Efficacy of pomegranate extract loaded solid lipid nanoparticles transdermal emulgel against Ehrlich ascites carcinoma

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Abstract

The purpose of this work was to incorporate an optimized pomegranate extract loaded solid lipid nanoparticles (PE-SLNs) formula in a transdermal emulgel to evaluate its anticancer effect. The prepared emulgel formulae were evaluated for their physicochemical properties. An ex vivo permeation study was done through mouse skin and the kinetic parameters were determined. Kinetic data showed that the ex vivo permeation of PE from SLNs transdermal emulgel through mouse skin followed non-Fickian diffusion transport. Further, in vivo study was done by applying the optimized PE-SLNs transdermal emulgel on mice skin bearing a solid form of Ehrlich ascites carcinoma (EAC) as well as free PE, control, placebo, and standard groups for comparison. In addition, histopathological examinations of the samples obtained from the EAC mice model were performed. The results proved that application of the selected PE-SLNs emulgel formulation on the mice skin bearing solid tumor revealed statistically significant anticancer effects.

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Key words: Cancer; Ehrlich; Pomegranate extract; Solid lipid nanoparticles; Transdermal

In recent times, natural products have been applied to inhibit and treat malignancy despite it still being a difficult medication objective. Numerous examinations have been done on many natural products to determine the molecular pathways for malignant growth prevention and treatment.1 Although it has been verified that numerous natural products have good therapeutic effect, their poor solubility and low bioavailability have limited their use.

Recently, researchers have used nanoparticle delivery systems that are synthesized naturally and that are intended for the inhibition and treatment of several malignancies.2 Solid lipid nanoparticles (SLNs) are a good choice for the delivery of anticancer drugs due to their high drug loading capacity, control release kinetics, improvement of blood circulation time, and enhancement of the overall therapeutic effectiveness of anticancer drugs because of their lipid core.3

Pomegranate fruit is a natural product that is commercially available as juice, sauce, or wine.4 Seeds, juice, and peels are the fruit parts whose extracts seem to have therapeutic properties. Promising results from studies of pomegranate have been described for the inhibition of specific malignant growths that include breast,5 prostate,6 liver,7 lung, colon,8 and skin tumors.2

The fruit of pomegranate is rich in phenolic compounds such as flavonoids (catechins, anthocyanins, and other complex flavonoids) and hydrolysable tannins named ellagitannins (punicalagin, punicalin, pedunculagin, gallic acid, and ellagic acid).9 Pomegranate extract (PE) contains ellagitannins, which are hydrolysable tannins, that on hydrolysis yield ellagic acid and other phenolics10; ellagic acid is measured as a characteristic indicator for standardization of the pomegranate peel extract.11 Ellagic acid has anticancer effects by means of its anti-proliferative and pro-apoptotic activities in addition to its action on subcellular signaling pathways. It was previously observed that ellagic acid reduces the multiplication of and prompts apoptosis of human osteogenic sarcoma cells as proved by the degradation of chromosomal DNA and apoptotic body appearance.12 It has also been shown that ellagic acid causes cell death by affecting the in vitro and in vivo kinase signaling as well as
alting and interacting with both estrogen receptor and tyrosine
kinase receptors pathways, which are inducers of cell prolif-
eration and also are critical to primary and recurrent breast cancers' development.13

The two fundamental delivery methods for anticancer drugs
are oral and intravenous, but transdermal delivery systems have
numerous points of interest over regular routes for their
avoidance of hepatic first pass metabolism, enhancement of
therapeutic efficacy and maintenance of steady plasma level of
drug.14 The transdermal route has been considered as an
inspiring developing approach for targeting solid tumors.15

Solid Ehrlich tumor is an undistinguishable solid tumor that is
commonly used as a tumor model for chemotherapy investiga-
tions.16 It is originated from breast adenocarcinoma of mouse,
which is a virulent, aggressive, and quickly developing tumor
indicating a high rate of malignancy.17 Use of this transplanta-
tion model is valuable due to quick growth of the tumor that
makes the investigation time shorter.18

The present study's main aim was to formulate PE loaded
SLNs transdermal emulgel to evaluate its anticancer effect
compared to free PE. Evaluation was done in vivo by applying it
on mice bearing Ehrlich ascites carcinoma in solid form using
PE-SLNs transdermal emulgel. Histopathological examinations
of the samples from EAC mice model were done to track the
modulation of the tumor environment.

