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**ABSTRACT**

Reactive molecules derived from molecular oxygen and free radicals are commonly known as Reactive Oxygen Species (ROS). These molecules produce oxidative stress in cells leading to many pathological problems like diabetes, cancer, inflammation, neurodegenerative disorders, and aging. Antioxidants can remove free radicals from the human body. Therefore, it is of remarkable interest to increase the bioavailability of antioxidant drugs by increasing transdermal delivery. Nonionic surfactant vesicles or niosomes are drug carriers used to improve the dermal/transdermal bioavailability of drug supplied to the skin. The present work investigates the efficiency of ex-vivo transdermal delivery of the antioxidant plant extract *A. squamosa* encapsulated into niosomes using 60 exponentially decaying electroporative pulses of field strength $100 \text{ V cm}^{-1}$ and maximum pulse duration 4 ms. Transdermal delivery was enhanced by applying electroporative pulses with the niosomes entrapping *A. Squamosa*. The suggested method would help in purifying the body from harmful impurities and oxidants by topical drug enhancers that can be applied directly onto the skin.

1. **Introduction**

Reactive Oxygen Species (ROS) is a term used to describe a number of reactive molecules and free radicals derived from molecular oxygen. Human skin is exposed to various types of stressors like environmental electromagnetic radiation from natural (cosmic radiation and radon) and artificial sources (chemicals and biological agents). The oxidative stressors can disturb the balance between the production of ROS and the antioxidant defense (Betteridge, 2000).

Human body produces about 1.72 kg of superoxide per year; with much more in people with chronic infections (Indo et al., 2015). Autophagy protects the body from ROS (Navarro-Yepes et al., 2014). By increasing the formation of free-radicals in the body, the damage is also increased by oxidative stress effect (Kaur & Halliwell, 1990) to the extent that our antioxidant defense system is not quite effective.

There are compounds extracted from plants that can act to diminish oxidative stress damage in cells. *A. squamosa* belongs to the family Annonaceae. This plant contains several medicinal properties (Gajalakshmi, Divya, Divya, Mythili, & Sathiavelu, 2011). Its extract from the leaves is reported to have activity as an anti-malarial (Jaswanth, Ramanathan, & Ruckmani, 2002), cytotoxic, anti-diabetic (Neha & Dushyant, 2011), anti-bacterial (El-Chaghaby, Abeer, & Eman, 2014), anti-inflammatory (Xiao et al., 2010), and an anti-cancerous activity (Ma, Chen, Chen, Li, & Chen, 2017; Vivek et al., 2015).

Gas chromatography-mass spectroscopy (GC-MS) analysis of *A. squamosa* leaf extract revealed the existence of Sodium benzoate (27.50%), 4, 4-Tert-Butylcalix(4)arene (12.34%), 4, 4-Dimethylcholsterol (10.30%), Butyloctylphthalate (9.67%), stigmasterol acetate (2.92%), isoamylacetyate (2.29%) justifying the use of this plant to treat many ailments in folk and herbal medicine (Vanitha, Umadevi, & Vijayalakshmi, 2011).

The extractions of different solvents of *A. squamosa* showed antioxidant properties (Nandhakumar & Indumathi, 2013). *A. squamosa* leaf extract has high antioxidant activity, and can scavenge free radical effectively (Sarma, Kashyap, Sarmah, & Choudhury, 2015; Surendra, Male, & Ratala, 2016).

Transdermal drug delivery avoids drug metabolism in the gastrointestinal tract. Also, it has the potential for sustained and controlled drug release. Thus, the present study investigates the possibility to deliver transdermally the antioxidant drug *A. squamosa* entrapped in nonionic surfactant-based vesicular system called niosomes. Niosomes enhance antioxidant efficacy by improving bioavailability and intra-follicular penetration of *A. squamosa*. However, it cannot contact well with the skin and may cause skin irritation. Therefore, *A. squamosa*-loaded niosomes were prepared and incorporated in the carbopol 934 gel to improve skin contact time to gain maximum benefits of the treatment.

High-intensity electric pulses can induce pore formation and other structural rearrangements in lipid...
membranes, including cell membranes and skin in a biophysical phenomenon called electroporation or electropermeabilization. Electroporation could increase the permeabilization of polar and charged molecules such as dyes, drugs, proteins, and peptide and provide a local driving force for these molecules (Tieleman, 2004). Electroporation is safe for the skin, so we choose it as a second enhancer technique with niosomes to increase the transdermal delivery of the antioxidant agent A. squamosa.

