Original Article

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Optimizing an Infusion Method of Producing Cosmos Caudatus Encapsulated In Exosomes as A Nanocarrier for Drug Delivery

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Abstract:

Objective: This study aimed to propose different encapsulation methods of a medicinal plant extract *Cosmos Caudatus* (CC), into HSC-2-derived exosomes.

Material and Methods: Encapsulation of HSC-2-derived exosomes was achieved via internal and external infusion. The internal infusion was achieved by treating HSC-2 cells with CC extract and isolating the exosomes from its media (M1_ExoCC). The external infusion was performed by dissolving CC extract with HSC-2 purified plain culture media (M2_ExoCC), direct incubation of isolated exosomes suspended in PBS with CC extract (M3_ExoCC), freeze-thaw incubation of purified HSC-2 media containing CC extract (M4_ExoCC), and freeze-thaw incubation of PBS suspended exosome with CC extract (M5_ExoCC).

Results: M1_ExoCC and M2_ExoCC were the most advantageous encapsulation methods in terms of yield, stability and solubility.

Conclusion: It was determined that the M1_ExoCC and M2_ExoCC methods yielded the most favorable outcomes across all parameters. The cost-effectiveness and simplicity of these methods make them particularly appealing for scaling up production and translating research findings into clinical practice.

Keywords: *Cosmos Caudatus*, drug delivery, exosomes, nanocarrier, solubility, stability

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Introduction

Oral cancer (OC) is one of the most diagnosed carcinomas in the world, with 377,713 cases being reported globally in 2020[1]. One of its subtypes, oral squamous cell carcinoma (OSCC), arising from the mucosal layer of oral epithelium, is the most common subtype of OC, occurring in 90% of all cases. It is categorized as a dangerous subtype of OC due to its effects on the function and appearance of the oral cavity[2].

Cosmos caudatus (CC) is an endemic plant from Mesoamerica, belonging to the aster family (Asteraceae), and has been extensively studied for its pharmacological activities. It exhibits many beneficial effects; including antioxidant, anticancer, antihypertensive, antidiabetic, and hypolipidemic properties[3–7]. These pharmacological activities are attributed to the rich phytochemical composition of CC extract, which includes flavonoids, phenylpropanoids, carotenoids, phenolic acids and vitamins[8].

Exosomes are nano-sized cellular secretions ranging from 30–200 nm in size. They are released by different types of cells as a means of communication and nano-carrying of biomolecules from one cell to another. As nanocarriers, scientists are employing this feature; especially in drug delivery[9]. In addition, naturally derived bioactive compounds are being encapsulated and delivered by exosomes, boosting their cytotoxicity against diseases[10,11].

Cancer-derived exosomes generally share common receptors with their parental cell, allowing them to return to their cell of origin. This feature is being exploited to deliver therapeutic cargo as a targeted delivery system[12]. This study aimed to harness this ability by optimizing an approach for encapsulating CC extract in exosomes derived from HSC-2 (OSCC cell line), so as to optimize and formulate a drug delivery system to combat oral cancer. The exosomal yield, solubility, and stability were factors considered when devising the most effective method for infusing CC extracts into exosomes.

Material and Methods

Cell culture

The HSC-2 cell line was cultured in T25 flasks within a complete culture medium consisting of minimum essential medium (MEM) (Gibco®, New York), and containing 10% fetal bovine serum (FBS) (Gibco®, New Zealand) and 1% of a penicillin/streptomycin antibiotic mixture (Gibco®, New Zealand). Cells were incubated at 37°C and 5% carbon dioxide. After 70% confluency, cells were washed three times with phosphate buffer saline (PBS) to remove traces of FBS. During experiments, cells were cultured with serum-free media to exclude contamination with exosomes from FBS.

