**Peganum harmala Alkaloids Self-Assembled Supramolecular Nanocapsules with Enhanced Antioxidant and Cytotoxic Activities**

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**ABSTRACT:** Amphiphilic macrocycles, such as p-sulfonatocalix[6]arenes (p-SC6), have demonstrated great potential in designing synthetic nanovesicles based on self-assembly approaches. These supramolecular nanovesicles are capable of improving the solubility, stability, and biological activity of various drugs. In the present study, the biologically active harmala alkaloid-rich fraction (HARF) was extracted from *Peganum harmala* L. seeds. Ultraperformance liquid chromatography–electrospray ionization–tandem mass spectrometry (UPLC/ESI-MS) analysis of HARF revealed 15 alkaloids. The reversed-phase high-performance liquid chromatography (RP-HPLC) analysis revealed three peaks: peganine, harmol, and harmine. The HARF was then encapsulated in p-SC6 nanocapsules employing a thin-film hydration approach. The designed nanocapsules had an average particle size of 264.8 ± 10.6 nm, and a surface charge of −30.3 ± 2.2 mV. They were able to encapsulate 89.3 ± 1.4, 74.4 ± 1.3, and 76.1 ± 1.7% of the three harmala alkaloids; harmine, harmol, and peganine; respectively. The in vitro drug release experiments showed the potential of the designed nanocapsules to release their cargo at a pH of 5.5 (typical of cancerous tissue). The IC₅₀ values of HARF encapsulated in p-SC6 (H/p-SC6 nanocapsules) were 5 and 2.7 μg/mL against ovarian cancer cells (SKOV-3) and breast adenocarcinoma cells (MCF-7), respectively. The prepared nanocapsules were found to be biocompatible when tested on human skin fibroblasts. Additionally, the antioxidant activity of the designed nanocapsules was 5 times that of the free powder fraction; the IC₅₀ of the H/p-SC6 nanocapsules was 30.1 ± 1.3 μg/mL, and that of the HARF was 169.3 ± 7.2 μg/mL. In conclusion, encapsulation of *P. harmala* alkaloid-rich fraction into self-assembled p-SC6 significantly increases its antioxidant and cytotoxic activities.

1. INTRODUCTION

Cancer is the second most common cause of death in developed and developing countries, with a steady increase in the number of new cases each year. The number of patients diagnosed with cancer is predicted to increase by 2-fold within the coming 30 years. Currently, synthetic chemotherapeutic agents are used in cancer therapy either alone or in combination with surgical resection and/or radiation therapy. However, direct use of classical chemotherapeutic agents and procedures has many drawbacks and generates severe adverse effects and resistance. However, encapsulation of the anticancer drugs into nanocarriers could provide effective drug delivery and minimize their side effects. Since more than 50% of the currently available anticancer drugs are from natural products or developed derivatives, natural products should be recognized as a potential source of potent and less toxic anticancer agents. Many studies have been reported on this path on employing natural products to treat several diseases.

*Peganum harmala* L. (Zygophyllaceae) is a small herbaceous plant known to grow as an invasive weed on poor soils in the Middle East and North Africa. The mature harmala seeds are rich in alkaloids (about 6% of total dry weight), including some β-carboline and quinazoline alkaloids, jointly known as harmala alkaloids. Major harmala alkaloids include harmine, harmane, harmol, harmaline, harmalol, and peganine. These alkaloids possess a broad spectrum of biological activities, including antibacterial, antioxidant, antiplatelet, immunomodulatory, and cytotoxic activities. However, their clinical applications in cancer therapy are hindered by their hydrophobic nature, and some studies reported toxic effects of harmala alkaloids on the central nervous system at high doses. Accordingly, carrying out encapsulation of harmala alkaloids could effectively reduce their harmful effects and/or enhance their anticancer activities.

