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Preparation of liposome encapsulated azadiradione

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Abstract
Azadiradione (AZD) is one of the four active components in Neem. AZD is commonly used in traditional medicine (Ayurveda) in all over the world. AZD showed potential biological activities such as anti-inflammatory, anti-cancer and anti-feedant. The aim of this study is to prepare and characterize liposomal formulation of the neem constituent AZD. The liposomal nanoform of AZD was successfully prepared and the high zeta potential (-38.13 ± 1.89) indicated the repulsion between the particles preventing the aggregation. Liposomal AZD showed an average size of 323.4 ± 9.79 and a narrow size distribution (0.594 ± 0.0331). In addition, AZD loaded liposomes had a zeta potential value of -38.13 ± 1.89. Furthermore, the encapsulation efficiency of the drug (EE %) was determined by using high performance liquid chromatography. The analysis showed that EE% was around 82.02 ± 9.01%. Additionally, the morphology of the particles was examined by scanning electron microscope. The results revealed that the nanoparticles have a roughly spherical shapes. In conclusion, the employed method produced a liposomal encapsulated form of the biologically active AZD which worse further analysis for the determination of the bioavailability and tissue distribution of AZD vs liposomal nanoparticles *in vivo* in order to increase the activity of AZD as anticancer potential drug. Key word: Azadiradione, Neem, liposomal formulation, scanning electron microscopy, HPLC.

Keywords: Azadiradione (AZD), traditional medicine (Ayurveda), liposomal

1. Introduction
Azadiradione (AZD) (Figure 1) is a limonoid natural product which is extracted from the neem tree (*Azadirachta indica*) grown in India, however, it is widely cultivated throughout the tropics (Kikuchi, Ishii *et al*. 2011) [24]. AZD is known to possess anti-nociceptive and anti-inflammatory activities (Ilango, Maharajan *et al*. 2013) [22]. More importantly, AZD shows variable anticancer and apoptotic activity against five human cancer cell lines; HL60 (leukemia), A549 (lung), AZ521 (stomach), SK-BR-3 (breast), and CRL1579 (melanoma) (Kikuchi, Ishii *et al*. 2011) [24]. Therapeutic properties of neem have been identified since ancient times and are extensively used in ayurveda, unani, and homoeopathic medicine. Literature reveals that neem possess anti-inflammatory, anti-arthritic, antipyretic, hypoglycemic, antigastric, antifungal, antibacterial, and antitumor properties and is being used for the same (Bandyopadhyay, Biswas *et al*. 2004, Sultana, Anwar *et al*. 2007, Ebong, Atangwho *et al*. 2008, Paul, Prasad *et al*. 2011, Mahapatra, Young *et al*. 2012) [8, 30, 15, 28, 26]. However, most of these types of compounds are not stable in the human body and will get easily eliminated from the blood. Additionally, it has been well established that liposomal encapsulated compounds are more stable in the human body. Recently, nanotechnology has received a great attention as a potential approach for cancer therapy. Over the last three decades, a large number of nanoparticles delivery systems have been developed for cancer management. In this regard, many liposomal, polymer–drug conjugates and micellar formulations are currently in the preclinical and clinical stages (Alexis, Pridgen *et al*. 2010) [1]. Among the various investigated delivery systems, liposomes have gained more attention owing to their unique characteristics. Liposomes are lipid-based vesicles that are widely used as drug nanocarriers (Vahed, Salehi *et al*. 2017) [31]. They are characterized by their biocompatibility, biodegradability, safety, and lack of immunogenicity (Deshpande, Biswas *et al*. 2013, Khan, and Saeed *et al*. 2017) [13, 24]. Moreover, liposomes have a strong impact on pharmacokinetics and tissue distribution of loaded drugs. This may lead to enhanced efficacy as well as reduced toxic effects of antitumor drugs (Huwyler, Drewe *et al*. 2008) [21]. Taken together, various liposomal formulations have clinically shown preferential accumulation in tumors, via the enhanced permeability and retention (EPR) effect of the cancer tissue. This may be contributed to the nanosize of liposomes which may help increase the bio-distribution and accumulation of drugs in targeted tumor sites (Durymanov, Kamaletdinova *et al*. 2017) [14].