Plant extraction

Clean, fresh leaves of CC were dried in an oven at 50 °C for 24 hrs, weighed, and blended using a blender (Panasonic®, Malaysia). The powder was then soaked in 70% ethanol, with a ratio of 7:1 (ethanol: grounded leaves), for three days on a reciprocating shaker (Stuart SSL2) at 150 strokes/min for 2 days. Then, the solvent was filtered with Whatman filter paper No. one. The extract was air-dried under an air current for 24 hours so as to allow evaporation of any water or alcohol. Finally, the extracts were freeze-dried (ScanVac CoolSafe) into a powdery form and stored at -20 °C.

Isolation of exosomes

At 70% confluency, HSC-2 culture media was collected and subjected to 20 minutes of centrifugation at 6,000xg at 4 °C to remove cell debris. Exosomes were isolated using ultracentrifugation (UC) at 110,000xg for 2 hours at 4 °C to obtain the exosome pellet. The supernatant was discarded and the pellet was suspended in 100 µl of phosphate-buffered saline (PBS) and stored at -80 °C. Furthermore, sucrose gradient (SG) was used to purify the exosomes (Figure 1).

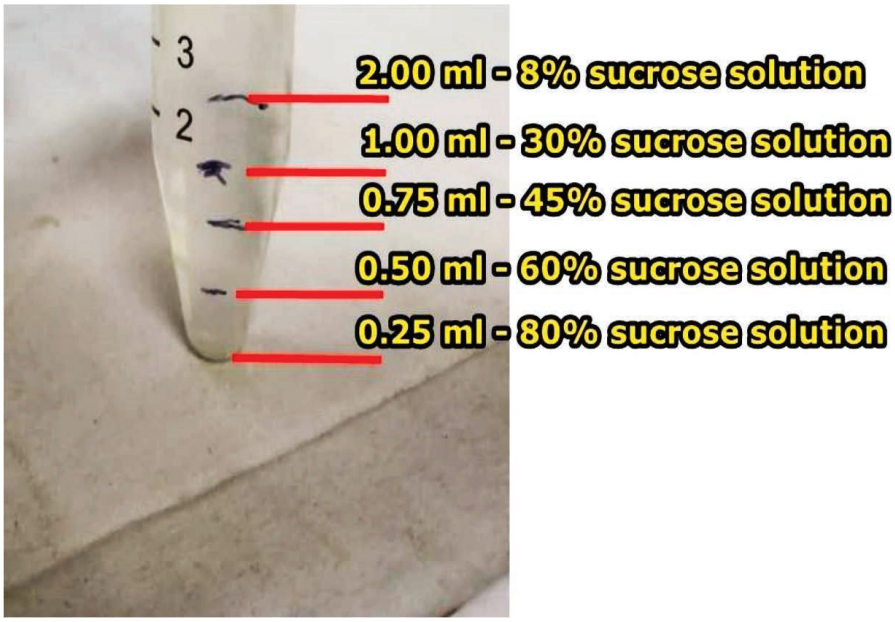


Figure 1 Illustrates sucrose gradient layers (8%–80%) for the process of exosome isolation

Characterization of exosomes

Atomic force microscopy

After preparing the specimen disk, by attaching adhesive tape and adding $NiCl_2$ solution, 100 µl of 1x PBS was pipetted on the mica disk and placed in a paraffin-wrapped petri dish for about 12–18 hours at 4 °C. When the incubation was completed, 80% of the sample was aspirated and hydrated with 1x PBS 3 times. After washing, about 80% of the sample was discarded and 40 µl of 1x PBS was added. The sample was then washed 3 times with deionized water dried via a nitrogen stream and viewed under atomic force microscopy (AFM) (Dimension Edge™).

Western blotting

Following the isolation, exosomes were suspended in 50–100 µl of RIPA lysis buffer (Bio-Rad, CA). Jess automated western blot system (Jess™ Simple Western system) was used for the detection of exosomal biomarkers, namely cluster of differentiation (CD63 and CD81) and heat shock protein (HSP70).

Infusion of CC extract into exosomes

M1_ExoCC

Confluent HSC-2 cells were treated with 0.1 mg/ml of CC extract for 24 hours. A culture media (serum-free) containing the treatment was collected and centrifuged to remove cell debris and excess extract particles. Then, the purified media was proceeded to UC and subjected to SG. The isolated exosomes were suspended in 100 µl of PBS.

