Niosomes and liposomes as promising carriers for dermal delivery of \textit{Annona squamosa} extract

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The leaf extract of \textit{Annona squamosa L.} has antibacterial, antidiabetic, antioxidant, and anticancerous activities. The present work aims to compare between liposomes and niosomes as carriers for \textit{A. squamosa} extract to improve its transdermal bioavailability. Physical characterization for niosomes and liposomes was performed using: transmission electron microscope (TEM), scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR). In addition, the encapsulation efficiency for \textit{A. squamosa} in both carriers was evaluated and in-vitro drug release experiments were performed. The results proved the potential of both carriers to penetrate the outer layer of the skin (stratum corneum) which is considered as a strong barrier against the diffusion of many compounds through the skin. Moreover, the results pointed out that niosomes and liposomes lasted long time through the skin, which ensures the presence of antioxidant extract in the skin for prolonged periods. This would have a benefit of targeting free radicals in the skin. The encapsulation efficiency of liposomes for \textit{A. squamosa} extract exceeded that of niosomes, however, niosomes demonstrated longer time of drug release through the skin. In conclusion, niosomes and liposomes are promising carriers for dermal delivery of the antioxidant extract \textit{Annona squamosa}.

\textbf{Keywords:} \textit{Annona squamosa}. Dermal delivery. Liposomes. Niosomes.

\section*{INTRODUCTION}
Plants are considered as a source of secondary metabolites (Phytochemicals) like flavonoids, phenols, phenolic glycosides, saponins and glycosides with a variety of structural arrangements and properties. These secondary metabolites are highly biological active (De Fatima \textit{et al.}, 2006). Phytochemicals have protective or disease preventive properties including - antibacterial, anticancer, antifungal, and antioxidant that can protect the health (Krishnaraju, Rao, Sundararaju, 2005).

Since chemotherapeutics have not increasingly failed and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo, Bosisio, 1996; Iwu, Duncan, Okunji, 1999).

Natural antimicrobials can be derived from seeds, stems, leaves, flowers and fruits of plants or from microorganisms. Even some therapeutic benefits can be traced to specific plant compounds, many herbs contain dozens of active constituents that, together, combine to give the plant its therapeutic value. \textit{Annona squamosa L.} (\textit{A. squamosa}), usually recognized as sugar apple, is a tree growing in dry climes. All parts of \textit{A. squamosa} have been traditionally utilized in the handling of many diseases (Suresh \textit{et al.}, 2006). \textit{A. squamosa} has been employed as anti-inflammatory agent (Kirtikar, Basu, 1987). The beneficial medicinal properties of \textit{A. squamosa} refer mainly to its contents of phytosterols, phenolic compounds, tannins, flavonoids, coumarin glycosides and terpenoids (Shalini, Sampathkumar, 2012). The extracts of different solvents of \textit{A. squamosa} showed antioxidant properties (Jagtap, Bapat, 2012). Acetogenins and phenol contents of \textit{A. squamosa} ethyl acetate leaf extract have high antioxidant activity and so, can scavenge free radical effectively (Sarma \textit{et al.}, 2015; Surendra, Male, Ratala, 2016). Transdermal drug delivery has many advantages over other established routes of drug delivery (Tuan-Mahmood \textit{et al.}, 2013). It can offer a non-invasive alternative to parenteral routes, thus circumventing issues such as needle phobia (Han, Das, 2015). A great surface area of skin and ease of
access allows many placement options on the skin for transdermal absorption (Schoellhammer, Blanlschtein, Langer, 2014). In addition, it can improve patient compliance due to the reduction of dosing frequencies and is likewise suited for patients who are unconscious or vomiting, or those who rely on self-administration (Prausnitz, Langer, 2008). Transdermal drug delivery avoids pre-systemic metabolism, and so, can improve bioavailability (Ita, 2014; Han, Das, 2015).

