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# Chinese herbal medicine-derived extracellular vesicles as novel biotherapeutic tools: present and future

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**Abstract**

Extracellular vesicles (EVs) are phospholipid bilayer-enclosed biological particles that are secreted by almost all living cells including animals, plants, and microorganisms. Chinese herbal medicines (CHM) have a long history of using plant-based remedies to treat and prevent human diseases. Chinese herbal medicine-derived extracellular vesicle (CHMEV) generic term refers to nanoscale membrane structures isolated from medicinal plants such as ginseng, ginger, and Panax notoginseng. In recent years, CHMEVs have garnered substantial attention as a novel class of functional components due to their high bioavailability, safety, easy accessibility, and diverse therapeutic effects, indicating their great potential for development as a new dosage form of CHM. Research on CHMEVs in traditional Chinese medicine (TCM) has become a prominent area of interest, opening new avenues for further exploration into the therapeutic effects and functional mechanisms of CHM. Nonetheless, as an emerging field, there is much unknown about these vesicles, and current research remains inconsistent. The review comprehensively summarizes the biogenesis, isolation methods, and physical, and biochemical characterizations of CHMEVs. Additionally, we highlight their biomedical applications as therapeutic agents and drug delivery carriers, including anti-inflammatory, anticancer, regenerative, and antiaging activities. Finally, we propose current challenges and future perspectives. By summarizing the existing literature, we aim to offer valuable clues and inspiration for future CHMEV research, thereby facilitating research standardization of CHMEVs in the treatment of human diseases and drug discovery.

**Keywords** Chinese herbal medicine-derived extracellular vesicles, Extracellular vesicles, Biological activities, Drug carrier

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## Introduction

The generic term extracellular vesicle (EV) generally refers to phospholipid bilayer-enclosed nanoscale membranous particles that encapsulate active substances including nucleic acids, proteins, lipids, and other small molecule compounds [1]. They can be naturally secreted by almost all types of cells including those of organisms, such as animals, plants, and microorganisms [2]. Based on their biogenesis, size, and biophysical properties, EVs are mainly classified into different subtypesincluding exosomes (30–150 nm) and microvesicles (50–1000 nm) [3]. In addition, autophagosomes and migrasomes also been used to denote EVs that arise during specific cellular processes such as cell migration or programmed cell death. As important mediators of intercellular communication, EVs are involved in surface signal transduction and specific cargo transfer across species, playing important regulatory roles in different pathophysiological processes, such as cell differentiation, tumor angiogenesis, and immune response [4]. The lipid bilayer structure of EVs enables the delivery of hydrophilic and hydrophobic drugs across the blood-brain barrier. As early as 2011, Wood et al. were the first to intravenously inject EVs loaded with siRNA into mice, and the EVs successfully delivered siRNA specifically to the mouse brain [5].

In recent decades, EVs, especially mammalian-derived EVs (MEVs) have been widely studied in different scientific fields, such as drug carriers, targeted therapy, disease diagnosis, and treatment. Nevertheless, further clinical development is hampered by the complex extraction process, potential side effects, limited yield, and high cost. In recent years, plant-derived EVs (PDEVs) isolated from edible plants (e.g., vegetables and fruits) have garnered extensive research interest not only for their pharmacological effects but also for lower immune risks, high biocompatibility, and lower production costs [6]. The first discovery of PDEVs dates back to 1967 when Halperin et al. used transmission electron microscopy (TEM) to observe multivesicular bodies (MVBs) in cultured carrot cells [7]. In 2009, Regente et al. detected exosome-like vesicles with apparent phospholipid membranes in the extracellular fluids of sunflower seeds and highlighted their central role in intercellular signal transfer [8]. Since then, EVs mostly derived from fruits, and vegetables have attracted much attention from scientists and have been comprehensively explored.