M2_ExoCC

Approximately 0.1 mg of CC extract was incubated with 1 ml of culture media collected from non-treated cells overnight at 4 °C in an upright position and proceeded to UC and SG. The isolated exosomes were suspended in 100 µl of PBS.

M3_ExoCC

Isolated exosomes from the culture media were incubated with 0.1 mg of CC extract diluted in 100 µl of PBS overnight at 4 °C. Then, the mixture was proceeded

for UC and SG. The isolated exosomes were suspended in 100 µl of PBS.

M4_ExoCC

Roughly, 0.1 mg of CC extract was mixed with 1 ml of culture media from non-treated cells. The mixture was first incubated for 30 minutes at room temperature (RT) in an upright position. Next, the mixture was placed in liquid nitrogen until it was fully solidified and let thaw at RT for 15 minutes. This freeze-thaw was performed repeatedly for 1-10 cycles and finalized with incubation of the mixture in an -80 °C refrigerator for 30 minutes, then thawed at RT. After this, the mixture was proceeded for UC and SG. The isolated exosomes were suspended in 100 µl of PBS.

M5_ExoCC

This method is similar to previous freeze-thaw techniques; however, the isolated exosomes were suspended directly in 100 µl of 0.1 mg of CC extract diluted PBS. Upon which, the mixture was proceeded for UC and SG. The isolated exosomes were suspended in 100 µl of PBS.

Wavelength analysis of CC extract

To study the solubility and stability of the CC extract, it was crucial to determine the wavelength of the CC extract using a microplate reader (Varioskan Flash, Thermo Scientific, USA) to be used as a comparison baseline. To do so, 0.1 mg⁄ml of CC extract was added to 1 ml of PBS and incubated on ice for 30 minutes with a quick centrifugation to remove excess and undissolved extract. Then, the supernatant was pipetted into a microplate, and absorbance was read at wavelengths ranging from 250-800 nm. The results were plotted as a wave scan graph to determine the highest peak wavelength. The next step was to determine the R^2 value of the CC concentrations using a bio-photometer. Serial dilutions of the stock solution, containing 1 mg of CC dissolved in 10 ml acetonitrile, ranged from 100-3.125 µg⁄ml (dilution factor 2).

Solubility and stability tests

Solubility and stability tests were carried out according to Sun et al., with modification[11].

In vitro solubility test was conducted by adding 1 mg of CC with 10 µl of each exosome sample (Exo_CON and M1-M5_ExoCC). Each sample was vortexed and spun for 5 seconds, followed by 30 minutes of incubation at RT. Then it was diluted with 1 ml of PBS and incubated on ice for 30 minutes, followed by a brief centrifugation (500 rpm for 5 minutes) to remove excess CC. The supernatant was then transferred into a sterile centrifuge tube, and absorbance was measured using a wave scan in the range of 250–800 nm (Shimadzu, EU).

The *In vitro* stability test was carried out with the same concentration for each sample as the stability test, however it had different incubation periods: as the samples were incubated for 0, 30, 60, 90, 120, and 190 minutes in the dark at 37 °C in a water bath. The results are presented in a fraction of the fold reduction point, wherein the beginning was set as 1.0, at 0 minutes. Absorbance was taken at 352 nm. The less fold reduction of a sample, the more stable it was in PBS (pH 7.4) against incubation time.

Bradford assay

Each sample's exosomal protein was quantified using Bradford protein assay (Pierce™ Coomassie Plus, Thermo Scientific™) and Nanodrop (Thermo Scientific™, USA). A standard curve, with R^2=0.995, was used to extrapolate the concentrations of exosome samples. The LINEST function in Microsoft Excel was used to auto-calculate the unknown concentration of the samples.