2. Material and methods

2.1. Chemicals

Methanol was purchased from Fisher Scientific UK, and polyoxyethylene-80 (tween 80) was supplied from Bio Basic Canada Inc. Phosphate buffer saline (PBS) pH 7.4 was purchased from Bio Shop Canada Inc. Diethyl ether and cholesterol were purchased from Sigma Aldrich (USA).

2.2. Preparation and identification of A. squamosa leaves extract

A. squamosa leaves were collected from local gardens of Giza (Egypt). Plant leaves were washed very well with tap water. Leaves were dried in a hot air oven, and then were crushed into powder using an electric blender. The powder was then stored in a plastic bag. Six grams of powdered A. squamosa leaves were dissolved in 20 mL (16 mL methanol and 4 mL distilled water). The extract was centrifuged at 3000 rpm for 15 min, and then the supernatant was collected and filtered using Whatman paper No. 1. Finally, the solvent was allowed to be evaporated and the extract was concentrated using a rotatory evaporator at 45°C (Rajendran, Selvaraj, Arunachalam, Venkatesan, & Subhendu, 2012). The extract was analyzed using GC-MSSystem (SHIMADZU QP2010, Japan) and High-Performance Liquid Chromatography (HPLC) system (Camag, Muttenz, Switzerland) to detect the main components of A. squamosa extract. The remnant extract was kept in the refrigerator until its usage.

2.3. Preparation of niosomes loaded by Annona squamosa extract

Niosomes were prepared by the thin-film hydration method. Tween 80 and cholesterol in the ratio (2:1) were dissolved in 10 mL ethanol in a round bottom flask with 1 mL of Annona squamosa extract. The ethanol was evaporated at 50°C under reduced pressure using a rotary evaporator at 50 rpm producing a dried thin film. The thin film was then hydrated using PBS (pH 7.4). The formed niosomes were then subjected to ultra-sonication (Q55 sonicator, Thomas scientific, USA) that leads to the formation of small vesicles. Finally, drug-free niosomes were precipitated using a high-speed cooling centrifuge (10,000 rpm for 30 min) (Hamdy, Sayed, Amal, & Raid, 2011).

2.4. Preparation of excised rat skin

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 scissors and forceps. The skin was taken out and then the fatty material was removed (Nayak, Biswaranjan, & Kalyan, 2010). All animal experiments were performed in accordance with the Guidelines for Ethical and Regulations for Animal Experiments as defined by Cairo University, Egypt.

2.5. Skin ex-vivo permeation experiments

Skin ex-vivo permeation studies were carried out using Franz diffusion cell. The cell consists of two chambers, the donor, and the receptor compartment with a diffusion area of 3.14 cm². The donor compartment was opened at the top and was exposed to the atmosphere. The excised mouse skin is going to be located between the compartments of the diffusion cell with stratum corneum facing the donor compartment and clamped well.

The electroporation protocol involved application of 60 exponentially decaying electroporative pulses with inter-pulse time of 1 s, pulse duration 4 ms, and field strength 100 V cm⁻¹ in the presence of niosomes loaded with antioxidant extract (Ebtsam, Elshemey, Elsayed, & Abd-Elghany, 2016). The electrical pulses were delivered using two rod shaped stainless steel electrodes (0.5 cm width and 2.7 cm length) placed onto the skin at a distance of 2 cm from each other.

A magnetic stirrer was added to the receptor chambers and filled with the receptor medium Phosphate buffer (PBS) pH 7.4. The setup was placed over magnetic stirrer and the temperature will be maintained at 37 ± 0.5°C. 1 mL of Niosomes-loaded suspension was collected from the receptor compartment at the study period intervals and replaced with the same amount of fresh buffer. The amount of penetrated niosomes encapsulating extract was measured using a UV-Visible spectrophotometer (Shimadzu UV-Visible Spectrophotometer, UV-1601) by measuring absorbance at 276 nm (Nayak et al., 2010). PBS was used as a blank for spectrophotometric measurements.