Methods

Materials

Pomegranate extract (PE) was purchased from Shaanxi
Ciyuan Biotech Co., Ltd. (Shaanxi, China). Stearic acid,
disodium hydrogen phosphate and potassium dihydrogen
phosphate were purchased from El-Nasr Pharmaceutical Che-
eicals Co. (Cairo, Egypt). Tween 80, lecithin and Carbopol 940
were generously donated by Egyptian International Pharmaceu-
tical Industries Co., EIPICO, (10th of Ramadan City, Egypt).
Ellagic acid was purchased from Sigma-Aldrich (St. Louis, MO,
USA). Normal saline was purchased from Egypt Otsuka
Pharmaceutical Co. SAE, ARE, (Nasr City, Cairo). Formalin
was purchased from Mansoura for Resins and Chemicals
Industries Co. SAE, Egypt. All other chemicals were of
analytical grade.

Methods

Preparation of PE-SLNs based emulgel

For the current work, an optimized formula of PE-SLNs
dispersion was prepared according to the previously published
method of Badawi et al, and its optimized parameters were:
dispersion of particle size 280 nm, PDI value 0.32, zeta potential
41.9 mV, entrapment efficiency 62.56% and cumulative % drug
release 72.5% (equivalent to 1.2 mg/ml of PE).19 This was
converted into emulgel carrier system using Carbopol 940 as a
gelling agent and propylene glycol and ethanol as penetration
enhancers. Carbopol 940 at certain concentrations was sprinkled
and stirred using a magnetic stirrer (Thennolyne Corporation,
Dubuque, IA, USA) into the optimized PE-SLNs dispersion until
they were uniformly mixed to form emulgel. Then the pH of the
dispersion was adjusted with triethanolamine to form emulgel
with good viscosity. Thereafter, propylene glycol and ethanol
were added to the prepared emulgel formulae.20 In addition, void
SLNs emulgel formulae (free from PE) were prepared with the
same above-mentioned steps to be used as blank. The
composition of the PE-SLNs emulgel in their optimized
formulae (equivalent to 1.2 mg/ml PE) each contained 5% w/w
propylene glycol and 5% w/w ethanol, but they differed in their
percent w/w Carbopol 940: G1 contained 0.5% and G2 contained
1.5%.

Evaluation of the prepared optimized PE-SLNs emulgel

Physical investigation

The prepared emulgel formulations were visually examined
for their color, texture and homogeneity.

pH

Measurement of the pH of each prepared emulgel base was
done via pH meter (410A, ORION). Preparation of a solution
consisted of 1 g of each prepared emulgel base in 100 ml of
distilled water and the pH was measured in triplicate and the
average reported.21

Spreadability

Examination of spreadability of the prepared emulgel was
done by setting 0.5 g emulgel within a pre-marked circle of 1
cm diameter on a glass plate over which another glass plate was
positioned. The emulgel was left for 5 min on the upper glass
plate and then the increase in the diameter because of spreading
of emulgel bases was measured.22 Spread circles’ diameters
were determined in cm and were considered as comparative
values for spreadability. Results were the average of three
measurements.

Drug content

One hundred milligrams of the optimized PE-SLNs emulgel
was dissolved in 100 ml of phosphate buffer, pH 7.4, and stirred
on a magnetic stirrer to completely solubilize the extract.
Subsequently, filtration of this solution was done through a
Millipore filter (0.45 μm) and spectrophotometrically assessed at
265 nm.23 This experiment was repeated three times and also
done for void SLNs emulgel formulae (free from PE) as blank.

