Green Synthesized Honokiol Transfersomes Relieve the Immunosuppressive and Stem-Like Cell Characteristics of the Aggressive B16F10 Melanoma

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Background: Honokiol (HK) is a natural bioactive compound with proven antineoplastic properties against melanoma. However, it shows very low bioavailability when administered orally. Alternatively, topical administration may offer a promising route. The objective of the current study was to fabricate HK transfersomes (HKTs) for topical treatment of melanoma. As an ultradeformable carrier system, transfersomes can overcome the physiological barriers to topical treatment of melanoma: the stratum corneum and the anomalous tumor microenvironment. Moreover, the immunomodulatory and stemness-regulation roles of HKTs were the main interest of this study.

Methods: TFs were prepared using the modified scalable heating method. A three-factor, three-level Box–Behnken design was utilized for the optimization of the process and formulation variables. Intracellular uptake and cytotoxicity of HKTs were evaluated in nonactivated and stromal cell–activated B16F10 melanoma cells to investigate the influence of the complex tumor microenvironment on the efficacy of HK. Finally, ELISA and Western blot were performed to evaluate the expression levels of TGF-β and clusters of differentiation (CD47 and CD133, respectively).

Results: The optimized formula exhibited a mean size of 190 nm, highly negative surface charge, high entrapment efficiency, and sustained release profile. HKTs showed potential to alleviate the immunosuppressive characteristics of B16F10 melanoma in vitro via down-regulation of TGF-β signaling. In addition, HKTs reduced expression of the “do not eat me” signal — CD47. Moreover, HKTs possessed additional interesting potential to reduce the expression of the stem-like cell marker CD133. These outcomes were boosted upon combination with metformin, an antihyperglycemic drug recently reported to possess different functions in cancer, while combination with collagenase, an extracellular matrix–depleting enzyme, produced detrimental effects.

Conclusion: HKTs represent a promising scalable formulation for treatment of the aggressive B16F10 melanoma, which is jam-packed with immunosuppressive and stem-like cell markers.

Keywords: honokiol, melanoma, transfersomes, heating method, tumor microenvironment, immunosuppressive, stem-like cell

Introduction

Recently, great advances in the field of pharmaceutical biotechnology have been achieved. However, cancer and infectious diseases still occupy the second rank in threats to humanity after cardiovascular diseases. Skin cancer (melanoma)
originates from melanin-producing melanocytes, presenting the subtype with the highest mortality and highest potential to metastasize throughout the systemic circulation and lymphatic system traveling to reach distant organs, such as the brain, lungs, and liver.\textsuperscript{2–5}

Nowadays, surgical resection is considered the first line of melanoma therapy, in addition to traditional chemotherapy, radiotherapy, immunotherapy, and biological therapy.\textsuperscript{6} The failure of most therapeutic agents is due to the immunosuppressive\textsuperscript{7–9} and stem-like\textsuperscript{10,11} nature of B16F10 melanoma cells, in addition to the complex structure of tumor tissue. The complexity of tumor tissue is due to the tumor microenvironment (TME) which includes a variety of cellular components that contribute to tumor invasion, metastasis, and drug resistance.\textsuperscript{12} On one hand, B16F10 melanoma cells exhibit high levels of the “do not eat me” ligand CD47. This is a ligand for SIRP\textalpha, an immunoinhibitory receptor, which is expressed on myeloid cells, including macrophages and dendritic cells. The binding of CD47 to SIRP\textalpha is a tumor-immunoevasion strategy via inhibition of phagocytosis of tumor cells by immune-system cells.\textsuperscript{7–9}

In addition, B16F10 melanoma cells exhibit high levels of CD133 (Prom1), which is a membrane glycoprotein characteristic of cancer stem cells (CSCs). CSCs exhibit high potential for tumor growth and metastasis, inhibition of apoptosis, recurrence, and resistance to different therapeutic approaches.\textsuperscript{10,11} The key cellular players in the TME are tumor-associated fibroblasts (TAFs) and tumor-associated macrophages (TAMs).\textsuperscript{13} TAFs perform multiple activities in tumor formation. They induce the proliferation of tumor tissue contribute to the secretion of collagen and tumor-associated proteins (growth factors), which constitute the dense extracellular matrix (ECM). They also regulate the innate and adaptive immune system by secreting TGF-\beta, leading to the suppression of antitumor immunoresponses.\textsuperscript{14} Furthermore, TAFs contribute to the regulation of polarization of TAMs to their immunosuppressive phenotype M2.\textsuperscript{15} This phenotype of TAMs maintains several protumoral functions represented in supporting the progression of tumor growth by increasing the secretion of growth factors and facilitating angiogenesis and metastasis.\textsuperscript{16} Various strategies have been employed for TME remodeling, eg, collagenase has been utilized as an ECM-depleting enzyme to digest collagen, facilitating the diffusion of therapeutic agents in tumor tissue.\textsuperscript{17–19} Furthermore, metformin, an FDA-approved first-line treatment of type2 diabetes, is currently receiving great attention for its anticancer effects and potential for targeting CSCs.\textsuperscript{20} It has been reported that metformin mediates macrophage polarization from the M2 to M1 phenotype\textsuperscript{21,22} and possesses a suppressive role on TGF-\beta signaling.\textsuperscript{23–26}

Lately, plant-derived compounds have been proven to have safe pharmacological chemotherapeutic action against various cancer types compared to synthetic chemotherapeutics, with lower toxicity.\textsuperscript{27}