For thousands of years, CHM has been using medicinal plant-based remedies to treat and prevent human diseases [9]. Evidence indicates that CHM is rich in active constituents such as flavonoids, alkaloids, polysaccharides, and saponins, which play a crucial role in the treatment of various diseases and have been extensively researched. However, the complexities of CHM therapy involving multi-components, multi-targets, and multi-pathways have posed a great challenge to the effective elucidation of the active compounds and underlying mechanisms of CHM [10]. As the role of PDEVs in cross-kingdom communication between plants and animals or microbes has been revealed, research on EVs as a new class of functional components present in CHM has gradually increased. According to a consensus statement on CHMEVs (2023 version) [11], CHMEVs pertain to EVs derived exclusively from herbal medicine extracellular fluid, and EV-like particles derived from herbal medicine sap. In general, CHMEVs generally demonstrate comparable or even broader pharmacological activity compared to their original plants, including anti-inflammatory, anticancer, regenerative, and anti-aging properties. For instance, ginger, a representative material of CHMEVs, has traditional effects on stomach aches, abdominal spasms, and vomiting and is used to treat gastrointestinal disorders [12]. In addition to intestinal diseases, numerous studies have confirmed that ginger-derived EVs (GDEVs) have shown promising therapeutic benefits in inflammatory, infectious diseases, cancer, and other conditions [13]. To date, GDEVs have been considered better therapeutic preparations than ginger extracts due to their excellent targeting effects. Furthermore, CHMEVs have also emerged as an exceptional carrier platform, exhibiting significant potential for efficient and targeted drug delivery.

Although PDEVs have been systematically described in several excellent reviews [14–19], most of them only partially involve EVs derived from medicinal plants and designated CHMEVs as PDEVs, disregarding their inherent pharmacological properties associated with CHM efficacy. In the review, we collectively refer to these EVs derived from medicinal plants as CHMEVs and shift our focus toward the current research status of CHMEVs. By collating and summarizing relevant literature, a comprehensive overview of generation pathways, preparation methods, and general characterization of CHMEVs is provided. Furthermore, emphasis is placed on the great potential of CHMEVs as therapeutic agents for various diseases. Finally, we present our unique view on current challenges and limitations in the field, hoping to provide new ideas and directions for further exploration into CHMEVs, thus giving a new impetus to the clinical application of CHMEVs. To the best of our knowledge, this review represents the first comprehensive overview of the potential benefits of CHMEVs in future clinical applications and their significant role in modulating pathological processes.

## Biogenesis pathways of PDEVs

Compared to that of MEVs, the presence of plant cell walls further complicates intercellular communication modes across plant cells [19]. According to existing

research, the possible pathways for the generation of PDEVs are primarily involved in the MVB pathway, exocyst-positive organelle (EXPO) pathway, vacuolar pathway, and autophagosomes [20], as depicted in Fig. 1. Among them, the MVBs pathway is considered the main route for the formation of PDEVs, sharing a high similarity to the production pathway of MEVs [21]. Specifically, early endosomes (EE) are formed through the inward budding of the plasma membrane, which are able to interact with the trans-Golgi network and undergo maturation into multivesicular endosomes (MVEs) or MVBs [22, 23]. Within MVBs, multiple intraluminal vesicles (ILVs) selectively internalize different components, such as RNAs, nucleic acids, and proteins [24]. Once the plant cell is infected with pathogens, the proliferation and fusion of MVBs with plasma membrane will occur, thereby releasing ILVs and EVs at specific sites of infection to fight against pathogens. The phenomenon has been observed in the leaves of *Arabidopsis thaliana* [25]. As EXPO was observed in *Arabidopsis*, another secretion pattern was also revealed [26]. The EXPO pathway mainly involves the generation of EXPO, a spherical double membrane structure resembling autophagosomes. It is closely related to Exo70E2 and does not colocalize with any known organelle markers, representing a form of unconventional secretion unique to plants [26]. After fusion with plasma membrane, EXPO can secrete single-membrane vesicles into the extracellular environment. Vacuoles are the largest organelle in plant cells. As another source of PDEVs, vacuoles also play essential roles in plant defense against fungal and bacterial pathogens [27]. During infection, MVBs initially undergo fusion with the vacuole and release intraluminal vesicles (ILVs), followed by the exocytosis of these vesicles to the extracellular environment through the fusion of the vacuoles with plasma membrane. These vesicles rupture and release defensive agents to suppress the proliferation of pathogens. Recently, Cui et al. utilized 3D electron tomography to discover that central vacuoles originated from MVB-to-small vacuoles transition and subsequent fusions of MVB-to-small vacuoles in *Arabidopsis* root cells [28]. The research uncovered a potential connection between vacuole-mediated secretion and MVB pathways. In addition, autophagosomes are typically formed and released into the extracellular space during apoptotic induced by programmed cell death. In comparison to exosomes and microvesicles, autophagosomes tend to contain complete organelles, chromatin, glycosylated proteins, etc., and possess larger diameters ranging from 50 nm to 5000 nm [29]. The autophagosome-mediated secretory pathway occurs in yeast cells [30]. Extensive literature has suggested the important