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Various nanocarriers such as polymeric nanoparticles, dendrimers, and liposomes were previously reported for the targeted delivery of different natural and synthetic drugs.\textsuperscript{15,20} Recently, macromolecules, such as \textit{p}-sulfonato-calix[\textit{n}]arenes [\textit{n} = 4 and 6, (\textit{p}-SC4 and \textit{p}-SC6)] have demonstrated considerable impact on the delivery of anticancer drugs.\textsuperscript{10} \textit{p}-SCns are water-soluble, safe (for doses up to 10\textsuperscript{5} \textmu g/kg), biodegradable, and biocompatible. They are very promising as a potential host molecule for various chemotherapeutics via host−guest inclusion complexation.\textsuperscript{21−24} Additionally, amphiphilic macrocycles can be used to develop nanocapsules relying on self-assembly methods. These assemblies are bonded together via weak and reversible bonds, making them very responsive. Therefore, their amphiphilic nature qualifies them to encapsulate both water-soluble and oil-soluble chemo-therapeutic agents within their central shells where host−guest complexation plays a role part in binding the hydrophobic and hydrophilic moieties.\textsuperscript{21,25} These assemblies were also reported to exhibit targeted and controlled release of drugs to the sites of action and hence minimize the undesirable effects on healthy cells. The first amphiphilic macrocyclic nanocapsules based on self-assembly advances were designed by Markowitz et al. (1989) by injecting tetrahydrofuran solution containing \textit{p}-SC6 into water resulting in the production of nanocapsules with size ranging from 500 to 1000 nm.\textsuperscript{26} Further studies have been conducted on the design of \textit{p}-SCn as potential drug carriers. For example, nanovesicles created by the self-assembly of tetrahexyloxy-\textit{p}-sulfonatocalix[4]arene were utilized to encapsulate paclitaxel (PTX) anticancer drug. The prepared nanocapsules exhibited much enhanced anticancer activities on human cervical cancer cells than the unencapsulated PTX, at concentrations of 1, 10, and 100 \textmu g/mL.\textsuperscript{27} In the present work, the harmala alkaloid-rich fraction extracted from \textit{P. harmala} L. seeds was encapsulated in \textit{p}-SC6 nanocapsules (H/\textit{p}-SC6) employing a thin-film hydration approach. Nanocapsules were physically identified in terms of average particle sizes, surface charge, polydispersity index (PDI), encapsulation efficiency %, and morphology (employing transmission electron microscope (TEM)). Additionally, the chemical structural features of the nanocapsules were investigated employing Fourier transform infrared spectroscopy (FT-IR). Additionally, the antioxidant (using DPPH) and cytotoxic activities against ovarian cancer cells (SKOV-3), breast adenocarcinoma (MCF-7), and human skin fibroblasts (using sulforhodamine B (SRB) assay) were evaluated for both encapsulated and unencapsulated harmala alkaloid-rich fraction.

2. RESULTS AND DISCUSSION

2.1. \textit{P. harmala} Alkaloids. The three major alkaloids of \textit{P. harmala} (peganine, harmol, and harmine) were detected using HPLC−DAD and confirmed by UPLC-ESI-MS analysis.

2.1.1. Detection of Harmala Alkaloids by HPLC−DAD. The alkaloids of \textit{P. harmala} seeds were analyzed on HPLC−DAD. The chromatogram showed three major alkaloids, which could be detected by HPLC−DAD at 320 nm and corresponded to peganine, harmol, and harmine (Figure 1) as previously described.\textsuperscript{10} These peaks were compared to standard harmine and the isolated alkaloids (peganine, harmol, and harmine).

Figure 1. HPLC chromatogram of \textit{P. harmala} seed total alkaloid extract using a Waters LC 2695 coupled to a Waters 996 diode array detector (DAD) at 320 nm. XTerra MS-C18 column (100 × 4.6 mm\textsuperscript{2} id, 5 \textmu m). Mobile phase: water containing triethylamine (pH 8.6) (A) and acetonitrile (B). Gradient elution from 0% B (100% A) to 20% B in 4 min, 30% B at 9 min, 50% B at 14 min, and 60% B at 16 min. Then, 2 min washing with 100% B and reequilibration for 4 min. A flow rate of 1 mL/min, column temperature of 25 °C, and injection volume of 20 \mu L were applied. The peaks were compared to standard harmine and the isolated alkaloids (peganine, harmol, and harmine).
standard harmine and the isolated alkaloids peganine, harmol, and harmine. The isolated ones were annotated by their mass and UV spectra (Figures S1 and S2). Moreover, these alkaloids, in addition to the other 15 alkaloids, were confirmed by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

2.1.2. Analysis of Harmala Alkaloid-Rich Fraction by Ultraperformance Liquid Chromatography−Electrospray Ionization−Tandem Mass Spectrometry (UPLC/ESI-MS).