A natural bioactive compound derived from species of the Magnoliaceae family(Magnolia officinalis, M. grandiflora, and M. obovata), HK has proven antineoplastic properties against melanoma,\textsuperscript{28,29} pancreatic,\textsuperscript{30} lung,\textsuperscript{31} colorectal,\textsuperscript{32} and ovarian cancer,\textsuperscript{33} head and neck squamous-cell carcinoma,\textsuperscript{34} breast cancer,\textsuperscript{35} and even hepatocellular carcinoma.\textsuperscript{36} HK has proved to have a lot of striking anticancer activities: it can inhibit angiogenesis, provide direct cytotoxicity, potentiate apoptosis, inhibit multiple-drug resistance, and deregulate cancer cell–signaling pathological pathways involved in growth and malignancy, which it can target.\textsuperscript{28,37} HK shows very low oral bioavailability (5%), due to the first-pass effect and low absorption. Alternatively, topical application may offer a promising route, as it is patient-compliant and it can augment HK therapeutic action.\textsuperscript{6,38,39} Nevertheless, topical delivery is still challenging, due to the presence of physiological barriers: the stratum corneum, the anomalous TME, and the interstitial matrix.\textsuperscript{40,41} Consequently, several nanocarriers have been developed to circumvent the stratum corneum, whereby they can deliver hydrophilic compounds and biomacromolecules to deeper skin layers, achieve a sustained local effect, and enhance topical drug delivery and thus provide high drug bioavailability.\textsuperscript{42,43} The structure of transfersomes (TFs) resembles liposomes in composition — both formed of phospholipids — but TFs show superior advantages due to the presence of an edge activator in their composition.\textsuperscript{44} An edge activator is a single-chain surfactant that destabilizes the vesicle’s phospholipid bilayer and increases its deformability, rendering it an ultradeformable elastic vesicle\textsuperscript{45} that can squeeze itself through the intercellular space of the stratum corneum and penetrate deeper skin layers.\textsuperscript{44}

Volatile organic solvents like chloroform, ether, or methanol are used in most TF-preparation methods, such as solvent evaporation\textsuperscript{46} and thin lipid–film hydration.\textsuperscript{47} This leads to the presence of residues of these organic solvents, which may cause toxicity and affect the stability of the final TF preparation. Accordingly, it is advised to avoid the usage of toxic solvents in preparation.
This has led to the evolution of the heating method, which can be conducted without the utilization of organic solvents. This method was introduced by Mozafari et al, and involves the application of heat, not lower than the phase-transition temperature (Tc) of the lipids, as below this temperature lipids are present in the gel state and cannot form a closed continuous bilayered structure. The heating method has proved to be a scalable, safe, less time-consuming, and more cost-effective method of nanovesicular preparation. Using drug-delivery systems of these plant-derived compounds formulated by a technique based on green synthesis is an eco-friendly and safe therapeutic modality for human use that can be applied to large-scale production with low cost.

The aim of this study was to follow a green optimized fabrication process of an HK TF–nanodelivery system in order to improve HK bioavailability and skin penetrability. Furthermore, the potential effect on modulating the TME of B16F10 melanoma cells was evaluated in monotherapy or in combination with two TME-remodeling agents — metformin and collagenase — focusing on evaluation of the immunosuppressive and stem-like cell characteristics of B16F10 melanoma cells in response to mono- or combinatory therapies via evaluation of TGF-β, CD47, and CD133.

**Methods**

**Materials**

HK of purity 98% (HPLC) was purchased from Stanford Chemicals, sodium deoxycholate (SDc) from Janssen Pharmaceuticals, soy lecithin and cholesterol from Alfa Aesar, metformin hydrochloride from Sohan Healthcare, and collagenase from Sigma-Aldrich, Germany. Fluorescein isothiocyanate was obtained from Biotium, absolute ethanol from Medico, and HPLC-grade acetonitrile from Thermo Fisher Scientific. All other chemicals were of analytical grade. Murine melanoma (B16F10, CRL6475), fibroblast (L929, CCL1), and macrophage (RAW264.7, TIB71) cell lines were purchased from the American Type Culture Collection.

**Experimental Design**

In the present study, three-factor, three-level Box–Behnken design was used to investigate the influence of formulation and process factors and interaction effects on TF characteristics. This design is an optimum approach for ascertaining the effects of formulation/process factors (independent variables) and their associated effects on measured responses (dependent variables). Based on preliminary studies, drug amount (X1), stirring speed (X2), and homogenization speed (X3) were chosen as the independent variables. Particle size (PS; Y1), PDI (Y2), Zeta-potential (ZP; Y3), and drug entrapment efficiency (EE%)(Y4) were selected as the dependent variables. Table 1 shows the levels of the independent variables and the constraints of the obtained responses/dependent variables. A design matrix comprised of 15 experimental runs with three central points was obtained, as listed in Table 2. After polynomial equations relating the dependent and independent variables had been generated, the process was optimized.

**Method of Fabrication**

Fifteen TF formulations (Table 2) were fabricated using the modified heating method. Firstly, lecithin (180 mg) and SDc (20 mg) were hydrated individually in 2 mL PBS (pH 7.4) for 1 hour at room temperature, then placed into a preheated mixture of glycerol (3% v/v) and HK (different amounts) at 70°C while stirring at different speeds (1,000, 1,250, and 1,500 rpm) for 1 hour on a hot-plate magnetic stirrer (MSH-20D, 280 V, 50/60 Hz, 660 W, Daihan Scientific). Then, the mixture was left to cool for 30 minutes, leaving the vesicles to anneal and stabilize. Later, the formulations were homogenized using a WiseTis homogenizer (280 W, 50/60 Hz, Daihan Scientific) for 5 minutes, with 60 seconds on and 30 seconds off cycles. Finally, the vesicles were stored in a refrigerator at 4°C for further investigations. The whole fabrication process was performed in a six-baffled homemade vessel (Figure 1) simulating the specially designed glass bottle introduced by Mozafari.55