Rheological studies

Viscosity of the prepared emulgel bases was measured using
a programmable viscometer (Brookfield's Apparatus, Inc. DV-II
+ Pro Viscometer, Boston, MA, USA) at room temperature with
one-minute time intervals. The measurement was done using
spindle (T-D), and then about 25 g of the base was placed inside
the sample holder. For each emulgel sample, continuous
variation of the speed rate from 1 to 100 s⁻¹ and then backward
from 100 to 1 s⁻¹ was applied. The rheological parameters
(viscosity, shear rate, and shear stress) were obtained.24 A
complete rheogram was accomplished by plotting the shear rate versus shear stress. Results were the average of three determinations.

**Ex vivo permeation studies**

Mouse skin was selected as a model to simulate human skin. The study protocol was accepted by the ethical committee of Faculty of Pharmacy, Cairo University. Sacrifice of male mice (weighing between 25 and 30 g) was done first. Subsequently, hair removal, followed by excision of the ventral and dorsal abdominal skins, was done. Then tweezers were used to remove the subcutaneous fat, and the fragments of skin were washed with buffer and observed for validity. Afterwards, the membrane fragments were kept at −20 °C until used.

The permeation experiment was done using Franz diffusion cells with a diffusional area of 1.76 cm². The skin was located between the receptor and donor compartments of the Franz cell after being defrosted and drenched in phosphate buffer saline pH 7.4. A receptor phase of 25 ml of phosphate buffer saline pH 7.4 was placed and magnetically stirred in which the water bath was kept at 37 ± 1 °C. One gram of each prepared PE-SLNs emulgel base containing a known amount of PE was transferred into the donor compartment. Samples of 1 ml were taken from the receptor medium after 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h time intervals, and the drug concentration was measured spectrophotometrically at λ max 265 nm. After each sample was withdrawn, an equivalent volume of the fresh buffer solution was replaced. This experiment was repeated in triplicate and done also for void SLNs emulgel formulae (free from PE) as blank.

**Estimation of ex vivo permeation parameters**

The calculated parameters of permeation for PE from SLNs emulgel bases were estimated from the permeation data: steady state flux (Js), permeability coefficient through the skin (Kp), diffusion coefficient within the skin (D), and lag time (tl). The Js of PE was determined from the slope of the proportion of the permeated amount through unit area of the skin as a function of time plot. Kp and tl (the x-intercept of the extrapolated linear portion of the amount of drug permeated through unit area of the skin) were calculated according to the following equations:

\[
Js = K_p C_d
\]

(1)

\[
K_p = \frac{Js}{C_d}
\]

(2)

\[
t_l = \frac{H^2}{6D}
\]

(3)

where Js is the steady state skin flux (μg/cm² h), Kp is the permeability coefficient (cm/h), C_d is the initial drug concentration in the donor compartment, D is the diffusion coefficient (cm²/h), H is the skin thickness and t_l is the lag time (h).

**Kinetic data analysis**

The kinetic parameters for all ex vivo permeation studies of PE from SLNs emulgel were determined to describe the mechanism of drug release according to the Korsmeyer Peppas Equation:

\[
\frac{Mt}{M_\infty} = Kt^n
\]

(4)

where Mt/M∞ is the fraction of drug release at time t, M∞ is the amount of drug incorporated in the emulgel, K is the diffusion rate constant depending upon structural and geometric characteristics of the drug/polymer system, and n is diffusional exponent used to characterize the transport mechanism. The values of K and n were estimated by linear regression of log Mt/M∞ versus log t where log K is the intercept and n is the slope of the straight line.

**In vivo anticancer activity study of optimized PE-SLNs emulgel**

This study was divided into two parts: Ehrlich ascites carcinoma study followed by histopathological study using the following emulgel formulae that are prepared as previously mentioned.

a) Formula I (optimized PE-SLNs emulgel) (amount equivalent to 60% ellagic acid).
b) Formula II (optimized ellagic acid-SLNs emulgel).
c) Formula III (optimized void-SLNs (without drug) emulgel).
d) Formula IV (free PE emulgel) (amount equivalent to 60% ellagic acid).

**Ehrlich ascites carcinoma study (EAC)**

Thirty Swiss albino female mice weighing 22-30 g from the animal house at the Faculty of Pharmacy, The British University in Egypt, were used in the current study. Animals were placed at room temperature under relative humidity conditions, and they had free food and water admission and were not subjected to any medications. The experiment was performed as stated by the Helsinki agreement protocol and approved by the ethical committee of Faculty of Pharmacy, Cairo University requirements. As well, we followed the Egyptian instructions and the particular local institutional rules for animals’ protection under the regulation of official examiners.