The phytochemical identification of the harmala alkaloid-rich fraction (HARF) was performed using UPLC-ESI-MS analysis. Fifteen alkaloids were timidly detected using their m/z, fragmentation pattern, their elution order from the HPLC column, and by comparing their mass spectral data with those reported. The annotated alkaloids are either β-carboline or quinazoline-type alkaloids. Alkaloids of both types are major compounds in this plant.28 The major β-carboline alkaloids annotated were harmine (m/z [M + H]+ 213.1546) and harmaline (m/z [M + H]+ 215.5396), with MS2 product patterns at 198 and 170 (for harmine) and 200 and 174 (for harmaline). However, quinazoline alkaloids were represented mainly by vasicine (peganine) and vasicinone (m/z [M + H]+ 189.2218 and 203.1254; MS2 ions at 171, 154, 144, and 117 “for vasicine”, and 203 and 185 “for vasicinone”). Other perceived major harmala alkaloids were peganine, hydroxylated harmine, and tetrahydroharmine. In addition, some

| peak no. | assignment                          | formula           | RT (min) | m/z [M + H]+ | product ions MS/MS ref | refs |
|---------|------------------------------------|-------------------|----------|--------------|------------------------|------|
| 1       | vasicine (peganine)                | C11H13N2O+        | 0.79     | 189.3991     | 189, 171, 154, 144, 130, 117 | 29, 30 |
| 2       | vasicine (peganine) isomer         | C11H13N2O+        | 1.20     | 189.2218     | 189, 171, 154, 144, 117 | 29−31 |
| 3       | vasicinone hexoside                | C12H14N2O+        | 2.06     | 365.1631     | 365, 203, 185, 82 | 32   |
| 4       | harmalol                           | C11H13N2O2+       | 2.18     | 201.1134     | 201, 184, 160, 67 | 29, 30 |
| 5       | peganine                           | C12H14N2O+        | 2.31     | 205.1031     | 187, 120, 74 | 29, 33 |
| 6       | vasicinone                          | C11H13N2O2+       | 2.62     | 203.1254     | 203, 185, 82 | 29, 30 |
| 7       | hydroxylated harmine               | C12H14N2O2+       | 4.00     | 229.1012     | 259, 214, 186, 211 | 29, 33 |
| 8       | deoxyvasicinone                    | C11H14N2O+        | 4.56     | 187.0925     | 187, 159, 144, 131 | 30 |
| 9       | tetrahydroharantine                | C13H16N2O+        | 4.83     | 217.1005     | 217, 199.9, 188, 173.9, 169, 157 | 29, 30 |
| 10      | harmaline                          | C13H16N2O+        | 5.40     | 215.5396     | 215, 200,174 | 29, 30 |
| 11      | harmine                            | C14H16N2O+        | 5.81     | 213.1546     | 198,170 | 29, 30 |
| 12      | hydroxylated harmine               | C12H14N2O2+       | 6.60     | 231.1150     | 214, 199, 187 | 34   |
| 13      | unknown                            |                   | 7.17     | 2. 2321      | 213, 196, 188, 160 | 28   |
| 14      | tetrahydroharantine isomer         | C13H16N2O+        | 7.77     | 217.1005     | 217, 199.9, 188, 173.9, 169, 157 | 29, 30 |
| 15      | harmol                             | C13H16N2O+        | 8.43     | 199.1732     | 199, 181, 171, 131, 105, 73 | 29, 35 |
| 16      | unknown                            |                   | 9.24     | 379.2151     | 364, 335 | 29, 35 |
| 17      | unknown                            |                   | 9.82     | 441.2976     | 268, 215, 201, 174 | 28, 35 |
| 18      | unknown                            |                   | 10.49    | 481.3096     | 28, 35 |
| 19      | unknown                            |                   | 11.81    | 274.2809     | 28, 35 |
| 20      | hexadecenal                        | C16H31O+          | 20.76    | 239.2334     | 239, 221, 195, 169, 155, 141, 113, 99, 85, 71, 57, 43 | 36 |
| 21      | harmalanine                        | C16H15N2O2+       | 23.48    | 267.2690     | 253, 236, 206, 186 | 37   |
| 22      | unknown                            |                   | 25.11    | 413.3079     | 268, 215, 174 | 28, 35 |