**Table 1 Independent and dependent variables of Box–Behnken design utilized to prepare honokiol-loaded transfersome formulations**

| Independent variables (factors)                  | Levels   |
|-------------------------------------------------|----------|
| X1: HK amount (mg)                              | 10       |
| X2: Stirring speed (rpm)                        | 1,000    |
| X3: Homogenization speed (rpm)                  | 0        |

| Dependent variables (responses)  | Desirability constraints |
|---------------------------------|--------------------------|
| Y1: Particle size (nm)          | Minimize                 |
| Y2: Polydispersity index        | Minimize                 |
| Y3: Zeta-potential (mV)         | Maximize                 |
| Y4: Entrapment efficiency (%)   | Maximize                 |
Mean Particle Size, Particle-Size Distribution, and ZP

PS and PDI of all formulations were analyzed using a Zetasizer Nano ZS (Malvern Instruments) using dynamic light scattering. ZP was determined from electrophoretic mobility with the same instrument by injecting the samples into a universal folded capillary cell with platinum electrodes at both ends. Freshly prepared TF dispersions were diluted tenfold by deionized water, then PS, PDI, and ZP were measured. Measurements were performed three times and mean PS, PDI, and ZP were calculated.

Determination of HK-Entrapment Efficiency

HK entrapment in the TF vesicles was determined using the indirect method. Briefly, a known volume of the sample was centrifuged at 15,000 rpm for 2 hours at 4°C (Centurion Pro-Research K241R), the supernatant was then separated, and

Table 2 Experimental runs, independent variables and observed responses of 15 honokiol transfersome formulations prepared according to Box–Behnken design

|        | X₁ (mg) | X₂ (rpm) | X₃ (rpm) | Y₁ (nm)  | Y₂     | Y₃ (mV) | Y₄ (%)  |
|--------|---------|----------|----------|----------|--------|---------|---------|
| HKT1   | 25      | 1,000    | 10,000   | 213.3±2.86 | 0.472±0.020 | −40.00±0.96 | 60.00±1.24 |
| HKT2   | 40      | 1,000    | 5,000    | 306.5±0.64 | 0.422±0.034 | −35.37±1.06 | 76.24±1.62 |
| HKT3   | 10      | 1,000    | 5,000    | 475.3±3.82 | 0.488±0.011 | −38.83±1.55 | 49.46±0.87 |
| HKT4   | 40      | 1,250    | 10,000   | 139.1±1.85 | 0.413±0.014 | −39.33±1.65 | 62.60±0.97 |
| HKT5   | 25      | 1,250    | 5,000    | 234.2±1.02 | 0.465±0.003 | −50.20±2.45 | 75.00±1.36 |
| HKT6   | 10      | 1,250    | 10,000   | 131.3±1.54 | 0.385±0.011 | −39.23±1.77 | 32.60±0.88 |
| HKT7   | 10      | 1,250    | 0        | 566.8±1.63 | 0.549±0.011 | −44.70±1.22 | 74.40±1.57 |
| HKT8   | 40      | 1,250    | 0        | 463.4±1.77 | 0.435±0.010 | −42.50±1.61 | 89.90±0.96 |
| HKT9   | 25      | 1,500    | 10,000   | 166.7±0.81 | 0.444±0.024 | −37.47±1.27 | 76.57±0.47 |
| HKT10  | 25      | 1,250    | 5,000    | 211.9±3.78 | 0.469±0.008 | −52.93±3.05 | 77.50±0.99 |
| HKT11  | 25      | 1,250    | 5,000    | 220.6±1.06 | 0.48±0.0150 | −49.86±2.12 | 75.75±1.78 |
| HKT12  | 40      | 1,500    | 5,000    | 314.3±1.87 | 0.364±0.030 | −36.53±0.15 | 84.38±1.69 |
| HKT13  | 25      | 1,500    | 0        | 416.2±1.46 | 0.503±0.011 | −44.57±1.52 | 81.23±0.75 |
| HKT14  | 10      | 1,500    | 5,000    | 252.20±0.97 | 0.565±0.007 | −43.00±0.34 | 67.99±0.69 |
| HKT15  | 25      | 1,000    | 0        | 506.0±2.46 | 0.588±0.019 | −42.93±2.37 | 84.64±0.73 |

Notes: X₁, HK amount; X₂, stirring speed; X₃, homogenization speed; Y₁, particle size; Y₂, polydispersity index; Y₃, Zeta potential; Y₄, entrapment efficiency (%).

Abbreviation: HKTs, HK-loaded transfersomes.
the absorbance of the free HK was determined with UV-visible spectroscopy (JASCO V-630) at a wavelength with maximum absorbance at 219 nm. EE% was calculated:\[^{57}\]

\[
EE\% = \frac{\text{Total HK} - \text{Free HK in supernatant}}{\text{Total HK}} \times 100
\]

**Optimization of Process Variables**

Based on the results obtained, optimization of the variables was conducted using Design-Expert 11 (Stat-Ease) based on the criterion of desirability. The desirability function was introduced by Derringer and Suich.\[^{58}\] It relies upon the concept that the quality of a novel formulation developed, which has many features, is totally unacceptable if one of the features is outside the desirable limit.\[^{59}\] The desirability function is used to corroborate compliance with the criteria selected for all involved responses and to provide the best value of compromise in a desirable joint response.\[^{60}\] This can be achieved by converting the multiple responses evaluated into a single one, combining the individual responses into a composite function, followed by its optimization. To confirm the validity of the optimization process, the suggested HK transfersome (HKT)-optimized formula (HKT\(_{\text{opt}}\)) with the highest desirability and predicted levels of formulation and process variables was prepared. It was then characterized, and observed values of the responses were compared to the predicted values. The HKT\(_{\text{opt}}\) formula was then subjected to further investigations.

**Transmission Electron Microscopy**

The morphology of the HKT\(_{\text{opt}}\) formula was investigated using transmission electron microscopy (TEM). A drop of the TF suspension was placed on a carbon-coated copper grid and then air-dried at room temperature. The sample was examined at 80 kV with a Hitachi H600.