2.6. Characterization of niosomes

2.6.1. Entrapment efficiency

The capacity of niosomes to entrap A. squamosa extract was determined by the centrifugation method (Pham, Jaafar-Maalej, Charcosset, & Fessi, 2012). Briefly, the entrapment efficiency was measured by determining
the amount of free drug (FD) in the separated supernatant obtained by centrifugation (10,000 rpm for 30 min) at 4°C and the total amount of the drug (TD) added at the beginning of the experiment. The amount of FD and TD was measured using a UV-Visible spectrophotometer (Shimadzu UV-Visible Spectrophotometer, UV-1601) by measuring the absorbance of 1 mL of each at 276 nm. A standard curve was made to determine the drug concentration by plotting known drug concentrations with their absorbance (Nayak et al., 2010). The entrapment capacity was calculated using the following formula:

\[
\text{Drug entrapment efficiency} = \frac{TD - FD}{TD} \times 100 \quad (1)
\]

2.6.2 Study of the morphology using transmission electron microscope (TEM)

Niosomes were analyzed using a transmission electron microscope (TEM) (JEOL JEM 1230, Japan). The TEM microscope was operating at an accelerating voltage of 100 kV. A niosomal suspension-entrapping extract was negatively stained with 1% aqueous solution of phosphor-tungstic acid. The samples were incubated for about 10 min on perforated carbon-coated grids and then examined (Pham et al., 2012).

2.6.3 Study of the surface morphology using scanning electron microscope (SEM)

Niosome samples were freeze-dried and coated by platinum for 5 min. Surface morphologies of the samples were visualized by SEM (Model Quanta 250 Field Emission Gun, Japan) with accelerating voltage 30 KV.

2.6.4 Fourier-Transform Infrared Spectrophotometry (FTIR)

FTIR is a helpful technique used to recognize the types of functional groups and chemical bonds found in compounds. Freeze-dried powders of niosomes were tested using FT-IR, NICOLET 6700 (Thermo scientific spectrometer, England) at a resolution of 4 cm\(^{-1}\) with a scan range from 400 to 4000 cm\(^{-1}\). Ten milligrams of the dried extract powder were mixed with potassium bromide (KBr) pellet for FTIR investigation.

2.7. In vitro extract release

In vitro release of \(A. \text{squamosa}\) extract from niosome was assayed using dialysis technique in phosphate buffer solution (PBS, pH 7.4) for separating the release extract from niosomes (Gopi et al., 2002; Mahale, Thakar, Mali, Walunj, & Chadhari, 2012). Briefly, 3 mL of niosomes entrapping 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). Two milliliters of immersing solution were taken at different time intervals (every 1 h) and replaced with equal volumes of fresh PBS. The absorbance of samples was measured at wavelength 276 nm using UNICO UV-2000 spectrophotometer, China. When the absorbance of the extract in the receiving medium became constant, the experiment was ended.

2.8. Antioxidant activity estimation

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in methanol (Garcia et al., 2012). This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution. The antioxidant activities of the extract along with standard were assessed on the basis of the radical scavenging effect of stable DPPH. A solution of DPPH of concentration 0.2 mM was prepared in 70% methanol and kept overnight. Stock solution (1mg/ml) of the extract was prepared in 70% methanol. Various concentrations of the extracts 10, 20, 50, 100, 150, 200, 300, 400, and 500 µl were taken in different test tubes and the volume was made up to 1000 µl. One-milliliter DPPH was added to each solution and kept at dark for 30 min. Ascorbic acid was taken as standard. The optical density of these samples was measured at 517 nm along with blank (1 ml methanol with 1 ml DPPH solution). The changes in color from deep violet to light yellow were observed. The extract concentration providing 50% inhibition (IC50) of DPPH activity was calculated by plotting percent inhibition (%) against extract concentration. The percent inhibition (%) of DPPH activity was calculated by the following formulae (Nooman et al., 2008):

\[
\text{Percent(%) inhibition of DPPH activity} = \frac{A - B}{A} \times 100
\]

where A = Optical density of the blank, and B = Optical density of the sample.

2.9. Cellular uptake of niosomes

The uptakes and distributions of the niosome in rat skin were visualized by confocal microscope using rhodamine B-loaded niosomes. Rhodamine-B was used as a fluorescence probe (Lymberopoulos, Demopoulou, Kyriazi, & Katsarou, 2017). The penetration efficiency depends on the carrier (niosomes) itself. Briefly, 1 ml of niosomes was mixed with rhodamine B (1 mg/5 ml of PBS). Rhodamine B-labeled niosome solutions were incubated on either the intact or electroporated skin for 2 h at 37°C. The skin sections were observed at emission wavelength 545 nm by confocal laser scanning microscope (Device Model: LSM 710; Software version: ZEN 2009; Carl Zeiss, Jena, Germany).
3. Results and discussion

3.1. Identification of A. squamosa main components

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 1 and its corresponding chromatogram (Figure 1). HPLC detected 15 nonvolatile compounds in methanol fraction of A. squamosa leaf extract as shown in Table 2 and its corresponding chromatogram (Figure 2).