Figure 2. UPLC-ESI-MS/MS chromatogram (in positive-ion mode) of the total alkaloids fraction of P. harmala separated on an ACQUITY UPLC-BEH C18 (1.7 μm, 2.1 × 50 mm²) column.
minor alkaloids such as vasicinone hexoside, harmalol, deoxyvasicinone, hydroxylated harmaline, harmol, and harmalanine are presented in Table 1 and Figure 2. The structures of the major detected alkaloids are depicted in Figure 3, and the MS2 fragmentation pattern of selected alkaloids is represented in Figure S3.

Figure 3. Major alkaloids detected in HARF by ultraperformance liquid chromatography–electrospray ionization–tandem mass spectrometry (UPLC/ESI-MS).

Table 2. Average Size, PDI, ζ-Potential, and Encapsulation Efficiency of H/p-SC6 Nanocapsules

| formula | average size (nm) | PDI | ζ-potential (mV) ± SD |
|---------|------------------|-----|----------------------|
| H/p-SC6 | 264.77 ± 10.56   | 0.26 ± 0.02 | −30.33 ± 2.15 |
| harmine | 89.34 ± 1.41     |    |                      |
| harmol  | 74.39 ± 1.30     |    |                      |
| peganine| 76.10 ± 1.66     |    |                      |

Figure 4. TEM image for H/p-SC6 nanocapsules at a scale of 200 nm showing sizes of (A) 275.43 nm and (B) 250.41 nm.

2.2. Identification of the Designed Harmala Alkaloid-Rich Fraction/p-SC6 (H/p-SC6) Supramolecular Nanocapsules. 2.2.1. Particle Size, Polydispersity Index (PDI), ζ-Potential, and Encapsulation Efficiency (EE). The average size and PDI of the designed H/p-SC6 system were investigated by dynamic light scattering and were found to be 264.77 ± 10.56 nm and 0.26 ± 0.02, respectively (Table 2). These values lie within the range of the particles with nano sizes of 200–600 nm previously reported as ideal chemotherapeutics carriers with boosted passive accumulation, due to enhanced permeability and retention (EPR), into the permeable vasculature of tumor cells.38−40 The ζ-potential of the designed nanocapsules was obtained via laser Doppler velocimetry and exhibited a high negative surface charge of −30.33 ± 2.15 mV. This is attributed to the presence of the anionic p-SC6 amphiphile, which is highly negatively charged.21 The increased surface negativity on the nanocapsules is expected to impart high stability as it inhibits the aggregation of the particles, and hence keeps them suspended.
for a longer period. Encapsulation efficiencies on the three alkaloids are presented in Table 2.

The high encapsulation efficiency of HARF is attributed to the unique chemical structure of the amphiphilic p-SC6 that possesses a para-substituent of a phenolic ring on the upper edge, a phenolic hydroxyl group on the lower edge, and a hydrophobic π electron-rich central cavity (central annulus). Thus, their amphiphilic properties qualify them to encapsulate the water-insoluble harmala alkaloids, within their central cavities.21,41 The ability to encapsulate high concentrations of drugs inside nanovesicles supports sustained drug release.42

2.2.2. Morphological Features. The TEM images presented in Figure 4 show HARF loaded in amphiphilic p-SC6 nanocapsules, which have spherical shapes with smooth surfaces.

The self-assembly of amphiphilic p-SC6 is capable of producing spherical nanovesicles in aqueous solutions. The unique amphiphilic nature of p-SC6 permits the solubilization of the harmala alkaloid-rich fraction in the inner hydrophobic core while revealing its hydrophilic sulfonate groups found at the upper rims to the exterior medium.21

2.2.3. FT-IR Analysis. The FT-IR spectra of unencapsulated HARF, p-SC6, and the designed nanocapsules were compared to demonstrate the encapsulation of HARF into the cavity of the p-SC6 (Figure 5). Three major sharp bands, for pure HARF, are observed at 3444.02 cm⁻¹ (OH stretching vibrations), 1639.55 cm⁻¹ (C=O asymmetry stretching vibration), and 1014.59 cm⁻¹ (C–O stretching). These spectral bands agree with those reported previously.43 Upon encapsulation of HARF in p-SC6 nanocapsules, it is observed that the C–O stretching band at 1014.59 cm⁻¹ had disappeared compared to HARF. Thus, this could indicate the encapsulation of HARF inside the cavity of p-SC6.27