**Deformability Index**

For determination of the elasticity of TFs in comparison to liposomes (of relevant composition), the HKT\(_{\text{opt}}\) and the corresponding liposomal suspension were extruded through a polycarbonate membrane of pore size 50 nm (Thermo Fisher Scientific) applying a constant pressure of 250 kPa (Haug Kompressoren). Deformability-index values were calculated as an indicator of elasticity:\[^{61,62}\]

\[
D = \frac{J}{t(r_v - r_p)}
\]

where \(D\) is the deformability index (mL/second), \(J\) the quantity of dispersion extruded (mL), \(t\) is the extrusion time (seconds), \(r_v\) the vesicle size after extrusion (nm), and \(r_p\) the pore size of the extrusion membrane (nm).

**Fourier-Transform Infrared Analysis**

To assess interactions among HK, lecithin, and SDc, Fourier-transform infrared (FTIR; JASCO FTIR-6200) spectroscopy was performed for these components individually and the HKT\(_{\text{opt}}\) formula in the 4,000–400 cm\(^{-1}\) wavelength range at room temperature with a resolution of 4 cm\(^{-1}\).

**In Vitro Release Study**

For the determination of HK in vitro release kinetics from TFs, 500 µg HK of the HKT\(_{\text{opt}}\) and the same amount of free HK in ethanol (1.64 mg/mL) as control were used to compare between the release kinetics of the pure drug and the HKT\(_{\text{opt}}\). Each of the HKT\(_{\text{opt}}\) and the free HK was filled in a dialysis bag made of semipermeable cellulose membrane with molecular weight 12–14 kDa (Spectrum Medical). Each dialysis bag was incubated in a beaker containing a dissolution medium formed of 50 mL 0.5% sodium lauryl sulphate in PBS (pH 7.4 to achieve sink conditions). The whole set was immersed in a shaking water bath (Sci FineTech, 220 V, 50 Hz) at 37°C±1°C and 100 rpm. The experiment was conducted for 24 hours. At predetermined time points, the samples were withdrawn and replaced by an equal volume of the freshly prepared medium. The concentration of the released HK was quantified spectrophotometrically at 219 nm, and the cumulative release profile with time was calculated. This experiment was performed in triplicate.\[^{63}\]

**Establishment of Tumor-Associated Macrophage– and Tumor-Associated Fibroblast–Activated B16F10 Melanoma Culture**

For the establishment of TAM-activated and TAF-activated melanoma cultures, B16F10 melanoma cells were cultured in 50% RAW246.7 macrophages or L929 fibroblast-derived conditioned media in fresh culture media for 48 hours. The conditioned media was obtained from confluent cultures of RAW246.7 macrophages or L929 fibroblasts. Successful activation of B16F10 melanoma cells was assessed via evaluation of the concentration of TGF-β from culture supernatants of activated and nonactivated melanoma cells via ELISA. TGF-β secreted by TAMs
and TAFs promotes several hallmarks of tumorigenesis.⁶⁴–⁶⁷

**Intracellular Uptake of Fluorescein-Loaded TFs and Mechanism of Endocytosis**

For determination of the intracellular concentration of TFs, a modified protocol by Sebak et al. was followed. A nontoxic concentration of 250 μg/mL fluorescein-loaded TFs was incubated with the activated and nonactivated B16F10 melanoma cells under 37°C and 5% CO₂. After the predefined incubation of 24 hours, the culture medium containing the noninternalized TFs was discarded. Cells were then washed three times with PBS and fresh medium containing the noninternalized TFs was discarded. After the predefined incubation of 24 hours, the culture medium was utilized for the calculation of the intracellular concentration of TFs.

For determination of the mechanism of endocytic uptake, activated and nonactivated B16F10 melanoma cells were pretreated with endocytic inhibitors in their respective concentrations for 1 hour prior to the addition of the TFs. A 15×10³ μM quantity of sodium azide, 10⁵ μM methyl-β-cyclodextrin (MβCD), and 450 μM sucrose were utilized for evaluation of energy dependence, cholesterol dependence, and clathrin-pit dependence of TF uptake, respectively. Similarly, 20 μM nystatin and 50 μM amiloride were utilized as inhibitors of caveolin-dependent endocytosis and micropinocytosis, respectively.⁶⁸–⁷¹

**Cytotoxicity of HKTs**

For determination of the cytotoxicity of HKTs on activated and nonactivated B16F10 melanoma, a standard MTT assay was used for 1–100 μg/mL of TFs-loaded HK after a predefined incubation time of 24 hours.⁷² For determination of the cytotoxicity of the combination therapy, cells were pretreated with the nontoxic concentration of metformin (100 μg/mL) or collagenase (50 μg/mL) for 24 hours prior to the addition of HKTs.

**Biomarker Analysis**

For evaluation of the alteration of different biomarkers in response to the proposed treatment options, activated and nonactivated B16F10 melanoma cells were treated with the half-maximal inhibitory concentration (IC₅₀) of HKTs for 24 hours under standard culture conditions. The biomarkers selected were CD47 and CD133 (in cell lysates), and TGF-β (in the culture supernatant).

**Enzyme-Linked Immunosorbent Assay**

TGF-β was analyzed from the cell-culture supernatant according to the manufacturer’s protocol using a mouse transforming TGF-β ELISA kit (MBS160136) from MyBioSource.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blot (WB) Analysis**

CD47 and CD133 (Prom1) were analyzed from cell lysates via SDS-PAGE and WB according to the previously published, well-established protocol in the lab of Professor Laila Rashed, Biochemistry Department, Faculty of Medicine, Cairo University. CD47 (Thermo Fisher Scientific, 14–0471-82), CD133 (ThermoFisherScientific, 14–1331-82), and β-actin (ThermoFisherScientific, MA1-140) monoclonal antibodies were used for the analysis. Bands for CD47, CD133, and β-actin proteins were visualized by enhanced chemiluminescence (ECL Plus) and quantified relative to β-actin using densitometry and Molecular Analyst software (Bio-Rad).