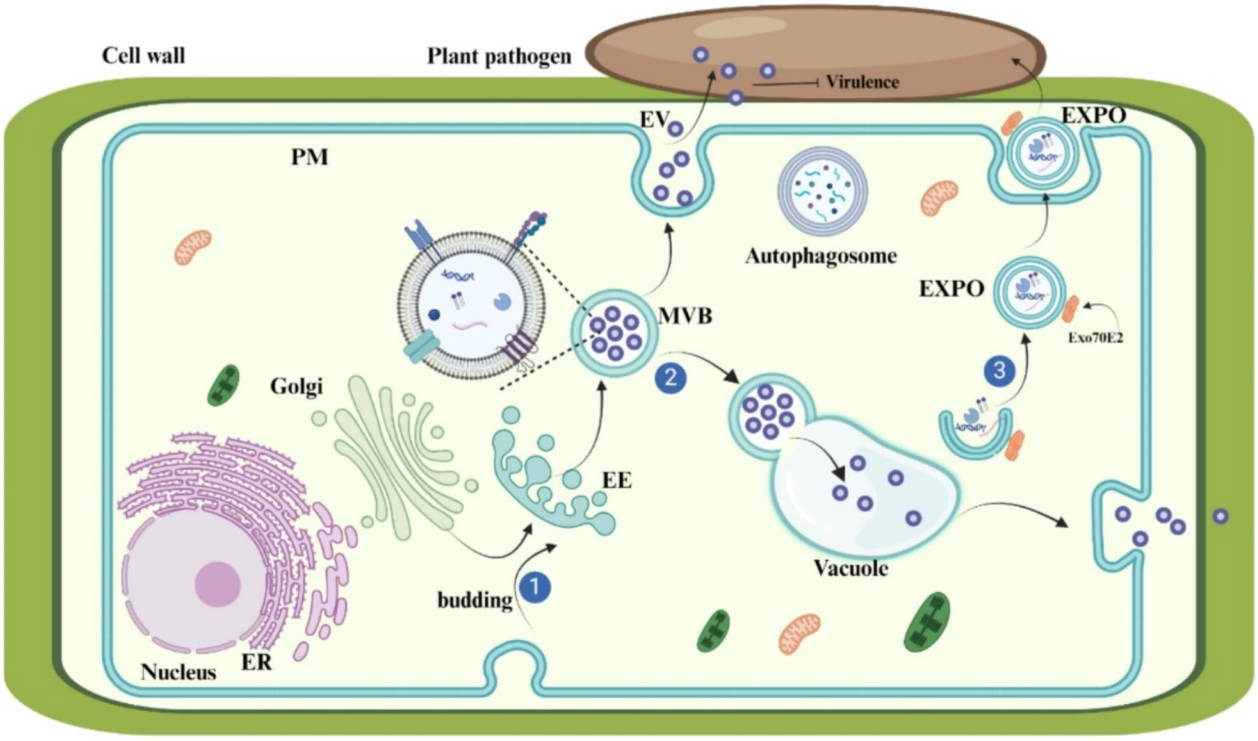


**Fig. 1** Biogenesis pathways of PDEVs. (1) MVBs are secreted and fusion with PM to fight against pathogen infection; (2) EXPO pathway; (3) Vacuole fuses with MVBs to release EVs. Abbreviations: ER, endoplasmic reticulum; EE, early endosomes; MVB, multivesicular body; EXPO, exocyst positive organelles; PM, plasma membrane; EV, extracellular vesicle

role of PDEV secretion in plant defense and intercellular and interspecies communication [31–33]. The biogenesis mechanisms underlying the biogenesis of CHMEVs have not been well-established. In the upcoming years, there should be a focus on elucidating formation pathways, which will facilitate improved quality control and clinical applications of EVs as therapeutic agents or drug carriers.

## Preparation of CHMEVs

Medicinal plants are widely distributed and diverse in species. Unlike edible plants such as fruits and vegetables, Chinese herbs encompass roots, stems, leaves, fruits, seeds, and dried ground components. The distinct parts have good therapeutic effects on their own and can be used in medicine. Therefore, due to the similarity of EVs to source cells, CHMs are an ideal source material for preparing EVs. For specific medicinal parts, it is necessary to select appropriate and effective isolation and purification protocols for stable and high-purity CHMEVs. The primary preparation methods, including the pretreatment of medicinal materials and the separation of CHMEVs as described in this section, are summarized in Fig. 2.

### Pretreatment of raw materials

At present, fresh herbs are the most commonly used raw material in the preparation of CHMEVs. Prior to extracting CHMEVs, specific pretreatment of different parts of different herbs, such as cleaning, crushing, and juicing, is required. Juicing is a primary pretreatment method to preprocess the majority of fresh herbs, exemplified by the harvest of fresh Rehmanniae Radix-derived EVs [34] and