an average particle size of 80 µm and, 25 µm respectively. Microspheres were found to the almost spherical, smooth and regular surface. Microspheres B2 exhibited small, regular, smooth, and a spherical surface, which is due to high permeability property of B2 microspheres as they have more quaternary ammonium groups which makes the polymer permeable as compared to B1 microspheres. The surface texture of the microspheres was investigated by Scanning electron microscopy. Microspheres of both the batches exhibited spherical porous surface. It can be seen that microspheres are almost spherical with a smooth surface and no drug crystals were found on the microsphere surface which might be attributed to the uniform removal of the solvent by evaporation to produce even polymer distribution. The in vitro drug release study by dialysis bag method was carried out which showed that the release rate of the drug from Eudragit
Table 2. Drug release kinetics from different models.

<table>
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<th>S. No.</th>
<th>Model</th>
<th>Correlation Coefficient ( (r^2) ) Batch B1</th>
<th>Correlation Coefficient ( (r^2) ) Batch B2</th>
<th>Rate Constant ( (k) ) B1</th>
<th>Rate Constant ( (k) ) B2</th>
<th>( n ) Value B1</th>
<th>( n ) Value B2</th>
</tr>
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<tr>
<td>1</td>
<td>Zero order</td>
<td>0.9476</td>
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<tr>
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<td>First order</td>
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<td>0.9394</td>
<td>0.003</td>
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<td>0.456</td>
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<td>0.9854</td>
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<tr>
<td>4</td>
<td>Peppas</td>
<td>0.9840</td>
<td>0.9873</td>
<td>3.048</td>
<td>4.862</td>
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</tbody>
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Fig. (6). Inhibition hales of usnic acid loaded microspheres along with usnic acid loaded B1 and B2 microspheres.

RL 100 microspheres was a little higher than that of Eudragit RS 100 microspheres because Eudragit RL 100 contains a higher amount of quaternary ammonium groups, which renders it more permeable and accelerates the drug release. The release rate of Eudragit RS 100 microspheres exhibited a lag time at the initial release and the best release was observed with formulation B2. In vitro diffusion study was carried out through dialysis bag method and for the various models applied, the highest regression coefficient was found in Peppas model with \( n \)-value less than 0.5 predicting Fickian diffusion of drug release from formulated microspheres [B2]. Antibacterial activity of formulated microspheres was carried out by the disc diffusion method. The purpose of the study was to evaluate and compare the potential of usnic acid loaded microspheres along with polymer Eudragit RS 100 [B1] and Eudragit RL100 [B2] loaded usnic acid microspheres. The in vitro antibacterial study revealed that usnic acid microspheres along with formulation B1 and B2 possess antibacterial activity against \textit{S. aureus} and are able to inhibit the growth of bacteria. In vitro disc diffusion analysis was done for the estimation of zone diameters of formulated microspheres (B1 and B2) demonstrating that \textit{S. aureus} is highly susceptible to bioactive usnic acid. Although a pronounced clear zone of inhibition surrounding the pure drug must be its great susceptibility towards this virulent bacterial strain which must be attributed to free diffusion of usnic acid into culture media.

CONCLUSION

Usnic acid loaded ERL 100 and ERS 100 microspheres were formulated by a solvent evaporation technique. Formulated microspheres were found to have a spherical and porous surface, facilitating high payload and controlled drug release. FTIR spectra of microspheres confirmed the presence of drug and polymer by predicting the wave number of their respective functional groups. TGA analysis showed better thermal behavior with no drug-polymer interaction. Batch 2 microspheres prepared from Eudragit RL 100 showed higher drug release, attributed to more permeability as compared to Eudragit RS 100 microspheres. The kinetic data of the in vitro diffusion release of usnic acid microspheres was found to follow Peppas model kinetic, based on \( r^2 \) values giving higher results of B2 than B1. The in –vitro
antibacterial study revealed that usnic acid microspheres along with formulations B1 and B2 possess antibacterial activity against S. aureus and are able to inhibit the growth of bacteria. In vitro disc diffusion analysis was done for the estimation of zone diameters of formulated microspheres (B1 and B2) demonstrated that S. aureus is highly susceptible to bioactive usnic acid. The formulated microspheres have the potential for the treatment of topical microbial infection.

ETHICS APPROVAL AND CONSENT TO PARTICIPE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

None.

CONFICT OF INTEREST

The authors declare no conflict of interest, financial or non-financial.

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