**Statistical Analysis**

All experiments were conducted at least three times, and results are expressed as means ± SE. One-way and two-way ANOVA or t-test analyses were utilized for statistical evaluation of the data using GraphPad Prism 8.3. Significance was regarded as P<0.05. IC₅₀ was determined by nonlinear regression analysis of the cytotoxicity data.

**Results and Discussion**

**Analysis of Responses**

After fabrication of the 15 HK transfersome (HKT) formulations mentioned in Table 2 by the modified heating method, the impact of the three independent variables — HK amount (X₁), stirring rate (X₂), and homogenization speed (X₃) — on PS (Y₁), PDI (Y₂), ZP (Y₃), and EE% (Y₄) was evaluated using Design Expert 11.

**Effect of Process Variables on Particle Size and PDI**

Y₁ ranged from 131.3±1.54 nm to 506±2.46 nm, corresponding to HKT6 and HKT15, respectively, as shown in Table 2. Vesicular Y₁ was greatly affected by X₃ (P<0.0001)
as speed increased from 0 to 10,000 rpm and decreased sharply from 506±2.46 nm to 131.3±1.54 nm, indicating that homogenization speed was a key parameter in controlling Y1. Figure 2A shows the response surface plot of the measured Y1 of the prepared formulations versus X2 and X3 respectively. The polynomial equation used relating the particle size to the independent variables was:

\[ Y_1 = 222.23 - 25.29X_1 - 43.96X_2 - 162.75X_3 \\
+ 57.72X_1X_2 + 27.80X_1X_3 + 10.80X_2X_3 + 57.22X_1 \\
+ 57.62X_2 + 45.70X_3 \]  

(3)

The findings obtained can be explained by the increase in the mechanical and hydraulic shear as a result of enhancing the homogenization speed, which in turn causes a dramatic decrease in PS. \(^7^5\) Similar findings were gained by Song et al, who discussed the effect of high-pressure homogenization on the PS of soy protein–isolate suspensions and found reductions in mean PS from 3,331.3 to 146.7 and 135.8 nm after being processed by high-pressure homogenization for 30 passes at 137 and 207 MPa, respectively. \(^7^6\) Stirring speed produced similar effect on PS whereas smaller PS was obtained at higher stirring speed (1,000-1500). This goes in accordance with previous studies elaborating the effect of stirring speed on surface area \(^7^7\) and droplet size. \(^7^8\) As shown in Figure 2A, the lowest PS, 131.3±1.54 nm (HKT6), was achieved at the maximum applied stirring and homogenization speeds — 1,500 and 10,000 rpm, respectively. The results proved that there was no significant effect of drug amount on PS (P>0.05), consistent with a study on paromomycin-loaded solid-lipid nanoparticles. \(^7^9\)

Regarding interactions among the independent variables, statistical interpretation of the design coefficients indicated that the combination of both HK amount (X1) and stirring speed (X2) had a synergistic effect on particle size of the TF vesicles. The PDI ranged from 0.364±0.030 to 0.588±0.019, represented by HKT12 and HKT15, respectively, as shown in Table 2. The polynomial equation relating PDI (Y2) to the independent variables can be shown as such:

\[ Y_2 = 0.4713 - 0.0441X_1 - 0.0118X_2 - 0.0451X_3 \\
- 0.0337X_1X_2 + 0.0355X_1X_3 + 0.0142X_2X_3 \\
- 0.0339X_1 + 0.0223X_2 + 0.0081X_3 \]  

(4)

The PDI was used as an indication of size distribution, where a value of 0 was considered as a completely homogeneous system and a value of 1 indicated a highly heterogeneous distribution of vesicles. \(^8^1\) In reference to Table 2, all samples showed relatively small PDI, indicating their homogeneity and the reliability of all measurements. It was clear...
that the homogenization speed had a significant impact on particle-size distribution ($P=0.0161$). The negative of $X_3$ indicated a negative relationship between homogenization speed and PDI, indicating more uniform size distribution and smaller PDI values with higher homogenization speed. Zidan et al affirmed similar outcomes. It was also found that drug amount had a significant effect on PDI ($P=0.0175$): its value was diminished by increasing the drug amount, as shown in Figure 2B. It was noticed that good PDI values were obtained in most of the formulations without the use of either sonication or extrusion, the most effective techniques for particle size–distribution reduction. This indicates that the modified heating method is an efficient preparation method that can be scaled up without using expensive organic solvents or energy-consuming processes.

Effect of Variables on ZP
ZP ranged from $-35.37\pm1.06$ to $-52.93\pm3.05$ mV for HKT2 and HKT10, respectively (Table 2), showing that the TF preparations possessed good stability as ZP $>30$ mV indicates the stability of the formulations. ZP was significantly affected by homogenization speed ($P=0.0184$). The polynomial equation relating particle size to the independent variables was:

$$Y_3 = -51 + 1.50X_1 - 0.555X_2 + 2.33X_3 + 0.7525X_1X_2 - 0.575X_1X_3 + 1.04X_2X_3 + 6.18X_1 + 6.38X_2 + 3.37X_3$$

(5)

The increase in homogenization speed from 0 to 10,000 rpm resulted in ZP decreasing from $-52.93\pm3.05$ to $-35.37\pm1.06$ mV, as shown in Figure 2C. This suggests that increasing the energy input in the system led to a decrease in the surface charge. In a study by Severino et al, it was observed that higher homogenization speed led to a reduction in ZP from 24 to 0 mV compromising the dispersion stability and promoting particle agglomeration.