2.2.4. In Vitro Release Study. The release of harmala major alkaloids from p-SC6-based nanocapsules was studied at pH 7.4 of healthy cells and pH 5.5 of cancer cells (Figure 6A–C).44 The released three alkaloids (peganine, harmol, and harmine) were determined using the above-mentioned HPLC–DAD method. As expected from ζ-potential measurements, H/p-SC6 nanocapsules exhibited outstanding stability at pH 7.4 with about 28.3, 39.9, and 35.2% of the loaded harmala alkaloids released after 48 h at 37 °C. At pH 5.5, 89.2, 88.9, and 90.8% of the loaded harmala alkaloids were released after 48 h at 37 °C. These results support the ability of self-assembled calixarenes to release their cargo selectively by a pH-triggered mechanism at a typical cancerous tissue pH of 5.5.45

These designed nanocapsules hold much promise for future cancer therapy because of their capability to release the encapsulated drugs inside the tumor tissue while shielding them from early decomposition in the systemic circulation of pH 7.4.

2.2.5. Antioxidant Activity. The antioxidant activity of free HARF and H/p-SC6 nanocapsules was investigated utilizing 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Trolox, a powerful antioxidant, was used as a positive control and showed an IC₅₀ of 56.82 ± 0.87 μg/mL (Figure 7). It was shown that the IC₅₀ of the H/p-SC6 nanocapsules (30.10 ± 1.30 μg/mL) exhibited about 5 times that of the free HARF (169.3 ± 7.2 μg/mL), as presented in Figure 7.

The nanocapsules’ enhanced antioxidant activity compared to harmala alkaloid-rich fraction might be attributed to enhancing the hydrophilic nature of harmala alkaloid-rich fraction upon its encapsulation within the p-SC6 self-assemblies.46,47 Additionally, calixarenes are reported to have antioxidant properties combined with the antioxidant abilities of harmala alkaloid-rich fraction, giving rise to more pronounced free radical scavenging activities.48 These findings are very promising because powerful antioxidants have many biomedical applications and might play a vital role in protecting normal cells, especially during cancer treatment.

2.2.6. In Vitro Cell Viability Assay. The cytotoxicity of p-SC6, free HARF, and H/p-SC6 nanocapsules was assessed by SRB assay and ovarian cancer cells (SKOV-3), breast adenocarcinoma cells (MCF-7), and normal human skin fibroblasts.23,24 p-SC6 was used as host control, and it showed a negligible decline in cell viability, and HARF was used as the positive control. After 48 h incubation, the harmala extract nanocapsules (H/p-SC6) exerted remarkable in vitro cytotoxicity compared to the free powder extract (positive control). The cytotoxic activities (IC₅₀ in μg/mL computed by Sigma plot) of p-SC6, free HARF, and H/p-SC6 nanocapsules against cancer noncancer cell lines are presented in Table 3. The IC₅₀
values of H/p-SC6 were about 1.2- and 3-fold less than that of the HARF against SKOV-3 and MCF-7 cells, respectively. On the other hand, testing H/p-SC6 nanocapsules on HSF noncancer cells confirmed their biocompatibility compared to free harmala seed fraction. The previous studies reported the ability of different host molecules to encapsulate anticancer drugs and selectively deliver them to cancer cells with minimal to no toxic effects on normal cells. These results agree with the in vitro release study demonstrating the pH-triggered release of the HARF from the self-assembled H/p-SC6 nanocapsules. Cancerous cells have an acidic pH of 5.7, normal cells possess a pH of 7.4, whereas late endosomes and lysosomes have pH values of 4.5–5.5. This pH variation is vital because the pH-sensitive carriers would selectively release their cargo in the acidic tumor microenvironment. Additionally, HARF showed increased cytotoxic activities as its encapsulation within the p-SC6 nanocapsules overcame the extract’s hydrophobic nature and improved its bioavailability.