EAC cells were taken from Swiss albino mice donor (22-30 g body weight). First, ascetic fluid holding about 3 × 10⁶ viable cells was diluted with normal saline (1:10)²⁹ and all inspected mice were injected in the left hind leg thigh intramuscularly with 0.2 ml of this diluted ascetic fluid.³⁰ Subsequently, mice were left for 5 days for the development of Ehrlich solid carcinoma (ESC) until the solid tumor volume reached 100 mm³ or above as measured with Vernier caliper. Studies were terminated when the tumor size of the negative control group attained 500 mm³ or above.

**Experimental design**

When the appropriate size of the solid tumor volume was reached, removal of the mice hair was done from the back portion of their body and the mice were divided randomly into five groups, each consisting of six animals as follows. All five groups had transdermal emulgel (0.5 g) twice daily applied with rubbing action on the hairless mice skin over tumor site and
groups had applied: group 1, Formula I; group 2, Formula II; group 3, Formula III; group 4, Formula IV; group 5, negative control group (received no drug or any external treatment).

**Determination of solid tumor volume and tumor inhibition rate**

The solid tumor volume was determined in all groups using a Vernier caliper in which the size of the normal hind leg (with no tumor) and the leg with tumor was measured once daily and the difference between the volumes of both legs (normal and tumor) was calculated to determine the tumor volume only without the muscle mass. The volume of the tumor (TV) was assessed using the following equation31:

\[
TV_v = \text{length} \times \text{width}^2 \times 0.52
\]

The inhibition rate of the tumor (TIR) was evaluated using the following equation32:

\[
TIR\% = \left(1 - \frac{TV_t}{TV_c}\right) \times 100
\]

where \(TV_t\) is the last tumor volume of the test group and \(TV_c\) is the final tumor volume of the control group.

In addition, differences between the values of normal leg volume and tumor leg volume were calculated for further statistical analysis. Results were displayed as mean ± standard deviation (SD) of different groups.

**Statistical analysis**

The differences between the mean values of the normal leg and the tumor leg volumes were evaluated with two-way analysis of variance ANOVA followed by Tukey’s multiple comparison test using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) to detect the effect of the applied emulgels on tumor size. Values of \(P \leq 0.05\) were considered significant.

**Histopathological examination**

At the end of the studies, sacrifice by cervical dislocation was done for all the animals and then they were dissected. Autopsy samples were collected from the Ehrlich tumor cells within the mice thigh muscle of all examined groups and kept in 10% formal saline for 24 h for pathological examination. Washing in tap water was done and then serial dilutions of alcohol were used for dehydration. Samples were cleared in xylene and embedded in paraffin wax in hot air oven at 56 °C for 24 h. Afterwards, preparation of paraffin bees wax tissues was done to be ready for sectioning by microtome at 4-μm thickness. The prepared tissue sections were transferred to glass slides, de-waxed, and stained with hematoxylin and eosin stain (H&E) for investigation under a light electric microscope. Histopathological investigation was done randomly and the examination of tumor segments was achieved by concentrating on the necrotic zone.33,34

**Results and discussion**

**PE-SLNs based emulgel**

To enhance the application and maximize the benefit of the optimized formula of PE-SLNs, it was incorporated into emulgel structure using Carbopol 940 emulgel forming agent in two concentrations with penetration enhancers propylene glycol and ethanol in order to enhance the percutaneous penetration of the drug.

**Physical characteristics**

The prepared PE-SLNs emulgel formulae were white with a smooth and homogenous appearance. The pH values were in the acceptable range as shown in Table 1 with the purpose of avoiding any skin irritation upon application, which indicates appropriateness of the formulations for application on the skin.35

Spreadability of the formulation that is applied topically is a vital characteristic when considering patient compliance. Formulations with high values of spreadability permit easy application and thus increased surface area presented for drug permeation. Results of the spreadability test are shown in Table 1. The prepared emulgel formulae of PE-SLNs gave an acceptable range, which indicates good spreadability.36 Results also revealed that formulations with low polymer concentrations have better spreadability than those with higher polymer concentrations (Table 1), which agrees with Bashir el al.23

The prepared emulgel showed an acceptable range of drug content and low standard deviations (Table 1). It shows that the drug is consistently dispersed in the emulgel formulation.

