Middle-East Journal of Scientific Research 14 (4): 544-548, 2013
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2013.14.4.7345

Nanoencapsulation of Withaferin-A Using Poly-(Lactic Acid) for Enhanced Anxiolytic Activity

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Abstract: A better formulation ensures a better delivery system. This study is based on this fact, having a greater belief in a natural product compared to chemical counterpart. Stress is a negative concept that affects the mental and physical well being. Stress is nowadays very common in the sedentary lifestyle. Drugs that are found in the market are chemically synthesized and might cause side effects. Owing to this reason, various studies have been made to discover natural products with anxiolytic properties, amongst which the phytochemicals, is a wide area of research. An effort was made to encapsulate the phytochemical Withaferin-A, using nanoparticles. Nanoencapsulation of this phytochemical is expected to have increased bioavailability and increased water solubility. Withaferin-A was nanoencapsulated using the polymer Poly-(Lactic Acid) by solvent evaporation method, the formation of nanoparticles were found using the Atomic Force Microscopy and the concentration of loaded particles were found using the diphenyl picrylhydrazyl assay.

Key words: Stress · Withania Somnifera · Bioavailability · Atomic Force Microscopy · Diphenylpicrylhydrazyl Assay

INTRODUCTION

Stress as already mentioned is a negative concept that affects the mental and physical well being. It alters the physiological homeostasis. Lack of time, tension in work place, relationship problems, other diseases suffered by an individual, life style etc can be attributed to causative reasons for stress. Stress causes various undesirable consequences such as increased breathing rate, insomnia, blood pressure increase, pulse increase and the digestive system and the immune system goes down.

As a consequence there is an increased demand for drugs to reduce stress. There are many drugs available in the market and are prescribed by the physicians. Drugs such as the Diazepam, Prozac, Paxil etc are used. These are chemically synthesized and will have side effects. Hence there is a need for an alternate source of medicine. The best option lies in the plant products [1] of which the phytochemicals are gaining importance and so Withaferin-A was chosen.

Withania somnifera Dunal (WS), is known as Ashwagandha, has been in use for medical practices since 3000 years. It has the ability to create a sense of mental wellbeing, arrest aging, defend against diseases and revitalize the body in exhausted condition [2].

Withaferin-A (Figure 1) [3] is an antioxidant, an adaptogenic, an aphrodisiac and possesses anti-inflammatory properties also being an anti ulcer [4]. It exhibits enhanced anxiolytic activity without tolerance *in vivo* [5]. It has been in use to treat stress, arthritis insomnia and age related disorders including neurodegenerative disorders [6, 7]. Active constituents such as Withaferin-A, Withanolide D, polyphenols, alkaloids, phytosterols, beta-sitosterol and 18 fatty acids [8, 9] are present in *Withania somnifera*.

Nanoencapsulation of the phytochemical was done in order to have increased bioavailability and increased water solubility, ensuring a better drug delivery system. The Withaferin A was nanoencapsulated with Poly-(lactic acid) (PLA).

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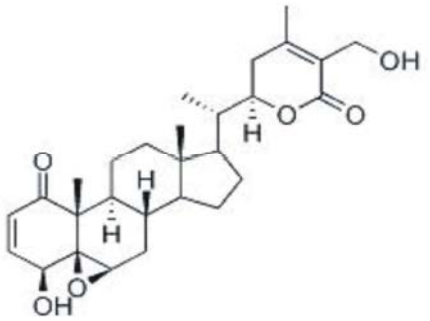

Fig 1: Chemical Structure of Withaferin-A

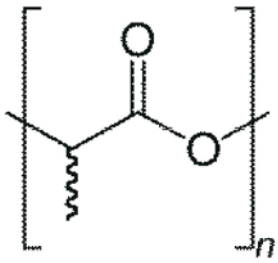

Fig 2: Structure of Poly-(Lactic Acid)

PLA (Figure 2), as the nano encapsulating material is chosen since it is a plastic substitute made from plant starch that has been fermented. It is usually taken from corn, since again it's a natural product it is supposed to have lesser negative impact. It is easily accepted by the human body. The advantages lie in the fact of helping by being safe, biocompatible and posses the ability to control time and rate of polymer degradation [10].