3.2. Electroporation enhances ex-vivo transdermal delivery of A. squamosa

The influence of electroporation and niosomes on ex-vivo transdermal delivery of A. squamosa was investigated by measuring the absorption spectrum at 276 nm of the penetrated niosomes loaded with...
A. squamosa at different time intervals. Figure 3 shows a significant increase in the absorption spectrum of A. squamosa entrapped in niosomes with respect to A. squamosa without enhancers. The highest absorption spectrum of A. squamosa was observed when loaded in niosomes and passed through electroporated skin. The increase in permeability started from 15 min to 45 min after skin electroporation and exhibited a plateau response above ~1 h.

### 3.3. Entrapment efficiency

The drug entrapment efficiency measured by Equation 1 in the method section showed that niosomes exhibited efficiency for entrapping A. squamosa extract about 60 ± 3.0%.

### 3.4. Morphology characterization

The transmission electron microscope revealed a positive image in which Nano-emulsion appeared dark with bright surroundings. The average droplet size of the sample was less than 150 nm. The study of the ultra-structure using TEM showed that the prepared niosome samples have non-aggregated spherical shaped particles (Figure 4), with sizes ranging between 100 and 130 nm. The morphology of each image indicates the homogeneity of the particle size. The niosome entrapping drug was larger than the empty ones so the magnification scale was

![Figure 2. HPLC chromatogram obtained for methanol fraction of A. squamosa leaf extract.](image2)

![Figure 3. Dependence of ex-vivo skin permeability on electroporation and niosomes. The optical density of the penetrated niosomes loaded with A. squamosa was measured at different times after exposure to transdermal delivery enhancers compared to control skin tissue exposed to A. squamosa only. Each data point represents the mean value ± standard error of three independent experiments, each experiment being performed in triplicate. The statistical significance was determined using student t-test.](image3)
reduced in (Figure 2(b)) (Carugo, Bott, Owen, Stride, & Nastruzzi, 2016).

Shape and surface characteristics of entrapping A. squamosa in niosomes were examined by scanning electronic microscopy (Figure 5). Scanning electron microscopy shows the smooth surface of niosomal formulation.

3.5. FTIR

Upon loading A. squamosa extract in niosomes, the FTIR spectrum of them did not change when compared to FTIR spectrum of free A. squamosa or niosomes without A. squamosa (Figure 6), which means that no additional chemical reactions took place and this proves that the reaction between A. squamosa and niosomes was electrostatic.

It was estimated that niosomes were able to load A. squamosa and it was located within the niosome membrane due to its hydrophobicity.

 FTIR spectrum of all samples showed characteristic peaks. They showed characteristic bands of CH$_2$ (2855 cm$^{-1}$), CH$_3$ (2925 cm$^{-1}$), C-O (around 1750 cm$^{-1}$), C-O-C (around 1124 cm$^{-1}$), Alkenyl C = C stretch (1644 cm$^{-1}$), Hydroxy group, H-bonded OH stretch (3210–3650 cm$^{-1}$; broad) (Vivek et al., 2015).

3.6. Sustained release of A. squamosa extract from niosomes

The release of A. squamosa from the dialysis bag was in burst mode and reached equilibrium within 4 h (Figure 7). Since A. squamosa was located within the niosome membrane, its release takes a long time because it depends on degradation of the carrier. The niosomes entrapping A. squamosa were suspended in PBS with pH 7.4 and the experiment was done at room temperature. A. squamosa released steadily and slowly over the time of 10 h which means that niosomes are stable carriers for the sustained release of A. squamosa and this could be beneficial in maintaining the therapeutic effects of A. squamosa longer.

3.7. Estimation of antioxidant activity

The inhibition concentration (IC50) of A. squamosa was found to be 26.41 ± 0.34U/ml and it was 22.1 ± 0.24U/ml for niosomes entrapping A. squamosa (Table 3).