Transdermal drug delivery has made a significant contribution to medical practice, but didn’t yet achieve its potential as an alternative to hypodermic injections and oral delivery. The penetration of therapeutic compounds into the skin is impeded by the impermeability of the outermost layer of the skin, named stratum corneum (Amjadi, Mostaghaci, Sitti, 2017). Liposomes are efficient for the transdermal drug delivery as they are capable to control the release of the therapeutic agent. In summation, they may offer a localized depot in the skin, which brings down the quantities of drug permeating through the skin, thus minimizing systemic effects. Further, liposome vesicles can enhance transdermal drug delivery, increasing systemic drug concentrations. Moreover, the use of liposomes in nanocosmetology also has many benefits, including improved penetration and dispersion of active constituents, transport of active components, extended discharge time, greater stability of dynamic components, reduction of unwanted side effects besides high biocompatibility (Siler-Marinkovic, 2016). It has likewise been demonstrated that in transdermal route of delivery, when the drug is incorporated in niosomes, the penetration of the drug through the skin is greatly enhanced (Muzzalupo, Tavano, 2015).

A. squamosa extract compositions are lipophilic, thus, it is insoluble in water, but is soluble in organic solvents; therefore, suitable carriers must be used to deliver it and improve its bioavailability through the skin. The present work aims to study the properties of two different carriers for A. squamosa extract: liposomes and niosomes, which are considered as popular carriers. The study extends also to evaluating the potential of the transdermal bioavailability and release of A. squamosa after being loaded in niosomes / liposomes.

**MATERIAL AND METHODS**

**Material**

Methanol was purchased from Fisher Scientific UK. Polyoxymethylene-80 (tween 80) (purity ≥ 99%) was supplied from Bio Basic Canada Inc. Phosphate buffer saline (PBS) pH 7.4 at 25 °C (purity ≥ 99%) was purchased from Bio Shop Canada Inc. Diethyl ether (purity ≥ 99.7%), cholesterol (purity ≥ 99.7%, M Wt. 386.65), 1,2-dipalmitoyl-Sn-glycero-3-phosphocholine (DPPC) (purity ≥ 99%), Rhodamine B (purity ≥ 95%, M Wt. 479.01) and chloroform solution (HPLC grade) were purchased from Sigma Aldrich. All chemicals were used without any further purification.

**Methods**

Preparation and Identification of Annona squamosa leaves extract

Leaves of A. squamosa (Family: Annonaceae) were collected during December 2017 from the medicinal garden of Ghabor Farm, Egypt. To clean them, plant leaves were washed very well with tap water. The leaves were left to dry in a hot air oven dried at 55 °C (Ashok et al., 2009; Kashyap et al., 2015), and then, they were crushed into powder using an electric blender. The powder was then stored in plastic bags until use.

An amount of 6 g of the powdered A. squamosa leaves were extracted using 20 ml of methanol: distilled water (8:2), the extract was then centrifuged at 3000 rpm for 15 min, then, the supernatant was collected and filtered using Whatman paper No. 1. Ultimately, the solvents were allowed to be evaporated and the extract was concentrated using a rotary evaporator (RV05-ST, Germini BV laboratory) at 45 °C. The extract was analyzed using gas chromatography-mass spectroscopy system (SHIMADZU QP2010, Japan) and High Performance Liquid Chromatography (HPLC) system (Camag, Muttenz, Switzerland) to detect the main components of A. squamosa extract. The remaining extract was kept in the refrigerator until being used. The extraction yield was dependent on the initially used powder (El-Chaghaby, Ahmad, Ramis, 2011). After the botanical identification, the exsicata was deposited in Faculty of Science, Cairo University herbarium under the number 1272.

Preparation of niosomes entrapping A. squamosa leaves extract

Niosomes were prepared by the thin film hydration method. 10 µg Tween 80 and 3 mg cholesterol in the ratio (2:1) were dissolved in 10 ml ethanol in a round bottom flask with 1 ml of A. squamosa extract. The ethanol was then evaporated at 45 °C under reduced pressure using a rotary evaporator at 50 rpm producing a dry thin film. The hydration of the thin film was then done using PBS (pH 7.4). The formed niosomes
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were then subjected to ultrasonic sonication (5 min) that led to the formation of small vesicles. Finally, niosomes were precipitated using a high speed cooling centrifuge (VS-18000M, Korea, power 220 V/50 Hz) (10000 rpm x 30 min) (Sathali, Rajalakshmi, 2010).