Over two decades, our research group in collaboration with multidisciplinary international researchers developed more selective and effective anti-cancer agents for various types of cancer including liver and breast cancers (Badria 1994, Badria, Mabed *et al*. 2000, Badria, Houssen *et al*. 2003, Badria, Houssen *et al*. 2003, Badria, Mikhaeil *et al*. 2003, Bar,

Khanfar *et al*. 2009, Ayyad, Abdel-Lateff *et al*. 2012, El-Naggar, Abdel-Bar *et al*. 2014, El-Senduny, Badria *et al*. 2016, H El-Far, A Badria *et al*. 2016, Barakat, Islam *et al*. 2017, El-Naggar, Mira *et al*. 2017, M Ghorab, SA El-Gaby *et al*. 2017, Bar, Elimam *et al*. 2018, Barakat, Islam *et al*. 2018) [5, 4, 7, 7, 7, 9, 2, 16, 20, 20, 12, 17, 25, 10, 11]. Taking into account that cancer treatment aims basically at killing cancerous cells with minimum adverse effects. Accordingly, there is still an increasing need to develop safe and efficacious agents for cancer therapy. Nevertheless, natural products have been proved as potential, effective and safe candidates for cancer therapy. Therefore, in this study, a liposomal formulation of azadiradione (AZD) have been formed and characterized by different techniques. To the best of our knowledge, there is no reported study on the liposomal formulation of AZD.

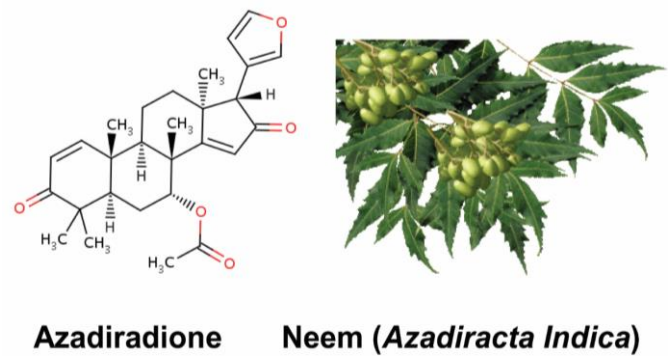

Fig 1: Chemical structure of AZD

2. Materials and methods

2.1 Reagents

Azadiradione was kindly provided by Dr. AJK (Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, Assam-781039, India), lecithin, cholesterol and chloroform were purchased from Sigma Aldrich, St. Louis MO, USA. HPLC grade water, acetonitrile and methanol were obtained from Fisher Scientific Co, Bridgewater, NJ, USA. All other solvents and reagents used in this study were of analytical grade.

2.2 Methodology

AZD liposomes were prepared by thin film hydration method as previously reported (Nkanga, Krause *et al*. 2017, Xu and An 2019) [23, 32]. Briefly, in a round bottom flask, known amounts of lecithin, cholesterol and AZD (500, 125 and 50 mg, respectively) were accurately weighed and dissolved in 3mL chloroform. Lipid film was formed by evaporating the organic solvent using rotary evaporator at room temperature for about 30 min. Thereafter, the flask was kept in a desiccator overnight for the removal of the organic solvent traces. Later, 5mL of water was added to the film to hydrate for a period of 60 min at 45°C using rotavapor. After hydration, liposomal dispersion of AZD was sonicated using bath sonicator for about 15 min. Finally, the dispersion was centrifuged at 15000 rpmfor 3 hrs at 5°C to separate the free drug. Supernatant was discarded and the pellet was re-dispersed in5 mL water for further experiments and characterization. Then, the liposomes were freeze-dried and kept for further characterizations.