This study also highlights the importance of using P. harmala seed alkaloid-rich fraction as a possible natural anticancer drug. Harmala alkaloid-rich fraction’s anticancer activity is attributed to the β-carboline alkaloids that inhibit DNA topoisomerases via DNA intercalation and induction of apoptotic pathways. Additionally, harmine alkaloid has been shown to possess antiangiogenic activity, reduce the expression of numerous proangiogenic factors, and diminish the propagation of vascular endothelial cells. Thus, these findings shed more light on the significance of using self-assembled p-SC6 as a possible supramolecular carrier for the natural promising anticancer agent, P. harmala seed alkaloid-rich fraction.

### 3. CONCLUSIONS

In the present work, the harmala alkaloid-rich fraction was extracted from P. harmala seeds, and 15 alkaloids were annotated using ultraperformance liquid chromatography–electrospray ionization–tandem mass spectrometry (UPLC/ESI-MS). Reversed-phase high-performance liquid chromatography coupled with diode array detector (HPLC–DAD) analysis of the alkaloid-rich fraction revealed three major peaks: peganine, harmol, and harmine. This fraction was encapsulated in amphiphilic p-SC6 self-assembled nanovesicles employing a thin-film hydration approach. The designed
nanocapsules possessed an average particle size of 264.77 ± 10.56 nm, a surface charge of −30.33 ± 2.15 mV, and were found to encapsulate 89.34 ± 1.41, 74.39 ± 1.3, and 76.1 ± 1.66% of the three harmala alkaloids, peganine, harmol, and harmine, respectively. In vitro drug release experiments showed the potential of the designed nanocapsules to release their cargo in mildly acidic media. This is very promising for future cancer therapy because of their ability to release the encapsulated drugs inside the acidic cancer tissue while keeping them stable in the systemic circulation of pH 7.4. The antioxidant activity of the H/p-SC6 nanocapsules was 5 times that of harmala alkaloid-rich fraction. Additionally, the cytotoxicity study revealed that the IC_{50} values of the designed nanocapsules were about 1.2- and 3-fold less than that demonstrated by harmala alkaloid-rich fraction against SKOV-3 and MCF-7 cells, respectively. The prepared nanocapsules were found to be biocompatible when tested on human skin fibroblasts. Thus, encapsulation of harmala alkaloid-rich fraction into self-assembled supramolecular nanocapsules dramatically enhances the natural anticancer and antioxidant effects of harmala alkaloids.

4. MATERIALS AND METHODS

4.1. Materials. p-Sulfocalix[6]arene was purchased from WuXi LabNetwork, China. Diphenyl-1-picylhydroxyl radical (DPPH), Harmine standard, HPLC-grade deionized water, chemicals, and solvents were obtained from Sigma-Aldrich (St. Louis, MO). Streptomycin, penicillin, fetal bovine serum, trichloroacetic acid (TCA), Dulbecco’s modified Eagle’s medium (DMEM) SRB, and tris(hydroxymethyl)-aminomethane (TRIS) were obtained from Lonza, Basel, Switzerland.

4.2. Plant Material. Dried mature seeds of *P. harmala* L. were purchased from the Egyptian local market. A voucher specimen was deposited (18.1.17) at the Department of Pharmacognosy Herbarium, Faculty of Pharmacy, Cairo University (Egypt).

4.3. Methods. 4.3.1. Extraction and Isolation of Major P. harmala Alkaloids. *P. harmala* seeds (2.5 kg) were ground to a fine powder and then extracted by soaking in 70% ethanol (3 x 5 L) at ambient temperature overnight. The ethanol extracts were filtered, combined, and evaporated under reduced pressure to yield a dark reddish-brown viscous residue (520 g). The concentrated extract was then dissolved in 5% HCl (2 L), filtered, and partitioned with dichloromethane (4 x 300 mL). The aqueous acid layer's pH was adjusted to pH 9 using NH₄OH and then extracted with dichloromethane (4 x 500 mL). The dichloromethane layer was washed with water and then evaporated under reduced pressure at 40 °C to yield 60 g of reddish-brown powder (harmala alkaloid-rich fraction).