Effect of Process Variables on HK-Entrapment Efficiency
The hydrophobic drug HK was entrapped in lecithin nanovesicles containing SDC as an edge activator. In the experimental design, we kept the quantities of lecithin and SDC fixed and varied the loading amount of HK to determine...
the effect of drug amount on EE%. EE% ranged between 32.6±0.88% to 89.9±0.96%, which were the values for HKT6 and HKT8, respectively, as shown in Table 2. Linear regression showed significance ($P=0.0008$), and the linear model was suggested to best describe the effect of variables on $Y_4$ as the difference between the adjusted $R^2$ and predicted $R^2$ being <0.2. Equation (6) describes the effect of the independent variables on EE%:

$$Y_4 = 71.22 + 11.08X_1 + 4.98X_2 - 12.3X_3 \quad (6)$$

Drug amount had a significant effect on HK EE% ($P=0.0026$). The positive $X_1$ in the equation indicated that increasing the drug amount resulted in an increase in EE%. Despite that our finding contradicts those observed in a previous study in which the EE% of resveratrol in solid lipid nanoparticles was observed to decrease as drug amount increases.\(^{84}\) This could be explained on the basis of the hydrophobicity of HK whereas the lipid bilayer represents a better solubilizing vehicle to HK than the external phase composed mainly of PBS (Figure 2D).\(^{85}\) Statistical analysis also revealed a significant effect of homogenization on drug EE% ($P=0.0013$), and the negative $X_3$ indicated an inverse effect of homogenization on EE%. This might be due to the ample shearing effect produced by the application of homogenization that caused disruption of the TF structure, leading to leakage of the drug and thus diminishing drug entrapment.\(^{86}\) These results are in agreement with a study on Pseudoephedrine HCl loaded nanoliposomes for transdermal delivery.\(^{87}\) In the current study, the maximum HK EE% (89.9%) was achieved by incorporation of 40 mg of drug in the TFs without the application of homogenization.

### Optimization of HKTs Formulation

The aim of the optimization process of HKTs formulation was to determine the levels of the factors studied required to produce a formulation of the highest-possible quality. According to the Box–Behnken model and based on criterion of desirability, the optimum values of the studied variables are suggested in Figure 2E. Desirability-function values range between 0 to 1. The function seeks the most favorable and compromising point in the design space that fulfills the goal for each dependent variable. The suggested values of the independent variables are shown in the optimization ramp (Figure 3). The desirability value of the suggested HKT\(_{opt}\) was 0.751.

To confirm the optimization process, the HKT\(_{opt}\) was prepared and characterized, then the actual observed responses were compared to the predicted responses of $Y_1$, $Y_2$, $Y_3$, and $Y_4$ that are listed in Table 3. As seen from the table, there was reasonably good agreement between predicted and observed responses. The adequate predictive performance of the design model justifies its application in the rationalization of an optimal formula and the validity of the optimization design.

### TEM of the Optimized HKT Formulation

Morphological examination of HKT\(_{opt}\) vesicles was done using TEM, as illustrated in Figure 4. As seen in the figure, it was noted that spherical, well-defined, uniformly sized, and unilamellar vesicles existed as discrete dispersed entities.

### Deformability Test

TF vesicles’ hallmark is their flexibility compared to liposomes, enabling the TFs to pass easily through the biological membranes.\(^{88}\) Therefore, the HKT\(_{opt}\) was formed of lecithin (the phospholipid) and SDC (the edge activator) at a ratio of 9:1, and its corresponding liposomal suspension consisting of lecithin (the phospholipid) and cholesterol, instead of SDC, at a ratio of 9:1 were selected to be tested for elasticity. The test was conducted by extruding each of the vesicular suspensions through a polycarbonate membrane of pore size 50 nm. The TFs exhibited a higher deformability index (13.9 ±0.9 mL/s) than the liposomes (3.94±1.2 mL/s) according to the deformability index as per equation (2). This is attributed to the presence of the edge activator in the TF structure, which rendered it higher elasticity than liposomes. The deformability index (D-value) of the TF preparation was in strong agreement with a previous study on the preparation of TFs encapsulating sildenafil, which found that the D-value of the best formula containing the optimum phospholipid: surfactant ratio was 14.88 mL/s.\(^{89}\) Another study done by Salama et al on olanzapine-loaded TFs containing SDC as edge activator showed that a phospholipid:edge-activator molar ratio of 10:1 achieved the highest deformability index in comparison to higher (100:1) or lower (5:1) ratios.

### Table 3 Predicted and observed responses of the optimized transfersomal formulation

| Independent variables | $X_1$ (mg) | $X_2$ (rpm) | $X_3$ (rpm) |
|------------------------|-----------|-----------|-----------|
|                        | 32.86     | 1,300     | 5,562     |

| Response | $Y_1$ (nm) | $Y_2$ | $Y_3$ (mV) | $Y_4$ (%) |
|----------|-----------|-------|-----------|-----------|
| Predicted | 208.5     | 0.431 | -47.98   | 76.65     |
| Observed | 189.9     | 0.471 | -53.4    | 78.92±1.22 |
| ±1.06 | ±0.0001 | ±1.63 |           |           |
as increasing the concentration of SDc in which the phospholipid:SDc molar ratio equaled 5:1 led to diminishing vesicle deformability owing to the steroid-like structure of the SDc, becoming bulkier and resulting in less flexible vesicles.\textsuperscript{90}