GDEVs [35]. It is worth mentioning that direct juicing may lead to the formation of flocculent fiber complexes for herbs that are rich in juice but have more fiber. The complex is difficult to remove during the centrifugation/filtration process, making squeezing a more feasible option for juice collection, such as dandelion [36]. Herbs with little juice or their dry forms, such as ginseng [37], Pueraria lobata [38], are hard and thus need to be pretreated by a combination of grinding and juicing. Before juicing, an appropriate amount of phosphate-buffered saline (PBS) should be added to increase the yield of juice such as yam [39]. Researchers have attempted to utilize cellulase and pectinase enzymes for the degradation of plant cells to extract EVs from the roots of Morinda officinalis (MO). One study validated that enzyme degradation is more efficient than grinding [40]. Despite its simple operation, high efficiency, and large yield, the tissue-disruption method can inevitably damage cells, thereby introducing contaminants into cells.

A novel pretreatment method was proposed to collect apoplastic washing fluid from plant tissues by infiltration centrifugation [41]. Liu et al. [42] conducted a comparative analysis of infiltration centrifugation and wall breakage and found that EVs obtained from apoplastic washing fluid had smaller particle sizes, lower potential, and density. PDEVs obtained from apoplastic washing fluid are much closer to meeting the definition of plant EVs. Compared to juicing, infiltration centrifugation is better able to maintain cell integrity and thus enables the harvest of higher-purity EVs [43]. However, this method is limited to plant leaves as raw materials; there are no related reports of infiltration centrifugation being conducted with other plant tissues. apoplastic washing fluid separation via infiltration centrifugation can be used as a reference method for preparing EVs from leaves of herbs in future research.