3.8. Ex-vivo skin electropermeabilization

Rhodamine-labeled niosome nanoparticles were applied onto the rat skin tissue to assure the existence of skin electropermeabilization. Rhodamine B-labeled niosomes permeate stratum corneum and deposited between epidermis/dermis during 2 h (Figure 8(a)) with more penetration depth in case of using niosomes + electroporation (Figure 8(b)). The penetration
of both carriers was significantly higher than the penetration of rhodamine only (Figure 8(c)) which means that niosomes conjugated with electroporation can efficiently transport the antioxidant drug, *A. squamosa*, transdermally. The fluorescence intensity of pure rhodamine B was much higher than the other 2 formulations due to the 60% encapsulation efficiency (as measured above) of niosomes to the rhodamine.

In this study, an antioxidant drug *A. squamosa* encapsulated in niosomes was delivered transdermally through rat skin ex-vivo in the presence of electroporative pulses. TEM and SEM results show good preparation of niosomal formulations manifested by homogeneity, lack of aggregations, and smooth surface of niosomes. Centrifugation method and FTIR studies revealed that *A. squamosa* extract was loaded properly in the niosomes without any chemical interaction between niosome membrane and the hydrophobic drug. The antioxidant activity of *A. squamosa* extract and niosomes entrapping *A. squamosa* was
measured and compared with ascorbic acid (vitamin C) as a standard used to improve the antioxidant status of the body (Arrigoni & De Tullio, 2002). The main driving force suggested for electropermeabilization of charged molecules across the cell membrane is electrophoresis (Anne-Rose, Vanbever, & Preat, 2004). The conductivity (10 mS cm$^{-1}$) of the exposure medium (PBS) potentiates the electrophoretic movement of the niosomes with charged polar heads. Molecular transport through electroporated skin is also due to enhanced passive diffusion. The increased permeability started after electroporation by ~15 min and persistent ~45 min. The prolonged permeabilization obtained in our experiments was confirmed by adding the drug after exposure to electric pulses or reversing the electrode polarity as done in previous studies (Regnier & Preat, 1999; Vanbever, LeBoulenge, & Preat, 1996; Vanbever, Leroy, & Preat, 1998). The obtained result is probably related to the shape of the exponential pulses used in this work. Exponentially decaying electric pulses were restricted to transdermal drug delivery to take advantage of the long voltage tail (Anne-Rose et al., 2004). Due to its long tail, the electrophoretic mobility of the niosomes encapsulating drug molecules was elevated and the skin permeabilization was preserved for a long time after exposure. The drug passed from the skin was confirmed by Rhodamine-labeled niosomes. The voltage drops steeply during skin electroporation such that the transdermal voltage is only a fraction (10–50%) of the voltage applied across the electrodes so, the electrical parameters should be strengthened. The field intensity should not exceed 100 V cm$^{-1}$ to avoid rupture of niosome membrane and therefore the electrical energy could be increased by increasing pulse number to 60 exponential pulses and relatively long duration 4 ms. The drug was released slowly from the niosomes which was an advantage for the therapeutic effects of the antioxidant drug.

4. Conclusions

It has been shown that the vesicular drug carriers called niosomes followed by electroporation could increase the ex-vivo permeability of the skin to the antioxidant agent; *A. squamosa*. Since *A. squamosa* composed of volatile oils, aliphatic alcohols, and fat-soluble sterols, the watery medium makes these compounds crystallized and decreases their absorption efficiency in the intestine and therefore resulted in low bioavailability (Rajendran et al., 2012). The use of niosomes for entrapping the essential oils is an attractive approach to overcome their physicochemical stability concerns; it improves the bioavailability of the drug and increases its duration inside

<table>
<thead>
<tr>
<th>Tested sample</th>
<th>IC50U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>27.3</td>
</tr>
<tr>
<td><em>A. squamosa</em></td>
<td>26.41</td>
</tr>
<tr>
<td>niosomes entrapping <em>A. squamosa</em></td>
<td>22.1</td>
</tr>
</tbody>
</table>

Figure 8. Confocal laser scanning microscope images of intact skin permeabilization of Rhodamine B loaded niosomes (a), electropermeabilized skin of Rhodamine B loaded niosomes (b), and intact skin permeabilizing Rhodamine B free without neither niosomes nor electroporation (c).
the body. The formed pores from the electroporation process extremely increase the passage of the A. squamosa entrapped niosomes through the skin. It is worth to be mentioned that prolonged and repeated exposure to electroporation disrupts the skin barriers and therefore increase the skin infections or dermatitis. It is recommended to electroporate the skin not more than once per day. The study gives hope to patients with chronic disorders arising from the oxidative stress accompanied by the formation of free radicals.

Disclosure statement
No potential conflict of interest was reported by the authors.

References