\textbf{Fourier-Transform Infrared Analysis}

The FTIR spectra of HK, lecithin, SDc, and HKT\textsubscript{opt} are shown in Figure 5. In the HK spectrum, the peaks in the range of 3,550–3,100 cm\textsuperscript{-1} were due to OH vibration. A band was located at 1,601 cm\textsuperscript{-1}, representing the alkene C=C present in HK.\textsuperscript{91} The intense bands at 1,495 and 1,426 cm\textsuperscript{-1} were assigned to the aromatic ring-stretching vibrations of the HK molecule. Other bands were found at 1,205 and 908 cm\textsuperscript{-1} (C–O) and 994 and 824 cm\textsuperscript{-1} (C–C).\textsuperscript{92} In lecithin, the broad band centered at 3,318 cm\textsuperscript{-1} was assigned to OH stretching, the principal bands at 2,861 cm\textsuperscript{-1} and 2,927 cm\textsuperscript{-1} corresponding to symmetric and antisymmetric stretching, respectively, in the CH\textsubscript{2} groups of alkyl chains, in addition to the scissoring vibrations at 1,404 cm\textsuperscript{-1} representing the CH\textsubscript{3} groups. The characteristic phosphate-group vibrational band assigned to the PO\textsubscript{2}\textsuperscript{-} antisymmetric stretching mode was centered at 1,232 cm\textsuperscript{-1} and the PO\textsubscript{2}\textsuperscript{-} symmetric stretching mode at 1,083 cm\textsuperscript{-1}.\textsuperscript{93} Regarding the SDc spectrum, the three distinctive features at 2,927, 2,861, and 1,556 cm\textsuperscript{-1} are infrared-absorption bands corresponding to CH stretching vibration and COO\textsuperscript{-} stretching vibration.\textsuperscript{94} The FTIR spectra of HKT\textsubscript{opt} showed that the peaks of the individual samples were shielded or absent and new peaks appeared at 3,268 cm\textsuperscript{-1} and 1,636 cm\textsuperscript{-1} indicating the occurrence of molecular interactions among the components during TF preparation.

\textbf{In Vitro Release Study}

The release profile (Figure 6) showed that only 50.1\%±2.673\% and 53.34\%±2.535\% of HK was released from the HKT\textsubscript{opt} after 24 and 48 hours, respectively, in contrast to the ethanolic solution of free drug, which showed 97.629\%±3.354\% release after 1 hour, indicating the sustained-release effect of the HKT\textsubscript{opt}. Similar patterns of sustained release were observed for lidocaine, a local anaesthetic, from transferosomes in comparison to the release profile of the free drug.\textsuperscript{63}

\textbf{Extent and Mechanism of Intracellular Uptake of TFs in Nonactivated and TAM- or TAF-Activated Melanoma Cells}

The extent of TF accumulation in B16F10 melanoma cells increased upon their incubation with stromal cell–derived conditioned media (Figure 7A). Similar results of higher uptake and retention of nanosystems in activated versus
nonactivated cells have been reported for TAFs versus normal fibroblasts\textsuperscript{95} and hypoxic versus normoxic phenotypes of breast cancer cells.\textsuperscript{96} The variation in the extent of intracellular accumulation of TFs can be explained on the basis of the mechanism of endocytic uptake. Treatment of B16F10 cells with sodium azide, an ATP-depleting agent, prior to the incubation of TFs caused a reduction in the extent of their accumulation, especially in nonactivated and TAF-activated cells (Figure 7B and D, respectively) suggesting the involvement of energy-dependent pathways in TFs uptake.\textsuperscript{68} Similarly, amiloride, which inhibits macropinocytosis,\textsuperscript{70} caused a very significant reduction in TF cellular concentration levels. Nystatin, which inhibits caveolin-dependent endocytosis,\textsuperscript{71} did not result in a significant change in intracellular concentration of the TFs. Regarding the involvement of cholesterol-dependent endocytosis, as suggested from the effect of pretreatment with MβCD, a reduction in TF intracellular uptake was observed in both nonactivated and TAF-activated cells (Figure 7B and D, respectively). Surprisingly, the extent of TF intracellular uptake increased in the case of TAM-activated cells (Figure 7C) upon utilization of MβCD and sucrose, which inhibit cholesterol-dependent\textsuperscript{68} and clathrin-dependent\textsuperscript{97} endocytosis, respectively. This suggests that blocking one endocytic mechanism could facilitate the uptake of TFs via an alternative pathway, which could be more efficient and/or more rapid.\textsuperscript{98}

Cytotoxicity of HKTs in Activated and Nonactivated Melanoma Cells
The effect of HKTs on the viability of B16F10 melanoma cells when cultured in fresh medium or conditioned medium

Figure 5 FTIR spectra of pure honokiol, lecithin, sodium deoxycholate, and the optimized formula.
derived from a culture of RAW264.7 macrophages and L929 fibroblasts was assessed. HK has been previously reported to possess appealing anticancer effects in hepatocellular carcinoma,\textsuperscript{99} triple-negative breast cancer,\textsuperscript{100} hepatoma,\textsuperscript{101} oral squamous-cell carcinoma,\textsuperscript{102} lung,\textsuperscript{103} thyroid,\textsuperscript{104} and ovarian cancer,\textsuperscript{105} and osteosarcoma.\textsuperscript{106} Upon activation of melanoma cells with conditioned media derived from stromal-cell cultures, a significant increase in IC\textsubscript{50} was observed (Figure 8). This contradicts the observed increase in the concentration of TFs in activated melanoma cells. This could be attributed to the development of resistance mechanisms upon induction of cell–cell interactions when cells were incubated with cytokine-rich conditioned media, as could be observed from the levels of TGF-β in TAM- and TAF-activated melanoma cells (Figure 10). TGF-β is a potent protumorigenic and immunosuppressive cytokine that regulates multiple functions in the TME.\textsuperscript{64–67}

Intracellular Uptake and Cytotoxicity of TFs upon Pretreatment with TME-Remodeling Agents (Metformin and Collagenase)