Preparation of liposome entrapping A. squamosa leaves extract

An amount of 5 mg DPPC lipids was first dissolved and mixed with 5 ml chloroform to ensure a homogeneous mixture of lipids with 1 ml of A. squamosa extract. 5 ml chloroform was then, removed by rotary evaporation to obtain a thin lipid film on the sides of a round bottom flask. The lipid film was thoroughly dried to remove residual organic solvent by placing the flask on a vacuum pump for almost 90 minutes. Hydration of the dry lipid film was achieved by adding an aqueous solution of 90% phosphate buffered saline (PBS) to the container of the dry lipid film and agitating at a temperature slightly above the phase transition temperature of the lipid (50 °C) (Torchilin, Weissing, 2003).

Characterization of niosomes and liposomes

• Entrapment efficiency
The capacity of niosomes and liposomes to entrap A. squamosa extract was determined by the centrifugation method (Bendas, Tadros, 2007). Briefly, samples were centrifuged at 12,000 rpm (VS-18000 M, Korea, power 220 V/50 Hz) for 30 min, to separate the free drug (supernatant) from the encapsulated one (pellet). The clear supernatant was then collected and vortexed to obtain a homogeneous solution, while the pellets obtained after centrifugation were diluted with 10 ml saline buffer (pH 7.4) and sonicated for 10 min for further use. This process was repeated three times for each sample.

The absorbance of A. squamosa was measured at different concentrations using a UV-visible spectrophotometer (JENWAY 6405, U.K.) at 270 nm (the resonance absorption of A. squamosa). The calibration curve of A. squamosa was made by plotting the absorbance against the concentration. The absorbance of the free A. squamosa in the supernatant was determined spectrophotometrically at 270 nm. The concentration of the free drug in the supernatant was calculated from the calibration curve made for A. squamosa and the encapsulation efficiency for noisome and liposome was calculated from the following equation:

\[ \text{Entrapment efficiency (\%)} = \frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}} \times 100 \]

• Study of the morphology using transmission electron microscope (TEM)
Niosomes and liposomes were analyzed using a JEOL JEM.1230, Japan. The TEM microscope was operating at an accelerating voltage of 100 kV. Niosome and liposome suspension-entrapping extracts were negatively stained with a 1% aqueous solution of phosphotungstic acid, left to air-dry about 1 sec then samples were incubated for approximately 10 minutes on perforated carbon-coated grids, and then analyzed.

• Study of the surface morphology using scanning electron microscope (SEM)
Niosomes and liposomes were freeze–dried and were coated with platinum for 5 min. The surface morphology of the samples was visualized by SEM (Model Quanta 250 FEG-Field Emission Gun), with accelerating voltage of 30 kV

• Fourier transform infrared spectroscopy (FTIR)
FTIR is a helpful technique used to distinguish the types of functional groups and chemical bonds found in compounds. Freeze-dried powder of liposome and niosome were tested using NICOLET 6700 FT-IR Thermo scientific spectrometer, England, at a resolution of 4 cm\(^{-1}\) with a scan range from 400 to 4000 cm\(^{-1}\). 10 mg of the dried extract powder was mixed with potassium bromide (KBr) pellet for FTIR investigation.

• In vitro extract release
The in vitro release of A. squamosa extract from niosome and liposome was assayed using the dialysis technique in phosphate buffer solution (PBS, pH 7.4) for separating the release extract from niosome and liposome (Trotta et al., 2002; Foco, Gašperlin, Kristl, 2005; Maestrelli et al., 2005). Briefly, 3 ml of niosome or liposome entrapped extract suspension was put into a cellulose acetate dialysis bag (Spectra/ Por, MW cutoff 12,000, Spectrum, Canada) immersed in 100 ml of PBS and magnetically stirred at 50 rpm (Model TK22, Kartell Italy). 2 ml of the immersing solution was taken at different time intervals (every 1 h), with replacing with equal volumes of fresh PBS. The absorbance of the samples was measured using at 270 nm using UNICO UV-2000 spectrophotometer, China. When the concentration of the extract in the receiving medium became constant, the experiment was stopped.

Cellular uptake of niosomes and liposomes
Penetration experiment was carried out using adult albino male rats weighing ~ 120 g. Rats were
sacrificed by sudden decapitation. The abdominal skin region was carefully removed using fine scissor and forceps. Skin was taken out and then, the fatty material was removed and the skin was freshly used (Nayak, Mohanty, Sen, 2010). All animal experiments were performed in accordance with the Guidelines for Ethical and Regulatory for Animal Experiments as defined by the Cairo University, Egypt.