**Rheological properties**

The rheological property determination of the prepared PE-SLNs emulgels is shown in Figure 1, A and B. The viscosity values were 88,500 cp for G1 and 190,700 cp for G2. Upon increasing the polymer concentration, there was an increase in consistency that was attributed to enhanced polymeric entanglement, thereby increasing the resistance to deformation.37

It can be seen from Figure 1 that all prepared formulae exhibited non-Newtonian pseudoplastic flow with shear thinning behavior, indicating that the disarranged viscosity (the slope of the curve) of the system decreases with the increase in shear rate.38 When the shear stress is raised, the gelling material molecules began to align their long axes in the way of flow; therefore, such orientation decreases the internal resistance of the gelling material and hereafter reduces the viscosity.39

<table>
<thead>
<tr>
<th>Formula code</th>
<th>Color</th>
<th>Homogeneity</th>
<th>Texture</th>
<th>pH ± SD</th>
<th>Drug content (%) ± SD</th>
<th>Spreadability (cm) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>White</td>
<td>Homogeneous</td>
<td>Smooth</td>
<td>5.793 ± 0.12</td>
<td>99.33 ± 0.85</td>
<td>6.3 ± 0.100</td>
</tr>
<tr>
<td>G2</td>
<td>White</td>
<td>Homogeneous</td>
<td>Smooth</td>
<td>5.65 ± 0.06</td>
<td>98.75 ± 0.94</td>
<td>5.75 ± 0.116</td>
</tr>
</tbody>
</table>

Table 1

Physical characters of prepared PE-SLNs emulgel formulae.
In addition, the figures illustrate that emulgel formulations had thixotropic behavior, showing a reduced shear stress at any rate of shear on the down curve compared to the up curve. Thixotropy, or time-dependent flow, happens because the emulgel needs a predetermined time to reconstruct its unique structure that breaks down throughout constant shear examinations.40

Ex vivo permeation studies

Ex vivo permeability of drug was done through mouse skin as a replacement for human skin as in earlier several studies.41,42 The ex vivo permeability of the prepared PE-SLNs emulgel formulae was studied using phosphate buffer saline pH 7.4 as the receptor medium for 48 h to define the effect of the concentration of the gelling agent on the permeation of drug.

The ex vivo permeation profile of the prepared PE-SLNs emulgel formulae is illustrated in Figure 2. Table 2 lists the PE steady-state flux (Js), diffusion coefficient (D), permeation coefficient (P), partition coefficient (K) of drug between skin and delivery system, and lag time (tL) for the emulgel formulations.

The prepared emulgel formulations are arranged according to the percentage of drug permeated in descending order, G1 > G2. Increasing polymer concentration was accompanied by a decrease in the percent of PE permeated. This may because at high polymer concentrations the drug is entrapped in polymer chains and is organized by its near proximity to those polymer particles, thus increasing the diffusional resistance.43 This could be ascribed to the increase of the viscosity of the emulgel by raising the polymer concentration. Therefore, high concentration of polymer and high viscosity mutually have a synergistic impact on reducing the release of drug from the formulation.44

Propylene glycol was used as a penetration enhancer to increase the permeation rate of the drug through the skin and this may be because propylene glycol increased the solubility and partitioning of the drug between the emulgel and skin barrier, which in turn increased the penetration rate of the drug.45 Ethanol showed an improving effect in the penetration of the drug through the skin that may be due to disordering the barrier structure of stratum corneum or improving the solubility and partitioning of the drug in stratum corneum.46

The data from the ex vivo drug permeation experiments were analyzed to find out the kinetics of drug release. Table 3 shows the kinetic analysis of the ex vivo permeation data of PE from the different investigated formulae.