2.3 Characterization of AZD liposomes

2.3.1 Particle size, polydispersity index (PDI) and zeta potential

Freeze-dried liposomes were characterized for size, size distribution (polydispersity index, PDI) and zeta potential by dynamic light scattering using Zetasizer Nano, Malvern, UK.

2.3.2 Determination of drug encapsulation efficiency (EE%)

The amount of AZD incorporated into liposomes was determined using an HPLC method (Gunasekaran and Anita 2010) [19] Briefly, Known amount of the prepared liposomes was taken and liposomes were digested (dissolved) using a mixture of acetonitrile: methanol (ACN: MeOH, 60: 40 v/v). The sample was sonicated for 15 using water bath sonicator. Later, the produced solution was filtered using syringe filter (0.2 µm). Drug concentration was determined using HPLC method. Mobile phase was a mixture of ACN: MeOH (60: 40 v/v). The sample (20 µL) was analyzed using C18 reverse phase column (150 mm x 4.6 mm, Agilent, USA). The detection wave length was 215 nm and flow rate was set at 1 mL/min. AZD calibration curve (1- 10 µg/mL) was constructed in ACN: MeOH (60: 40 v/v). Figure 2 shows HPLC chromatogram using the previously mentioned mobile phase.

The encapsulation efficiency (EE%) was calculated as described in Equation 1. The experiment was carried out in triplicate.

Equation 1: Calculation of encapsulation efficiency

$$EE\% = \frac{\text{Actual drug co}}{\text{Theoritical drug c}}.$$

2.3.3 Scanning electron microscopy (SEM)

The shape of freeze-dried liposomes was investigated using SEM (JOEL-JSM- 5400 LV scanning electron microscope, Japan).

2.3.4 Statistical analysis

Statistical analysis was done using Minitab 15 Statistical Software using one-way ANOVA followed by Tukey's post hoc test at a significance level of $p<0.05$. The data is presented as mean ± standard deviation. All experiments were carried out in triplicates.

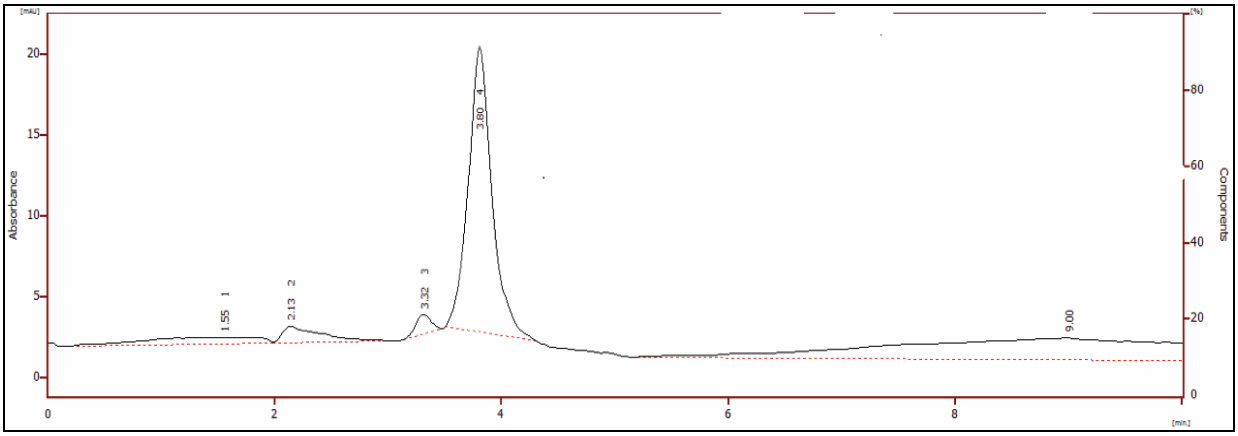

Fig 2: HPLC chromatogram of AZD (t3.8 min); (20 µL of AZD, column: C18 reverse phase column (150 mm x 4.6 mm, Mobile phase: ACN: MeOH (60: 40 v/v), flow rate: 1 mL/min UV: 215 nm)