4.3.2. HPLC−DAD Analysis of the Three Major Harmala Alkaloids. The analysis of *P. harmala* alkaloids was performed, according to our previously reported method, by RP-HPLC using a Waters LC 2695 coupled to a Waters 996 diode array detector (DAD). A 100 x 4.6 mm² id, 5 μm, XTerra MS-C18 column (Agilent Technologies) was used for analysis. Chromatographic conditions were water supplemented with triethylamine (pH 8.6) (A) and acetonitrile (B). The gradient was programmed from 0% B (100% A) to 20% B in 4 min, then 30% B at 9 min, 50% B at 14 min, and finally to 60% B at 16 min. The separation was followed by a 2 min washing procedure with 100% B and a reequilibration period of 4 min. Flow rate: 1 mL/min, column temperature: 25 °C, injection volume: 20 μL, and absorbance detection: 320 nm. Under these conditions, the elution order was peganine, harmol, and harmaline. These peaks were compared to standard harmine, and the isolated alkaloids were peganine, harmol, and harmine when injected individually on HPLC (Figure 1). The isolated ones were annotated by their mass and UV spectra (Figures S1 and S2). Calibration curves were constructed for peganine, harmol, and harmine to be used in the measurement of encapsulation efficiency and in vitro release studies.

4.3.3. UPLC−MS/MS Analysis of Harmala Alkaloids. The harmala alkaloid-rich fraction was analyzed using UPLC/ESI-MS. The sample was dissolved in methanol for HPLC at a final concentration of 100 μg/mL. Then, it was filtered using a membrane disk filter (0.2 μm) before subjecting to LC-ESI-MS analysis on UPLC/ESI-MS, ACQUITY UPLC System (Waters Corporation) using ACQUITY UPLC-BEH C18 (1.7 μm, 2.1 x 50 mm²) column and injection volume of 10 μL. The solvent system consisted of (A) water containing 0.1% formic acid and (B) methanol containing 0.1% formic acid. Elution was done using gradient mobile phase starting from 90% A: 10% B that was maintained for 2 min, it reached 70% A at 5 min, then 30% A at 15 min, 10% A at 22 min which was maintained for 3 min and then was changed to reach 100% B at 26 min and was upheld for 3 min and then back to the initial composition at 32 min. The flow rate was 0.2 mL/min. The parameters for analysis were carried out in positive-ion acquisition mode using a XEVO TQD triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). The source temperature, cone voltage, capillary voltage, and desolvation temperature were 150 °C, 30 eV, 3 kV, and 440 °C, respectively. The cone gas flow rate was 50 L/h, and the desolvation gas flow rate was 900 L/h. Mass spectra were detected in the ESI between m/z 100 and 1000. The peaks and spectra were processed using the Maslyx 4.1 software and tentatively identified by comparing their retention times (R) and mass spectra with reported data.

4.3.4. Preparation of Harmala Alkaloid-Rich Fraction-Loaded p-Sulfocalix[6]arene Nanocapsules (H/p-SC6). H/p-SC6 nanocapsules were produced using the thin-film hydration approach as described elsewhere with some modifications. Briefly, p-SC6 (57 mg) was mixed with HARF (1 mg) in a sufficient volume of methanol in a round-bottom flask. The round-bottom flask was exposed to evaporation under vacuum for 60 min at 40 °C, utilizing a Laboratoy 4000 rotary evaporator (Heidolph Instruments, Schwabach, Germany) equipped with a vacuum pump (KNF Neuberger GmbH, Freiburg, Germany), leaving a thin film. Then, hydration was conducted using phosphate-buffered saline (PBS, pH 7.4) in a rotary evaporator without using vacuum for 30 min at 40 °C. Finally, sonication for 1 min of the produced suspension was carried out employing a bath sonicator (Elmasonic P30 H, Elma Hans Schmidbauer, Singen, Germany).

4.3.5. Characterization of the Designed H/p-SC6 Nanocapsules. The average particle size and polydispersity index (PDI) of the prepared nanocapsules were determined using dynamic light scattering employing Zetasizer Nano ZS (Malvern Instruments Herrenberg, Germany). The instrument is equipped with a 10 mW HeNe laser allowing for the measurements to be performed at a wavelength of 633 nm and a detection angle of 173° backscatter. The ζ-potential of the nanocapsules was determined via laser Doppler velocimetry in...
a clear disposable folded capillary cell (DTS1070, Malvern Instruments).