Table 2
Permeation parameters of PE-SLNs from the prepared emulgel.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Steady-state flux (Js) (µg cm⁻² h⁻¹)</th>
<th>Lag time (tL) (h)</th>
<th>Permeability coefficient (Kp) (cm h⁻¹)</th>
<th>Diffusion coefficient (D) (cm² h⁻¹)</th>
<th>Partition coefficient (K)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>107.27</td>
<td>1.07</td>
<td>0.022</td>
<td>1.22</td>
<td>162.06</td>
<td>0.9982</td>
</tr>
<tr>
<td>G2</td>
<td>98.45</td>
<td>2.52</td>
<td>0.021</td>
<td>5.22</td>
<td>349.26</td>
<td>0.9817</td>
</tr>
</tbody>
</table>

Kinetic data analysis.
In the case \( n \) is equal to 1, then the release is either zero order or Case II transport, and \( n > 1 \) for Super Case II transport. If \( n \) is equal to 0.5 then the drug release is superlative described by Fickian diffusion, and in the case of \( 0.5 < n < 1 \) at that time the release is through inconsistent diffusion or non-Fickian diffusion transport. Regarding this model, a plot of drug percent permeated against log time is linear diffusion rate constant.

The kinetic treatments for the \textit{ex vivo} permeation of PE from different emulgel bases had \( n \) values indicating non-Fickian diffusion transport, thus showing that the drug release mechanism may include a merge of both chain relaxation and diffusion mechanisms. Consequently, the release of the drug from the emulgel formulations is restrained by the polymer swelling, followed by drug diffusion through the polymer and slow erosion of the polymer.\(^{47}\)

\textbf{In vivo anticancer activity study of optimized PE-SLNs emulgel}

\textit{In vivo Ehrlich Ascites Carcinoma study.} The anticancer activity of PE from its chosen formulae was investigated utilizing the Ehrlich Ascites Carcinoma study. Ehrlich carcinoma is an undistinguishable carcinoma that is primarily hyper-diploid; has high transplantable ability, no regression, rapid propagation, short life span, and 100% malignancy; and also does not have tumor-specific transplantation antigen.\(^{48}\)

\textbf{Tumor volume and tumor inhibition rate.} The solid tumor volume was measured for both legs of all tested mice and the difference between them was obtained in the study to determine the tumor volume only without the muscle mass. All groups received 12 doses in 6 days.

Results are shown in Table 4 and Figures 3 and 4 and are stated as the mean ± SD.

Generally, it was observed from Table 4 and Figure 3 that the progression and growth of the tumor in all groups decreased, in contrast to the control group. After six days' application of PE-SLNs optimized formula transdermal emulgel (group 1) the tumor volume was significantly decreased in comparison to the negative control group (group 5) at \( P < 0.01 \), reaching a maximum average tumor volume of 237.42 ± 93.77 mm\(^3\) and 589.21 ± 198.52 mm\(^3\), respectively.

After application of ellagic acid-SLNs optimized formula transdermal emulgel (group 2), void-SLNs optimized formula transdermal emulgel (group 3), and free PE transdermal emulgel (group 4) throughout 6 days, the tumor volume was insignificantly (\( P > 0.5 \)) decreased in comparison to the control group, reaching maximum average tumor volume of 373.34 ± 170.91 mm\(^3\), 495.31 ± 132.73 mm\(^3\), 526.05 ± 85.91 mm\(^3\) and 589.21 ±

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Tumor volume for the EAC study, number of mice in each group = 6.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment day</td>
<td>Tumor volume (mm(^3))</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>1</td>
<td>160.34 ± 66.42</td>
</tr>
<tr>
<td>2</td>
<td>196.00 ± 53.32</td>
</tr>
<tr>
<td>3</td>
<td>198.89 ± 57.00</td>
</tr>
<tr>
<td>4</td>
<td>220.56 ± 99.01</td>
</tr>
<tr>
<td>5</td>
<td>238.51 ± 104.96</td>
</tr>
<tr>
<td>6</td>
<td>237.42 ± 93.77</td>
</tr>
</tbody>
</table>

\(\textbf{Table 3} \) Kinetic analysis for the \textit{ex vivo} permeation of PE-SLNs from the prepared emulgel.