Data analysis

Data processing was performed using Excel (Microsoft Office version 16). Holm-Sidak's multiple t-tests

using GraphPad Prism (version 8.4.3) were performed to compare the means of stability; reading at a significance level of p-value<0.05. All experiments were done in duplicate.

Results

HSC-2 derived exosomes

Visual characterization of HSC-2-derived exosomes via AFM showed individually and clumped exosomes; ranging from 30 to 200 nm (Figure 3. A1). Higher magnification power showed distorted exosome membranes (likely due to mechanical stress) and ovally shaped exosomes (Figure 3. A2). On the other hand, western blot analysis showed that HSC-2-derived exosomes only expressed tumor proteins (CD63 and CD81), but no expression of HSP70 (Figure 3. B).

Exosomal encapsulation of CC extract

Post incubation with plant extracts, all the methods were subjected to a sucrose gradient (Figure 1). It was observed that the encapsulated CC formed sedimentation at the 80% layer of the sucrose gradient (Figure 2).

Moreover, spectrophotometer analysis showed enhanced solubility of CC post-encapsulation as compared to free CC. The highest CC solubility was obtained from methods involving freeze-thaw (M5_ExoCC and M4_ExoCC). In contrast, direct incubation of CC with exosomes (M3_ExoCC) had the lowest solubility (Figure 4).

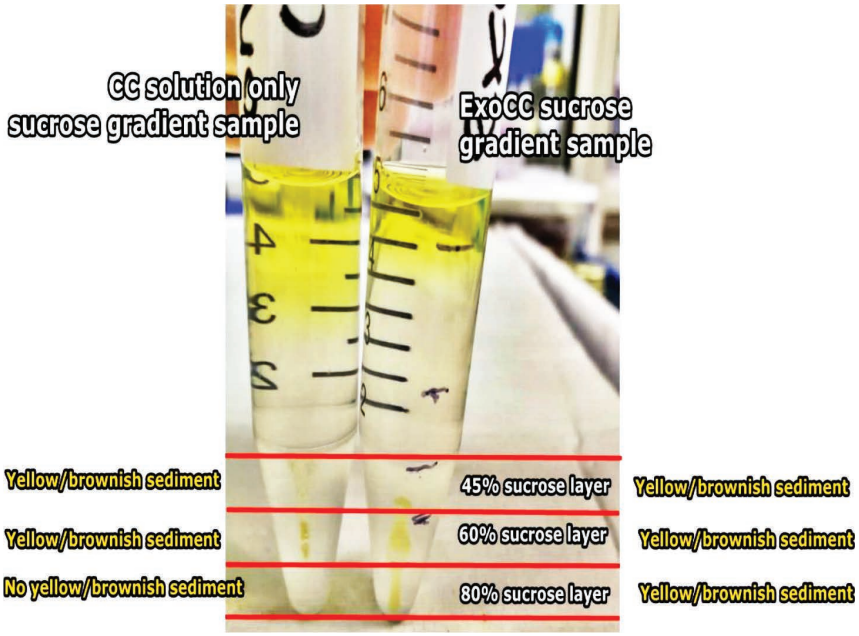


Figure 2 Illustrates the sucrose gradient purification process used to isolate exosomes. The gradient tube is depicted with layers of varying sucrose concentrations. Exosome-containing CC are loaded onto the top of the gradient and subjected to ultracentrifugation. As centrifugation proceeds, exosomes migrate through the gradient, leading to their separation, based on buoyant density. Fractions collected from the gradient 80% sucrose layer contain purified exosome-containing CC. This purification method enables the isolation of exosomes with high purity and yield, which is crucial for subsequent analyses and applications in drug delivery and biomarker discovery