Pretreatment with either metformin or collagenase did not result in any contribution to the extent of the intracellular accumulation of the TFs in nonactivated or TAF-activated B16F10 melanoma cells (Figure 9A). However, collagenase pretreatment significantly increased the intracellular concentration of TFs in TAM-activated B16F10 melanoma cells. This could be explained by the ability of collagenase to digest dense ECM components, eg, collagen which could have facilitated uptake of the TFs. Enhancement in the uptake of magnetic nanoparticles in pancreatic cancer cells has been reported with pretreatment with collagenase.\textsuperscript{107} A similar observation was recorded for permeability of nanoparticles in 3-D spheroids.\textsuperscript{18} Unexpectedly, the susceptibility of nonactivated melanoma cells to HK-mediated cytotoxicity decreased significantly upon pretreatment with collagenase (Figure 9B). However, this could be explained on the basis of the ability of collagenase to increase TGF-β activation,\textsuperscript{108} consistent with our results elaborated in Figure 10, which has multiple implications in cancer resistance to different chemotherapeutics.\textsuperscript{109} On the contrary, metformin and collagenase pretreatment were shown to increase the efficacy of HKTs in TAM- and TAF-activated melanoma (Figure 9C and D, respectively), pointing to a possible synergistic effect whereas both agents previously showed multiple roles in the TME.\textsuperscript{17–26}

Role of HKTs on Immunosuppressive and Stem-Like Cell Characteristics of B16F10 Melanoma Cells in Monotherapy or Combined with TME-Remodeling Agents (Metformin and Collagenase)

TAM- and TAF-activated melanoma cells exhibited higher levels of all the immunosuppressive (TGFβ and CD47) and stem-like cell (CD133) markers relative to
nonactivated melanoma cells (Figure 10A and B, respectively). It is well known that TGF-β can be secreted from tumor cells, TAMs, and TAFs, promoting proliferation, angiogenesis, immunosuppression, ECM remodeling, invasion, and metastasis. In addition, TGF-β has been reported to regulate cancer stemness and induce the expression of stem-like cell markers like CD133. CSCs can then secrete more TGF-β, regulating all immunohallmarks in the tumor, including the expression of CD47. This shows how the TME is highly orchestrated and that all the three markers are interrelated.

Treatment of TAM-activated cells with HKTs reduce the levels of TGF-β, CD47, and CD133 (Figure 10A and Figure S1A–C). This could be explained on the basis of the previously reported role of HK in exhibiting an immunogenic effect in gastric cancer via inducing endoplasmic reticulum stress, blocking epithelial–mesenchymal transition, and suppressing calreticulin. A phagocytic marker associated with poor prognosis of some cancer types, calreticulin has been positively correlated with the antiphagocytic CD47 for maintaining cellular homeostasis by balancing pro- and antiphagocytic signals. Therefore, the suppressive effect observed for HK in our study on CD47 could have been regulated indirectly via calreticulin. Another possible explanation for the reduced levels of CD47 in HK-treated cells is the downregulation of TGF-β signaling: CD47 expression has been previously reported to be associated with TGF-β expression. Moreover, HK possesses CSC-targeting potential, reduces the proliferation of CD133+CSCs, and reverts the conferred resistance to chemotherapy in glioblastoma multiforme and oral squamous-cell carcinoma.

Further improvement in the therapeutic outcome of TAM-activated melanoma cells was observed upon pretreatment with metformin. Metformin has been reported
to reduce TGF-β expression and attenuate TGFβ-mediated tumorigenesis.26 It also possesses a repressing role on tumor initiation, self-renewal, and chemoresistance conferred by the breast cancer stem cells (BCSCs). Metformin was found to cause miR-708 mediated repression of the gene CD47 facilitating phagocytosis of BCSCs by macrophages.125 Metformin also mediates the downregulation of CD133 in hepatocellular carcinoma126 and pancreatic cancer,127,128 affecting higher chemosensitivity.

In an opposite manner, pretreatment of TAM-activated melanoma cells with collagenase produced an undesirable impact. This could be explained by the previously reported anti-inflammatory role of collagenase: it was found to upregulate anti-inflammatory cytokines, IL10, and TGF-β and downregulate the proinflammatory cytokines TNFα and IL1β.129

Figure 8 Cytotoxicity of HKTs in activated and nonactivated B16F10 melanoma cells. *p<0.05; **p<0.01; ***p<0.001.

Figure 9 Intracellular uptake of fluorescein-loaded TFs in nonactivated and TAM- or TAF-activated melanoma cells upon combination with TME remodeling agents (metformin and collagenase) (A). Cytotoxicity of HKTs in nonactivated (B), TAM-activated (C), and TAF-activated (D) B16F10 melanoma cells upon combination with TME remodeling agents (metformin and collagenase). *p<0.05; **p<0.01.
Treatment of TAF-activated cells with HKTs reduced levels of all the tested markers (Figure 10B and Figure S1A–C). However, the combination therapy produced detrimental effects on the level of the immunosuppressive and stem-like cell characteristics of melanoma. While the effect of collagenase pretreatment was not surprising, the detrimental effect of metformin was not expected. Investigation of the literature for a possible explanation revealed that metformin has a positive role on the secretion of TGF-β in supernatants of 4T1 triple-negative breast cancer cells.

Conclusion

Atoxic HKTs were successfully fabricated using a simple, scalable, green, modified heating method. Box–Behnken surface analysis proved to be an efficient tool to optimize the HKT formulations. HKTs possessed uniform nanosize, high negative charge, high EE%, and sufficient elasticity, and provided sustained release of HK over 48 hours. Moreover, HKTs reduce levels of TGF-β, CD47, and CD133, alleviating the immunosuppressive and stem-like cell characteristics of melanoma cells. Further improvement in therapeutic outcome was observed upon pretreatment with metformin, while pretreatment with collagenase produced an undesirable effect. With the view that the TME is highly orchestrated, it is necessary to investigate the role of the proposed topical HKT therapy on other key players of the TME.

Acknowledgment

We would like to thank Professor Laila Rashed, Professor of Medical Biochemistry and Molecular Biology, Cairo University for helping with the ELISA and WB experiments.

Disclosure

The authors reported no conflicts of interest for this work.

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