<table>
<thead>
<tr>
<th>Formula</th>
<th>(r)</th>
<th>(n)</th>
<th>(K)</th>
<th>Mechanism of transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>0.95922</td>
<td>0.5356</td>
<td>0.001315</td>
<td>Non-Fickian</td>
</tr>
<tr>
<td>G2</td>
<td>0.96095</td>
<td>0.8316</td>
<td>0.01327</td>
<td>Non-Fickian</td>
</tr>
</tbody>
</table>

\(\textbf{Figure 3. Tumor volume for the EAC study versus the days of treatment.} \) Values are presented as means ± SD. *Significant compared to the control group.

\(\textbf{Figure 4. Tumor inhibition rate (TIR %) for the EAC study, indicating the effect of treatment administration in all groups.} \)
and metastasis. Stearic acid inhibits epidermal growth factor (EGF) receptor-mediated proliferation in Hs578t breast cancer cells, inhibits attack of HT-1080 fibrosarcoma cells, and causes stearate has been associated with a reduction in mammary tumor estrogen-induced mammary cancers, and induced apoptosis in cancer cells in vitro. It was observed that ellagic acid-SLNs optimized formula transdermal emulgel decreased the tumor volume. However, the value obtained from ellagic acid was not as good as that obtained from PE and this is in accordance to the findings of Hong and coworkers, who stated that pomegranate juice and PE were more effective in cell growth prevention than separated specific polyphenols in vitro in cell lines, recommending synergistic and/or additive properties of numerous phytochemicals that consist of pro-anthocyanidins, anthocyanins, and flavonoid glycosides. In addition, Seeram et al revealed that the in vitro actions are more noticeable on condition of a collective tannin-rich extract in preference to the single isolated bioactive compound. This can be ascribed to the likely additive and synergistic influence of several isolated bioactive compounds in the whole extract. Additionally, ellagitannins can reduce tumor development and evolution due to their capability to constrain cell growth. Our findings are in accordance with some reports that PE in particular prevent the growth of breast cancer, prostate cancer, lung cancer, and skin cancer in both cell culture plates and in mice xenograft model.

Regarding group 2, it was previously reported that ellagic acid reduced the occurrence of chemically induced lung and mammary cancers, decreased the volume and progression of estrogen-induced mammary cancers, and induced apoptosis in cancer cells in vitro. It was observed that ellagic acid-SLNs optimized formula transdermal emulgel decreased the tumor volume. However, the value obtained from ellagic acid was not as good as that obtained from PE and this is in accordance with the finding of Hong and coworkers, who stated that pomegranate juice and PE were more effective in cell growth prevention than separated specific polyphenols in vitro in cell lines, recommending synergistic and/or additive properties of numerous phytochemicals that consist of pro-anthocyanidins, anthocyanins, and flavonoid glycosides. In addition, Seeram et al revealed that the in vitro actions are more noticeable on condition of a collective tannin-rich extract in preference to the single isolated bioactive compound. This can be ascribed to the likely additive and synergistic influence of several isolated bioactive compounds in the whole extract.

Regarding group 3, there was a slight inhibition for the placebo, which may be due to the presence of stearic acid. Stearic acid is a saturated fatty acid that is present in comparatively high concentrations in many diets. It has been stated to prevent in vitro human breast cancer cell propagation and in vivo breast tumorigenesis. Stearic acid has similarly been revealed to cause breast cancer cell apoptosis and also to arrest breast cancer cell-cycle. It has also been discovered that stearic acid caused a significant inhibition of tumor growth, carcinogenesis, and metastasis. Stearic acid inhibits epidermal growth factor (EGF) receptor-mediated proliferation in Hs578t breast cancer cells, inhibits attack of HT-1080 fibrosarcoma cells, and causes apoptosis of MDA-MB-231 breast cancer cells, and dietary stearate has been associated with a reduction in mammary tumor growth and occurrence in unconstrained carcinogenesis models. Mechanisms illuminated for a direct apoptotic impact of stearic acid on breast cancer cells include incorporation of stearic acid into diacylglycerol, stimulation of protein kinase C, and Rho inhibition. It has also been stated in another study that stearic acid causes apoptosis by increasing levels of proapoptotic molecules, for example BAX, and reducing antiapoptotic molecules, for example cIAP2, in pre-adipocytes but not in developed adipocytes.