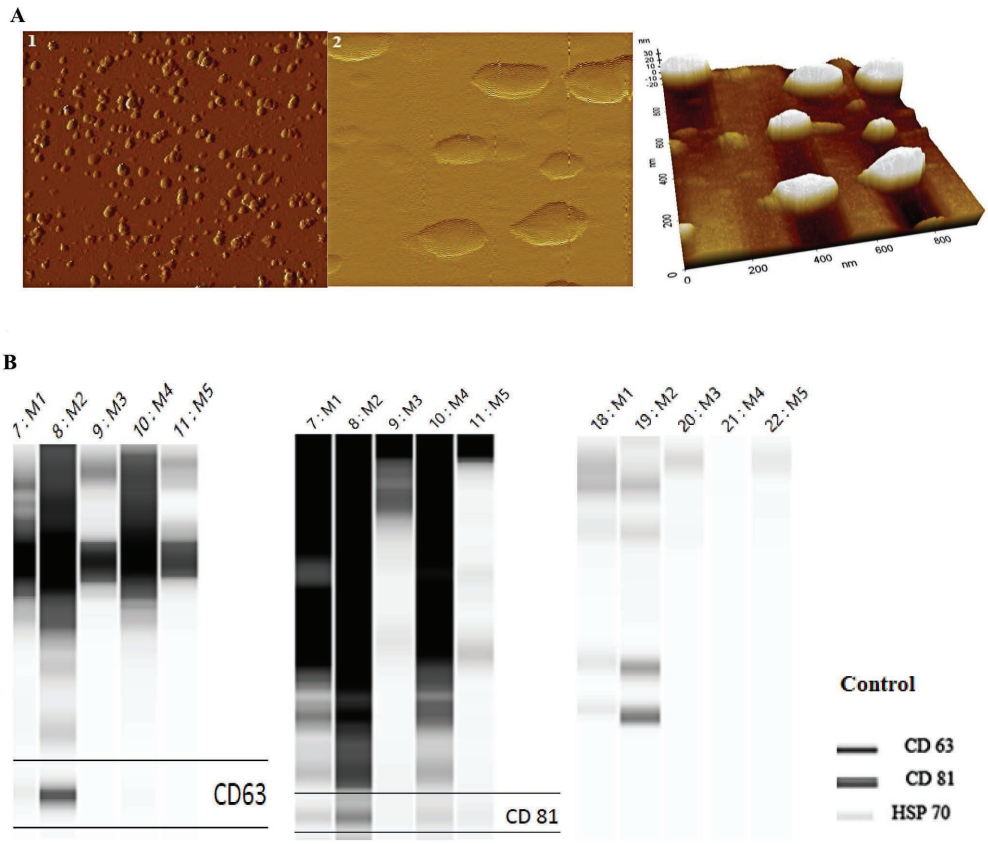


Figure 3 Characterization of HSC–2 derived exosomes. (A) Atomic force microscopy images showing round to oval–shaped exosomes; ranging from 30–200 nm in size. (B) Western blot analysis of protein expression in exosomes isolated from different encapsulation methods. CD63 expression is observed in M1_ExoCC and M2_ExoCC methods, while CD81 expression varies among methods; except for M3_ExoCC and M5_ExoCC. No expression of HSP–70 was observed via any method

Similarly, CC extracts from M1_ExoCC proved to be the most stable, with less fold reduction over time (statistically not significant) as compared to free CC as well as the other encapsulated exosomes (Figure 5).

Protein analysis was conducted to ensure which of the methods recovered more intact exosomes. M2_ExoCC recovered the highest concentration of exosomes (639.036 µg/ml), while M5_ExoCC and M3_ExoCC recovered the lowest exosome concentrations (111.137 µg/ml and 125.029 µg/ml), respectively (Table 1).

Table 1 Protein analysis of exosomes infused CC extract

Samples	Optical density	Protein concentration (µg/ml)
CC Extract only	0.000	0.000
Exo_CON	0.283	513.403
M1_ExoCC	0.285	517.027
M2_ExoCC	0.353	639.036
M3_ExoCC	0.069	125.029
M4_ExoCC	0.144	260.930
M5_ExoCC	0.061	111.137