3. Results and Discussion

3.1 Characterization of AZD loaded liposomes

3.1.1 Particle size, polydispersity index (PDI) and zeta potential

Particle size, PDI and zeta potential values of AZD loaded liposomes are presented in Table 1. As can be seen, the liposomal formulation showed average particle size of 323.4 ± 9.79 with narrow size distribution as indicated by the PDI value (0.594 ± 0.0331). In addition, AZD loaded liposomes had a zeta potential value of -38.13 ± 1.89. This high zeta potential value accounts for liposomes stability and prevents particle aggregation. Also, the high negative charge helps cell internalization. This finding is in accordance with results reported in literature (Kelly, Jefferies *et al*. 2011, Nkanga, Krause *et al*. 2017) [23, 27].

3.1.2 Determination of drug encapsulation efficiency (EE%)

In order to obtain the highest entrapment of a hydrophobic drug, lipid film method was preferred for the preparation of AZD liposomes (Sezer, Baş *et al*. 2004) [29]. Additionally, a preliminary study was carried out in order to determine the lecithin: cholesterol mass ratio that achieves the best entrapment of AZD (Data is not shown). It was found that liposome formation was best achieved at a lecithin/ cholesterol mass ratio of 4:1. Accordingly, the best EE% was found to be 82.02 ± 9.01%. This high EE% of AZD may be contributed to the relatively high lipophilicity of AZD and high lecithin/ cholesterol mass ratio. Similar results were previously reported by (Sezer, Baş *et al*. 2004) [29].

Table 1: Average particle size, polydispersity index (PDI), Zeta Potential (ZP) and EE% of AZD loaded liposomes. ± Standard deviation from three independent experiments.

Average particle size	PDI	Zeta potential	EE%
323.4 ± 9.79	0.594 ± 0.0331	-38.13 ± 1.89	82.02 9.01

3.1.3 Scanning electron microscopy (SEM)

Scanning electronic microscopy presented in Figure 3shows the morphology of AZD loaded liposomes. As can be seen, AZD loaded liposomes showed particles with roughly spherical shapes. Moreover, no particles aggregation was noticed during the samples microscopic observation. This is probably due to the negative charges on the surface of particles. These charges prevent particles aggregation via repelling each other. This is in agreement with the finding of Nkanga *et al* (Nkanga, Krause *et al*. 2017) [27]. This is the first report on the liposomal formulation of one of the constituent of neem plant and further studies is required in order to evaluate the effectiveness of the liposomal form versus the AZD alone(*in vivo* study).

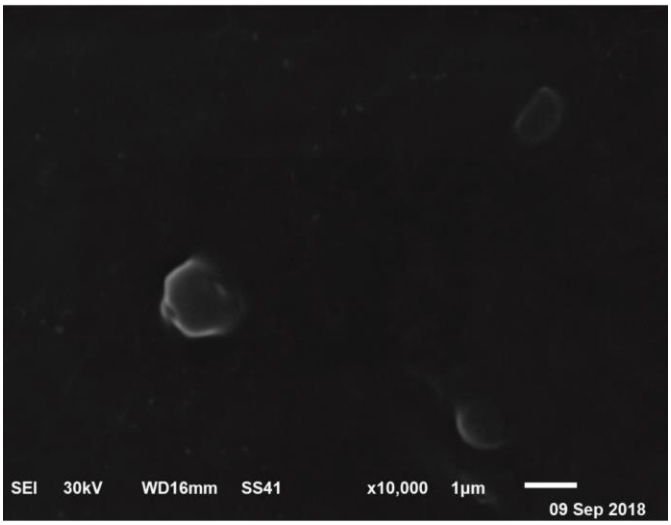

Fig 3: SEM photograph of AZD loaded liposomes

4. Conclusion

Here in this study, liposome encapsulated azadiradione formulation was successfully achieved. The size, polydispersity index (PDI) and zeta potential of the obtained nanoparticles were analyzed. Moreover, seta potential, part nanoparticles and characterized by different techniques such as SEM,

5. Acknowledgment

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