The uptake and distribution of niosomes and liposomes in the rat skin was visualized by confocal microscope using Rhodamine B dye. Briefly, 1 mL of niosome / liposome was mixed with Rodamine B (1 mg/5 mL of PBS). Rhodamine B-labeled niosome and liposome solutions were incubated on the skin for 2 h at 37 °C, then, the skin was sectioned with forceps. The skin sections were observed at emission wavelength of 545 nm by confocal laser scanning microscope (CLSM), Device Model: LSM 710, Software version: ZEN 2009, (Carl Zeiss, Jena, Germany).

RESULTS AND DISCUSSION

Identification of A. squamosa main components

Gas chromatography mass spectroscopy (GC/MS) was used to analyze the polar volatile compounds. Eight volatile compounds were identified in methanol fraction of A. squamosa leaf extract. Retention Time (RT), area of the peak, height, and peak width at half maximum (W05) were presented in Table I and its corresponding chromatogram (Figure 1).

| Peak | RT [min] | Area [mV.s] | Height [mV] | Area [%] | Height [%] | W05 [min] | Compound Name                                                        |
|------|----------|-------------|-------------|----------|------------|-----------|---------------------------------------------------------------------|
| 1    | 1.8      | 110395.3    | 12423.546   | 99.7     | 99.7       | 0.16      | Solvent (methanol)                                                   |
| 2    | 2.11     | 82.864      | 21.746      | 0.1      | 0.2        | 0.04      | Limonene                                                            |
| 3    | 2.84     | 168.324     | 14.521      | 0.2      | 0.1        | 0.18      | R-Carvone                                                           |
| 4    | 3.26     | 12.954      | 1.474       | 0        | 0          | 0.14      | Spermidine                                                          |
| 5    | 20.19    | 3.141       | 0.248       | 0        | 0          | 0.1       | 5-Methyl 1-5-(4,8,12,trimethytridencyl) dihydro-2 (3H)-furanone      |
| 6    | 20.89    | 12.257      | 0.458       | 0        | 0          | 0.28      | 16-Hentriacontanone                                                 |
| 7    | 23.223   | 5.937       | 0.435       | 0        | 0          | 0.15      | Adenosine 3 phosphoric acid                                         |
| 8    | 23.61    | 9.586       | 0.421       | 0        | 0          | 0.14      | Midacamide                                                          |

TABLE I - GC/MS analysis shows the main volatile components for the methanol fraction of A. squamosa leaf extract

FIGURE 1 - GC/MS Chromatogram obtained for the methanol fraction of A. squamosa leaf extract.

Entrapment efficiency

In the present study, liposome exhibits higher efficiency for entrapping A. squamosa extract (67±3%) when compared to that of niosome (24±3%). Encapsulation was optimized by investigating various processes and formulations.

Morphology and characterization

SEM surface morphology

As seen in Figure 2, the scanning electron microscope (SEM) images of A. squamosa extract-entrapped niosome and liposome were smooth having spherical surfaces with no pores. Particle size ranged from 160 up to 300 nm in the case of niosome and from 300 up to 500 nm in the case of liposome.
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Transmission electron microscope

The examination of the ultrastructure using TEM revealed that the prepared *A. squamosa*-entrapped niosomes/liposomes were non-aggregated spherical shape particles (Figure 3), with sizes in agreement with those obtained by SEM. The morphology of each image indicated the homogeneity of the particle size as SEM focuses on the sample’s surface and its composition whereas TEM provides the details about internal composition. Niosome and liposome suspension-entrapping extracts were negatively stained with a 1% aqueous solution of phosphotungstic acid. Samples were incubated for about 10 minutes on perforated carbon-coated grids, and then examined.

Figure 3a shows blank niosomes having spherical-shaped particles with size slightly lower than the two other formulations, niosomes entrapping *A. squamosa* and liposomes entrapping *A. squamosa*, the entrapping drug in nanocarrier is larger than the blanks (Carugo *et al.*, 2016).