In this study, the Withaferin-A stock was made, the nanoencapsulation was done by the solvent extraction method and the loading efficiency was estimated by the DPPH assay. The release study also was made using the DPPH assay. Morphological study of Withaferin-A loaded PLA nanoparticles by AFM.

MATERIALS AND METHODS

Chemicals: Withaferin-A was purchased from Natural Remedies, Bangalore. Poly-(lactic acid) (PLA), dichloromethane, Poly-vinyl alcohol (PVA), ethanol and DPPH were purchased from Sigma-Aldrich, Bangalore and Hi Media, Mumbai.

Preparation of Withaferin-A Stock: A quantity of 1mg of Withaferin-A was dissolved in 1ml of 100% ethyl acetate. (1 mg/ml).

Synthesis of Withaferin-A–Poly (Lactic Acid) (PLA) Nanoparticles by Solvent Evaporation Method: A quantity of 200 mg of PLA was dissolved in 2 ml dichloromethane (DCM) by vortexing. Withaferin-A of three different concentrations viz., 125 µg/ml, 250 µg/ml and 500 µg/ml were added to 500 µl of PLA stock separately. Sonication was done in room temperature at 40% amplitude for 1 minute. PVA of 3% concentration was added to make up to 1 ml and was sonicated again under same conditions. A quantity of 1 ml of 0.1% PVA was added. These 2 ml Withaferin-A loaded-nanoparticle suspensions were stored in refrigerator. The basic principle of this technique is emulsification of aqueous phase (PVA solution) and organic phase (PLA + DCM + Withaferin-A) [11].

Morphological Study of Withaferin-A Loaded PLA Nanoparticles by AFM: The pellets obtained by centrifuging the nanoparticle suspensions were taken. A quantity of 100 µl of each pellet was taken into a new 2 ml eppendorf tube and was made up to 1ml using DCM. Sonication was done in room temperature at 40% amplitude for 1 minute. Smear of these three samples were made on glass slides and AFM was carried out to study the morphology of the nanoparticles.

Standard Graph for DPPH Assay: A quantity of 100 ml of sodium acetate buffer was prepared by mixing 35.7ml of 0.1M acetic acid and 64.3ml of 0.1M sodium acetate. Buffered methanol was prepared by mixing 40ml of sodium acetate buffer and 60ml of methanol and the pH was adjusted to 5.5. A quantity of 25 mg of DPPH was dissolved in 50ml of buffered methanol (1mM).

DPPH of 1mM stock concentration was diluted to a working concentration of 0.1mM using sodium acetate buffer. A quantity of 1ml of 0.1mM DPPH was added to 0.5 ml of different concentrations of Ascorbic acid i.e. from 5µg/ml to 500µg/ml. Incubated in dark at room temperature for 30min and absorbance was read at 517nm. A standard graph was plotted between the concentration of ascorbic acid and the absorbance value [12].

Study of Loading Efficiency of Withaferin-A in PLA: A quantity of 1 ml of the nanoparticle suspensions were taken and centrifuged at 12000 rpm for 10minutes. The pellets were stored in refrigerator. The supernatant has the unloaded Withaferin-A whose amount has been calculated using DPPH assay. The % loading efficiency was calculated using equation 1.

$$\text{Loading Efficiency (\%)} = \frac{\text{(Initial amount of Withaferin-A} - \text{Amount of unloaded Withaferin-A)}}{\text{(Initial amount of Withaferin-A) (Equation 1)}} \times 100$$

Release of Withaferin-A from the Nanoparticle in the Solvent: A quantity of 1 ml of the Withaferin-A PLA nanoparticle suspension was divided into 100µl in ten 2ml Eppendorf tubes. To this 900µl of 50% ethyl acetate was added and vortexed. This was done for all the three concentrations in 30 Eppendorf tubes. After 30 minutes first tube of each concentration were taken and centrifuged at 15000rpm for 15min. The supernatant was collected and the concentration of Withaferin-A was estimated by DPPH assay. This was repeated for every 1 hour till 10th hour and for 20th and 26th hour.