In group 4, a slight inhibition was also detected for the free PE transdermal emulgel, and this may be due to the low systemic bioavailability, poor absorption, and short retention time of ellagitannins and their metabolites, which may weaken their chemo-preventive potential despite the documented beneficial effects of PE and its ellagitannins. Therefore, encapsulation of ellagitannins into biodegradable and biocompatible nanoparticles may help to surmount their susceptibility to poor absorption, gastrointestinal hydrolysis, low systemic bioavailability, and short half-life time. Accordingly, these results highlight the effectiveness of the therapeutic value of the PE optimized nanoformulation over the free PE formulation.

**Histopathological examination**

Ehrlich solid tumor induction was characterized by the existence of fragments of chromatophilic tumor cells that are different in shape, indicating cell proliferation. The surrounding tumor zones established areas of differentiated cells and necrosis. The occurrence of high necrosis is commonly a sign for reduction in tumor evolution. The microscopic examination of Ehrlich solid carcinoma (ESC) tissues revealed high-grade tumor cells inside the skeletal muscle bundles in the non-treated group tissues (control group) with limited small area of necrotic tissue (Figure 5, A). On the other hand, group 1 that received Formula I (PE-SLNs optimized formula transdermal emulgel) showed the highest abundance of necrotic area, in which the necrosis increased to more than 50% per field in ESC (Figure 5, B). This finding is in good correlation with the tumor volume and TIR results of group 1, which were 237.42 mm$^3$ and 59.71%, respectively.

Group 2 received Formula II (ellagic acid-SLNs optimized formula transdermal emulgel) and showed less necrosis than that of group 1, about 30% per field in ESC (Figure 5, C). This result is also related to that of the tumor volume and TIR results of group 2, which were 373.34 mm$^3$ and 36.63%, respectively. The ESC tissues of the placebo group (group 3) were characterized by metastatic cancer cells inside the skeletal muscle bundles with few areas of necrosis (about 20% per field, Figure 5, D), which are in accordance to the findings of the tumor volume and TIR of group 3, which were 466.31 mm$^3$ and 15.98%, respectively.

Finally, the tumor tissues in the group that received Formula IV (free PE transdermal emulgel) had about 20% necrosis per field (Figure 5, E), which agrees with the results of the tumor volume and TIR of group 4, which were 526.05 mm$^3$ and 10.71%, respectively.

Based on the histopathological examinations associated with the results of EAC study, the significant variation in tumor volume between the group receiving Formula I (PE-SLNs optimized transdermal emulgel) leads to the conclusion that PE as well as its optimized formula had a remarkable necrotic result on cancer cells that resulted in a reduction in the growth and progression of the tumor.
Conclusion

PE-SLNs optimized formula emulgels were successfully prepared and the selected formulation (Carbopol 940 0.5% in addition to 5% w/w propylene glycol and 5% w/w ethanol) was chosen to be subjected to anticancer activity study of PE based on the higher permeation rate. Additionally, the in vivo study results ascertain the effective delivery and the significant therapeutic efficacy of the optimized PE-SLNs emulgel form with observable tumor tissue necrosis. Therefore, the developed optimized PE-SLNs transdermal emulgel formulation is presented as a promising delivery carrier for PE to be applied on solid breast carcinoma.

Funding

This research did not receive any specific grant from funding agencies.

References


Figure 5. A photomicrograph of Ehrlich solid carcinoma sections (H&E × 16) (A) from control mice showing high-grade tumor cells inside the skeletal muscle bundles (line) with limited small area of necrotic tissue, (B) from mice of group 1 that received Formula I shows the highest abundance of necrotic area (arrow), (C) from mice of group 2 that received Formula II showing limited necrotic area (arrow), (D) from mice of group 3 that received Placebo (Formula III) showing metastatic cancer cells inside the skeletal muscle bundles (star) with few areas of necrosis (arrow) and (E) from mice of group 4 that received (Formula IV) showing the least necrotic area (arrow).


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