All formulations exhibited a more narrow size distribution. The electrostatic charge repulsion between the similar charge (either positive or negative) particles prevents the aggregation and thus ensure a dispersed state of the nanosuspension in all the three formulations. The agglomerates in Figure 3c are not aggregated, it just a closely behind particles.

FTIR

FTIR spectrum (Figure 4) demonstrates the successful entrapment of *A. squamosa* into niosomes and liposomes. Upon adding *A. squamosa* extract in liposome / niosome, their FTIR spectra didn’t change, which means that no additional chemical reactions took place. *A. squamosa* and liposomes entrapping *A. squamosa*, the entrapping drug in nanocarrier is larger than the blanks (Carugo *et al.*, 2016).

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A. squamosa was entrapped in the niosomes and liposomes bilayer membrane due to its hydrophobicity.

FTIR spectra of all samples showed characteristic bands of $\text{CH}_2$ (2855 cm$^{-1}$), $\text{CH}_3$ (2925 cm$^{-1}$), C-O (around 1750 cm$^{-1}$), C-O-C (around 1124 cm$^{-1}$), Alkenyl C=C stretch (1644 cm$^{-1}$), Hydroxyl group in addition to H-bonded OH stretch (3210-3650 cm$^{-1}$ broad) (Coates, 2016).

In vitro A. squamosa extract released from niosomes and liposomes

The release of A. squamosa from the dialysis bag was in burst mode and reached equilibrium within 4 h (Figure 5), since its molecules were unrestricted and have free movement as the accumulative percentage release was estimated. For A. squamosa-entrapped niosomes/liposomes, since A. squamosa was located within the niosome and liposome membranes, release from niosomes and liposomes takes longer time interval. The present formulations have been designed to prolong the release of A. squamosa as nano-encapsulation protects the molecules from premature degradation, improves their solubility, and promotes controlled drug release (Akl et al., 2016).

Figure 5 shows a sustained release of A. squamosa from niosome/liposome within 12 h in PBS buffer, room temperature and pH 7.4. A. squamosa released steadily and slowly over the time of the experiment which means that niosomes and liposomes are stable carriers for the sustained release of A. squamosa and this may enhance to save the therapeutic effects of A. squamosa. Liposome and niosome leading to a higher diffusion resistance compared to diffusion resistance seen in A. squamosa alone, which promotes their prolonged release. Values of the diffusion resistance in liposome and niosome dispersions are higher of those associated with unencapsulated A. squamosa. This implies that liposomes and niosomes are promising vehicles for protection and sustained release of A. squamosa (Pravilovic et al., 2015).

Cellular uptake of niosomes and liposomes using ex-vivo skin tissue

Rhodamine-labeled niosome/liposome nanoparticles were applied onto the rat skin tissue to test the penetration of both formulations across the different skin layers. In Figure 6, Rhodamine B-labelled niosome/liposome penetrate the stratum corneum and deposit between epidermis/dermis in 2 h. These results suggested that niosomes and liposomes could be used as promising skin delivery carriers for A. squamosal, in addition, they led to the deposition of A. squamosa extract in epidermis and dermis, which leads to save the skin from accumulated oxidants by enhancing the hydrophilicity of the extract. These carriers were able to transmit A. squamosa extract to the skin by reasonable concentrations as the efficiency of liposomes entrapping A. squamosa is 67±% and the intensity of penetrated labeled liposomes was high (Figure 6b). Whereas liposome leads to a higher diffusion resistance that provides long periods of release. As shown in Figure 6a&b, A. squamosa-entrapped liposome penetrated skin slightly deeper than squamosa-entrapped niosomes and the penetration of both carriers was significantly higher than the penetration of Rhodamine only (Figure 6c).
CONCLUSION

Niosomes and liposomes act as biological carriers by entrapping A. squamosa in their membrane, at the same time save its structure. Since A. squamosa extract rapidly oxidizes like most natural plant extracts and its structures break through the digestive system. These carriers significantly decrease the burst release of the extract as they have high extract diffusion resistance.

Additionally, niosomes and liposomes can be deposited in the epidermis and dermis, with more intense dermal distribution of A. squamosa in the case of liposome carriers and therefore could lead to antioxidant effect to the skin over a controlled release period. The two carriers penetrate skin in significant depth and this recommends the external use of A. squamosa entrapped niosomes and liposomes to scavenge skin from accumulative oxidants.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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