CC=*Cosmos Caudatus*, µg=microgram, ml=milliliter, M1_ExoCC=Method 1_Exosomes Cosmos Caudatus, M2_ExoCC=Method 2_Exosomes Cosmos Caudatus, M3_ExoCC=Method 3_Exosomes Cosmos Caudatus, M4_ExoCC=Method 3_Exosomes Cosmos Caudatus, M5_ExoCC=Method 5_Exosomes Cosmos Caudatus

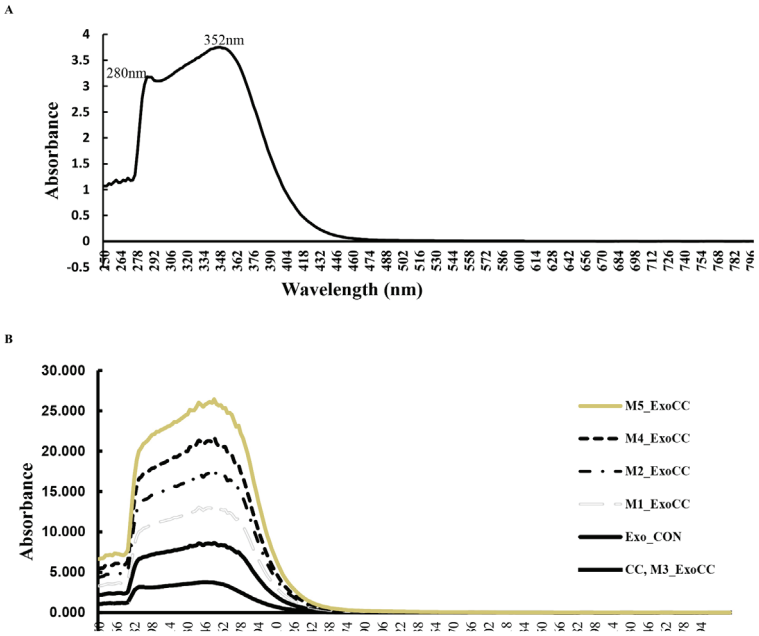
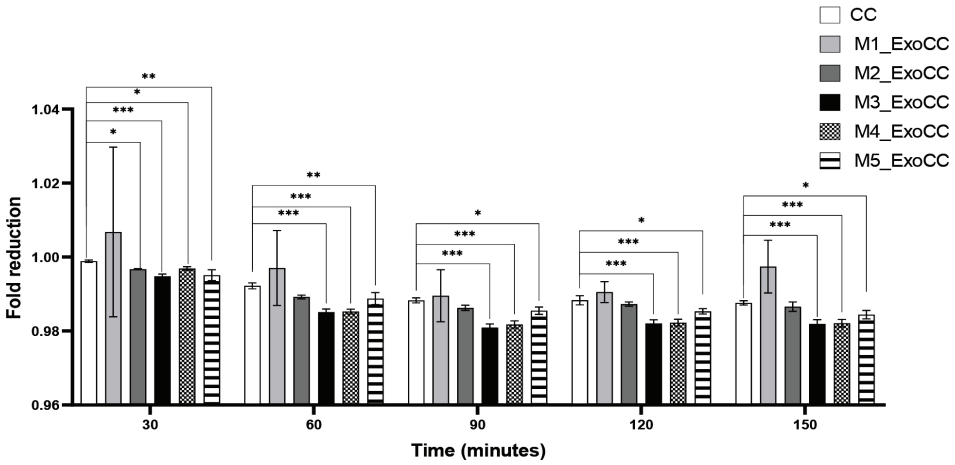


Figure 4 Solubility Analysis of CC Extract and Different Methods of CC Encapsulation. (A) Graph depicting the solubility of CC extract. (B) Comparative solubility analysis of various methods employed for CC encapsulation. The encapsulation of CC extract enhanced the solubility of CC as compared to free CC. Each method's solubility is visually represented, providing a comparative assessment of their efficacy in facilitating CC encapsulation. The highest solubility obtained was generated by freeze-thaw encapsulation (M5_ExoCC and M4_ExoCC). Direct encapsulation of CC (M2_ExoCC and M1_ExoCC) generated moderate solubility