Decoctosomes, a new kind of heat-stable exosome-like membrane structure, are extracted from Chinese herbal decoction [44]. The pretreatment procedures of dandelion and *Aucklandia lapp* decoctions offer important reference significance for the preparation of CHMEVs from Chinese herbal decoction [45]. In general, researchers typically prioritize juicing for more starting materials to work with in experiments [15].

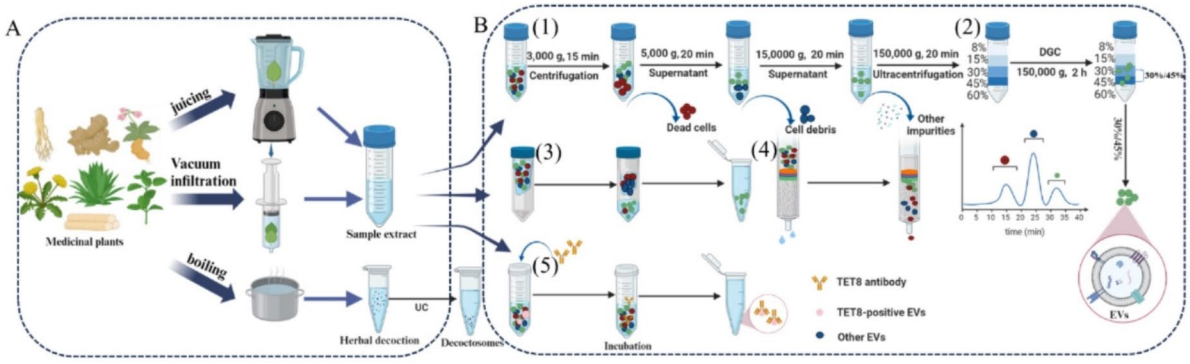


**Fig. 2** Extraction and purification of CHMEVs. **(A)** Pretreatment of raw materials; **(B)** Isolation and purification of CHMEVs. (1) differential centrifugation (UC), (2) sucrose gradient centrifugation (DGC), (3) ultrafiltration (4) size exclusion chromatography, and (5) immunoaffinity isolation

### Separation and purification of CHMEVs

After obtaining the heterogeneous EV mixture by the aforementioned crude treatment, further extraction and purification are required for different subtypes of vesicles. There are five common approaches available for the isolation and purification of CHMEVs. They are divided into five major categories on the basis of their separation principles, namely, ultracentrifugation, ultrafiltration, immunoaffinity capture method, size exclusion chromatography (SEC), and polymer precipitation methods [18], but there is not yet a standardized protocol for isolation and purification. Table 1 provides a summary of the principles, advantages, and disadvantages of these techniques. Currently, there is no comprehensive approach that simultaneously considers both recovery and specificity. Figure 2 describes the corresponding separation process in detail.

#### *Ultracentrifugation*

Differential centrifugation (DC) stands as the most popular and the earliest ultracentrifugation technique due to its easy operation and abundant yield [46]. The separation mechanism mainly involves the fact that particles with different sizes have different sedimentation coefficients under the action of centrifugal force. Initially, plant fibers and larger cellular debris in the sample solution are removed by successive low-speed centrifugation (<10,000 g). As the speed and duration of the centrifugation gradually increase, small-sized EVs are precipitated and accumulated due to the high centrifugal force (>100,000 g) [47]. Moreover, it is worth noting that long-term and multiple ultracentrifugation may damage the morphology and structure of EVs, resulting in elevated soluble protein contamination [29]. To address the issue, a sucrose cushion is added to the bottom of the centrifuge tube as a buffer layer [48]. The high centrifugal force may also lead to coprecipitation of protein or protein/RNA aggregates. To further distinguish different subtypes of EVs and protein aggregates with similar size, a more specific method, density gradient centrifugation (DGC) is usually combined with DC. A density gradient consisting of different sucrose solutions (8%, 15%, 30%, 45%, and 60%) is usually constructed after DC [49, 50]. EVs are separated according to their density and size and mainly accumulated in the 30%/45% layer of sucrose solution, as shown in Fig. 2B [51]. Compared with DC, DGC offers the advantage of high-purity separation of EVs [52]. Apart from sucrose, iodophor is also a widely used gradient matrice in the study of PDEVs and has already been commercialized [41, 53]. However, given the potential impact of density gradient media on EV composition, coupled with their inherent time-consuming nature, it may not be an ideal choice for large-scale production of PDEVs [19, 54].