RESULTS

Synthesis of Withaferin-A PLA Nanoparticles: The antioxidant molecule Withaferin A was encapsulated by PLA using solvent evaporation technique.

Morphological Study of Withaferin-A Loaded PLA Nanoparticles by AFM: The sizes of the nanoparticles are 198nm and 263nm for 125µg/ml and 250µg/ml nanoparticle suspensions respectively (Figure 3 & 4).

Standard Graph for DPPH Assay: Standard graph was generated with different known concentrations (µg/ml) of ascorbic acid vs. OD at 517nm (Figure 5). Standard graph for DPPH of ethanolic extract of *Withania somnifera* with the regression equation being y=0.027x+5.077 was taken from Panchawat [13] (Figure 6) and compared with the ascorbic acid standard graph (Figure7). OD at 517nm for negative control = 1.061.

Loading Efficiency of Withaferin-A in PLA: The amount of unloaded Withaferin-A was determined using the standard graph of DPPH. The loading efficiencies of the three different concentrations of Withaferin-A are given in the Table 1. With increase in the concentration of Withaferin A, the loading efficiency was also found to increase.

Table 1 % Loading efficiency of Withaferin-A at three different concentrations estimated using equation 1

Release of Withaferin-A from the Nanoparticle in the Solvent: The release study for three different concentrations of Withaferin-A (125µg/ml, 250µg/ml and 500µg/ml) was done and results were obtained from the standard graph of DPPH (Figure 8).

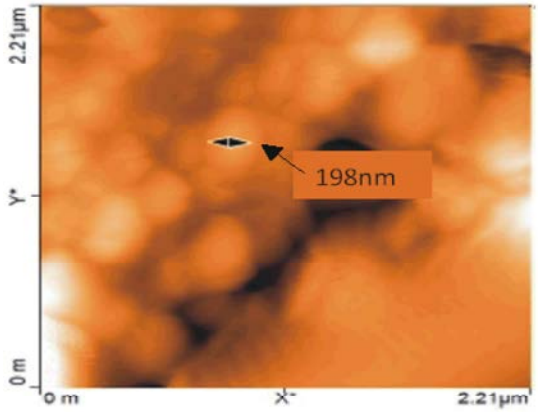

Fig. 3: AFM image of 125µg/ml concentration Withaferin A- Poly-(Lactic Acid) suspension

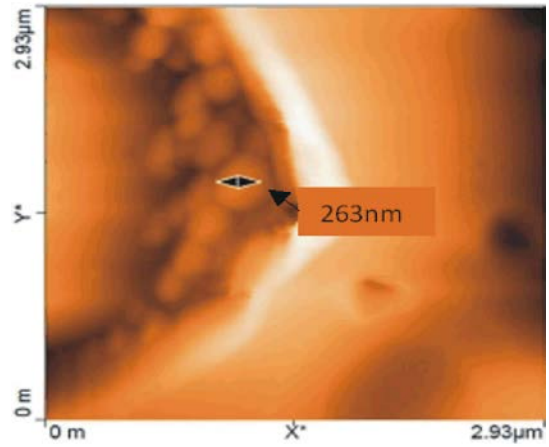

Fig. 4: AFM image of 250µg/ml concentration Withaferin A-Poly-(Lactic Acid) Suspension

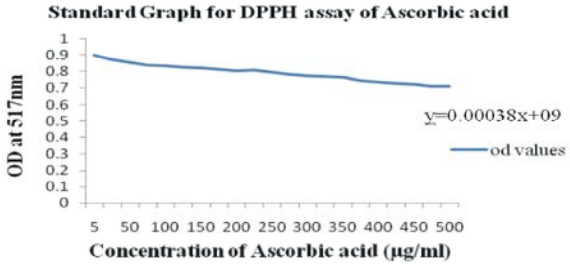

Fig. 5: Optical density measured at 517nm corresponding to the known concentrations of Ascorbic acid (µg/ml)