Statistical significance levels are indicated: *significant at p-value<0.033, **significant at p-value<0.002, ***significant at p-value<0.001. CC-encapsulated exosomes isolated from M1_ExoCC enhanced the stability of CC when compared to free CC (statistically not significant). In addition, it showed a slight decrease in stability between 30 to 120 minutes and remained the most stable method at 120 minutes as compared to other approaches

Figure 5 Comparison of stability methods at different incubation time points

Discussion

Based on their flotation density, the sucrose gradient effectively isolates and purifies exosomes from unwanted protein aggregates and apoptotic bodies[13]. Sun et al. applied the SG method to exo-curcumin and observed the development of a layer of exo-curcumin within 30% to 45% gradients[11]. When exosomes incubated with CC were subjected to SG, a clearly defined ExoCC pellet was specifically found at the 80% gradient (Figures 1, 2)

Characterization of isolated exosomes showed intact, round to oval-shaped exosomes, ranging from 50 to 100 nm[14]. Tumor-derived exosomes are reported to express transmembrane proteins, namely CD81 and CD63[15]. On the other hand, HSP70 is not specifically expressed by exosomes; it is expressed by heat-stressed exosomes (Figure 3)[16].

Formation of an encapsulated layer within exosomes occurs through membrane fusion or passive diffusion, allowing for the loading of compounds into the vesicles. While the specific mechanisms underlying compound loading into exosomes are still being elucidated, it is believed that hydrophobic and hydrophilic molecules can be encapsulated within the lipid bilayer or the lumen of exosomes; depending on their physicochemical properties[11].

Direct incubation of CC extract with parent cells (M1_ExoCC) allows the internalization of CC extract into the cells. The encapsulation of drugs into exosomes occurs during their biogenesis and stimulates the production of exosomes by cells[17]. Also, toxic drugs stimulate the production and secretion of exosomes by parent cells. However, the hypersecretion of exosomes is temporary due to the apoptosis effect induced by the toxic drugs on the cells[17]. We ensured the concentration of CC used was not toxic or high enough and did not induce the effect of cellular stimulation to release exosomes, hence the low exosomal yield.

Direct incubation utilizing pre-isolated exosomes allows the spontaneous passive diffusion of cargoes to occur due to the hydrophobic nature and lipid-enriched exosome membrane without the need to apply external force to infuse[18]. This approach provides better control over harvesting and the process of drug delivery[19]. However, increasing the suspension of desired cargo to be loaded can form large particles, which might damage the exosomes, resulting in a lower encapsulation and yield[20]. Cargo loading into exosomes generally favors small-sized particles[21].

The application of freeze-thaw cycles allows the encapsulation of particles by disrupting the exosomal membrane. However, this disruption can destroy, aggrege, or affect the membranal integrity resulting in lower exosomal yield[9,22]. Another drawback of freeze-thawing encapsulation is the formation of non-exosomal or polymeric materials, which increases the absorbance, resulting in high false-positive solubility[23].

Generally, exosome encapsulation enhances the solubility and stability of loaded particles[11,24]. CC extract is a hydrophobic compound with moderate stability (Figures 4 and 5). Nevertheless, successful encapsulation of CC extract enhanced its solubility and stability. In agreement, Sun et al. encapsulated curcumin in exosomes and observed increased solubility and stability[11]. Furthermore, exosomal encapsulation of CC extract can potentially extend its pharmacological activity since exosomal encapsulation of curcumin improves cellular uptake, bioavailability, and anti-inflammatory action of curcumin[25].

Conclusion

In conclusion, this study investigated five distinct approaches for infusing CC leaf extracts into exosomes, with a focus on identifying the most effective methods in terms of exosomal yield, stability, and solubility. It was determined that the M1_ExoCC and M2_ExoCC methods yielded the most favorable outcomes across all parameters. The cost-effectiveness and simplicity of these methods makes them particularly appealing for scaling up production and translating research findings into clinical practice.

Conflict of interest

None exist.

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