#### *Ultrafiltration*

Ultrafiltration is usually utilized to filter herbal juice under fluid pressure through ultrafine nanofiltration membranes [11], including two filtration modes, ultrafiltration centrifugation and tangential flow filtration (TFF). Similar to traditional membrane filtration, the separation method can further purify PDEVs based on particle size or molecular weights. Small molecule particles will pass through the filter, while large molecular weight particles will be retained by the membrane [55]. The use of TFF mode partially prevents the formation of a constrictive layer, also known as "filter cake", thereby facilitating the smooth flow of sample solution through membrane pores [14, 56]. EVs separated by ultrafiltration possess higher yield and superior biological activity compared with those obtained via ultracentrifugation [57]. The advantages of TFF in separation speed and efficiency make it more appropriate for large-scale sample separation [58]. However, it should be noted that some particle impurities, such as protein aggregates and other vesicles, are difficult to separate by relying solely on the micropores

**Table 1** Principles, advantages, and disadvantages of different extraction techniques for CHMEVs

| Techniques | Principles | Advantages | Disadvantages | Literature |
|---|---|---|---|---|
| Differential centrifugation | Sedimentation coefficients | High quantities, low cost, simple to operate, and suitable for large-scale sample processing | Low purity, possible damage to vesicle structures | [145, 146] |
| Density gradient centrifugation | Density and particle size | Higher purity and recovery | Time-consuming, lower recovery, strict operation | [46] |
| Ultrafiltration | Particle size and molecular weight | Higher separation efficiency, operational simplicity | Possible clogging | [55] |
| Size exclusion chromatography | Particle size | Higher purity, simple conditions, expanded production, and low cost | Difficulty in large-scale production | [147] |
| Immunoaffinity isolation | Antigen-antibody specific binding | High specificity and efficiency | High cost, the dearth of protein markers and specific commercial antibodies | [21] |
| Polymer Coprecipitation | Solubility reduction | Lower equipment requirements and cost, high output | Possible coprecipitation of soluble proteins and other impurities | [66] |

of the ultrafiltration membrane [59]. Therefore, ultrafiltration is typically integrated with other techniques to enhance EV purity. For example, a recent study suggested that cabbage EVs isolated by a combination of size-exclusion chromatography and ultrafiltration exhibited a more uniform peak than EVs isolated solely by ultracentrifugation or polyethylene glycol-based precipitation [60].

#### *Size exclusion chromatography (SEC)*

SEC is another method for separating molecules or particles based on their differences in particle size. When herbal solution passes through a chromatography column filled with porous resin stationary phase, EVs will exhibit different retention times owing to their varying size [52, 61]. Therefore, under the action of the elution buffer PBS, soluble contaminants, such as free proteins and small molecule substances, are temporarily sequestered within the column matrix, whereas larger EVs swiftly pass through and emerge in the initial fractions [19]. At present, commonly utilized polymers encompass glucose polymers (Sephadex), agarose (Sepharose), and polyacrylamide (Sephacryl or BioGel) [62]. In contrast to DC and ultrafiltration, SEC is often more effective in maintaining the biological activity and integrity of EVs. This could be attributed to the absence of external forces acting on the sample solution [63]. As an additional method, SEC can be integrated with other separation methods such as DC, ultrafiltration, and polyethylene glycol (PEG) precipitation. Researchers have employed ultrafiltration combined with SEC to isolate EVs from cabbage, and the results indicated this combination method provided higher purity without losing yield compared to ultracentrifugation and PEG-based precipitation methods. Moreover, higher-purity EVs have been isolated from cucumbers, peppers, and tomatoes using SEC-ultrafiltration [55, 60]. Thus, SEC is considered a relatively standard separation strategy due to its high purity, simplicity, and minimal damage to the structures and functions of CHMEVs.