Table 1: Loading efficiency of Withaferin A

Initial Concentration of Withaferin A (µg/ml)	OD at 517nm DPPH assay	Concentration of unloaded Withaferin A (µg/ml)	% Loading Efficiency
125	0.871	77.5	38
250	0.850	130	48
500	0.813	230	54

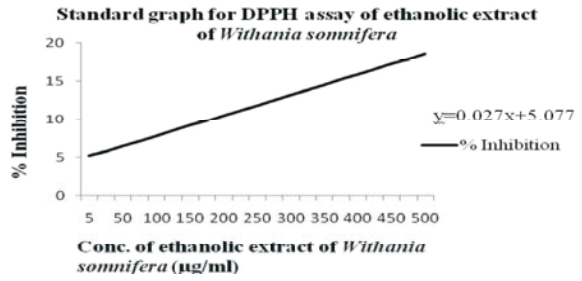

Fig. 6: Standard graph for ethanolic extract of *Withania somnifera* using DPPH assay

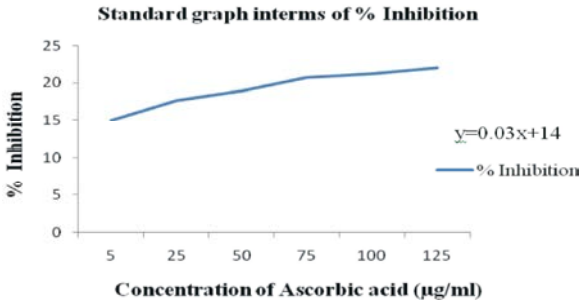

Fig. 7: Standard graph for Ascorbic acid using DPPH assay

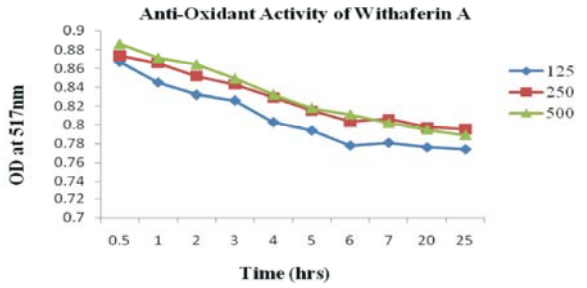

Fig. 8: Release study of Withaferin-A of three different concentrations (125 µg/ml, 250 µg/ml and 500 µg/ml) using DPPH assay

DISCUSSION

Anxiety being the challenging psychiatric disorder to be treated without causing any side effects, the focus has turned to the natural products. In this present study, the phytochemical Withaferin-A was chosen for treating anxiety. A better drug delivery system was needed to increase the bioavailability of Withaferin-A. So it was encapsulated in PLA nanoparticles. Nanoparticles are submicron sized solid particles, biodegradative properties depends on the materials [14, 15]. Nanoencapsulation increases bioavailablity [16] and enhances entry into the cell [17]. From the AFM images it is quite evident that Withaferin A has been loaded with PLA successfully.

To scrutinize the release of the phytochemical from the nanoparticle, in vitro study was done and a steady release was observed. This was done by measuring its free radical scavenging activity using DPPH assay. It was proved that its antioxidant property was retained even after encapsulation. This is a proof that drug delivery by nanoparticles would increase its bioavailability *in vivo*. Degradation of PLA in human body is possible [18] and it does not post any side effects. This preliminary work will support further studies on animal model (mice), to ensure the enhanced anxiolytic activity of Withaferin-A.

Having water soluble PLA and water insoluble Withaferin A, encapsulation by micelle formation method would give even higher encapsulation efficiency [19-21].

CONCLUSION

In conclusion, from this study it is quite evident that nanoencapsulation of Withaferin-A is possible and its antioxidant activity is retained even after encapsulation. Further areas of research, such as checking the release of Withaferin A in vivo should be concentrated.

AKNOWLEDGEMENT

We would like to thank the management of VIT University for providing funding for this research work. We would also like to extend our sincere thanks to Centre for Nanotechnology Research, VIT University for helping us with Atomic Force Microscope facility.

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