The morphological features of the nanocapsules were studied using transmission electron microscopy (TEM), employing a JEOL-JEM 2100 electron microscope operating at 160 kV. A 50 μL aliquot of the nanovesicles diluted to 1:2 (v/v) with PBS were stained with 2% aqueous phosphotungstic acid. This mixture was placed and dried over a carbon-coated copper 200 mesh grid, imagined, and photographed.

The FT-IR spectra of unencapsulated HARF, p-SC6, and the designed nanocapsules were subjected to Fourier transform infrared (FTIR) spectroscopy employing FTIR-8400s (Shimadzu, Japan). Samples were first compressed with KBr into disks, scanned, and spectra were recorded in the range of 500–4000 cm⁻¹.

4.3.6. Encapsulation Efficiency. The encapsulation efficiency (EE) of H/p-SC6 nanocapsules was determined by centrifugation at 14 000 rpm for 90 min at 4 °C, followed by ultrafiltration to eliminate the harmala alkaloids loaded pellet. The quantity of the harmala alkaloids unencapsulated in the ultrafiltrate was detected using the HPLC protocol described earlier. The EE of the H/p-SC6 nanocapsules was determined using eq 1:

\[ EE \% = \frac{\text{total amount of drug} - \text{amount of drug unencapsulated}}{\text{total amount of drug}} \times 100 \]  

4.3.7. In Vitro Release Study under Different pH Conditions. The release rate of harmala alkaloids from H/p-SC6 nanocapsules was studied using the dialysis membrane method at pH 5.5 and 7.4. Briefly, 1 mL of the sample was loaded to a dialysis bag (cutoff molecular weight, 3500 Da). The dialysis bag was inserted into 50 mL of PBS at pH 7.4 or 5.5, with 1% Tween and 0.5% FBS in a proper jar. The whole system was fixed in a shaking incubator (Jeio Tech SI-300, Seoul, Korea), rotating at 100 rpm with temperature adjusted to 37 °C. At specific time intervals, a 1 mL aliquot of the sample was withdrawn for analysis by the above-described HPLC method and immediately replaced with another equal volume of warmed buffer.

4.3.8. Determination of Total Antioxidant Activity. The antioxidant activity of HARF and H/p-SC6 nanocapsules was evaluated using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging assay as previously reported by Boly et al. with minor modification. Briefly, 100 μL of freshly prepared DPPH reagent (0.1% in methanol) was added to 100 μL of the sample in a 96-well plate (n = 6) and the reaction was left at room temperature for 30 min in the dark. Afterward, the color intensity of the DPPH resulting from its reduction was recorded at 540 nm using a microplate reader Fluostar Omega (Ortenberg, Germany). Data are represented as mean ± SD according to eq 2.

\[ \text{DPPH scavenging activity} \% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \]  

where \( A_{\text{control}} \) is the absorbance of the DPPH and \( A_{\text{sample}} \) is the absorbance of the sample.

Trolox was considered a reference standard, and DPPH solution was used as the control. Data were analyzed using Microsoft Excel, and the IC₅₀ value was calculated using GraphPad Prism 5 by converting the concentrations to their logarithmic value and selecting nonlinear inhibitor regression equation [log (inhibitor) versus normalized response – variable slope equation]..

4.3.9. Cell Viability Assay. 4.3.9.1. Cell Culture. Ovarian cancer cells (SKOV-3), breast adenocarcinoma cells (MCF-7), and human skin fibroblasts healthy cells were obtained from American Type Culture Collection, (University Boulevard, Manassas, VA 20110) and maintained in DMEM supplemented with streptomycin (100 mg/mL), penicillin (100 units/mL), and 10% heat-inactivated fetal bovine serum. Cells were incubated in humidified 5% (v/v) CO₂ at 37 °C.

4.3.9.2. Sulforhodamine B Colorimetric Assay. Ovarian cancer cells (SKOV-3), breast adenocarcinoma cells (MCF-7), and human skin fibroblasts were treated with different concentrations of p-SC6, free HARF (positive control), and H/p-SC6. The cell viability of either cancerous or non-cancerous cells was tested using the SRB assay, and the IC₅₀ (in μg/mL) value was computed using our methods described previously.

All trials were conducted in triplicates, and data are demonstrated as mean ± standard deviation.

ASSOCIATED CONTENT

 Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00455.

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