#### *Immunoaffinity isolation*

Immunoaffinity isolation represents the most precise and advanced technique for obtaining specific subgroups of EVs [64]. The technique can further isolate EVs utilizing specific antibodies to capture corresponding protein markers present on the EV surface such as tetraspanin proteins, CD63, CD9, and CD81, and prevent cytoplasmic proteins or RNA from contaminating EVs (Fig. 2B) [65]. The high specificity and rapid separation rate of immunoaffinity isolation render it an optimal method for purifying EVs [21]. Recently, He et al. successfully obtained TET8-positive EVs from *Arabidopsis* using magnetic beads coated with antibodies that could specifically recognize the EC2 domain of TET8 [3]. However, antibodies or beads may interfere with downstream communication between EVs and target cells. The application of immunoaffinity isolation is constrained by the lack of specialized antibodies that can identify PDEV surface proteins [15]. In the CHM field, CHMEV surface protein markers are undergoing extensive exploration and commercial antibodies targeting PDEVs are needed to be designed. Nonetheless, immunoaffinity isolation provides a meaningful reference for establishing specific methodologies for the purification and production of CHMEV.

#### *Polymer coprecipitation*

Coprecipitation is an efficient separation method that boasts simplicity in operation and significant profitability. The method typically uses PEG as a precipitation agent [66]. The highly hydrophilic PEG interacts with the water molecules surrounding vesicles, thereby creating a hydrophobic microenvironment within which EVs are precipitated due to the reduction of solubility. Researchers have employed PEG 6000 to successfully purify EVs derived from ginger which was comparable to that achieved by DC in terms of biochemical composition and biological activities [67]. Nevertheless, it is worth noting that the purity is relatively low owing to possible coprecipitation of soluble proteins and other impurities [68]. Recently, extraction kits based on polymer coprecipitation such as ExoEasy Midi and ExoQuick systems have been developed and implemented in the laboratory. For instance, EVs from ginseng and ginger were successfully extracted via an exosome isolation kit combined with low-speed UC, which greatly improved their separation purity [69, 70]. Polymer coprecipitation using PEG represents a promising approach for large-scale isolation of CHMEVs.

At present, the techniques available for the isolation and purification of CHMEVs are notably restricted, and most of them are based on MEVs. Given the advantages and disadvantages of each separation method, most laboratories use a combination of multiple methods. Appropriate and standardized extraction methodologies play a crucial role in subsequent investigations into the structure, functions, and biological activities. Hence, more basic experiments are needed to conduct standardized and systematic isolation for CHMEVs.

## Characterization of CHMEVs

CHMEVs derived from different medicinal plants display variations. Thus, physical and biochemical characterization is crucial for quality control. A comprehensive analysis not only helps distinguish CHMEVs from other impurities but also confirms their effective separation and purification. Additionally, it provides valuable insights into their biological activities and potential future clinical applications. The detailed guidelines for

the characterization of EVs are described in the Minimal Information for Studies of Extracellular Vesicles (MISEV) inventory released in 2024 [1]. The physical and compositional characterization of CHMEV is illustrated in Fig. 3.

## Physical characterization

### *Morphological features*

Electron microscopy (EM) is extensively used to capture the morphology and ultrastructure of CHMEVs in high resolution. Among these, TEM and scanning electron microscopy (SEM) are two of the most commonly adopted technologies due to their low cost and ease of operation. Cumulative EM studies have indicated that CHMEVs usually exhibit typical saucer-shaped and nearly spherical morphology. However, complex sample pretreatment such as dehydration, fixation, and staining, potentially impacts the structural integrity of EVs [71]. Without any pretreatment, cryo-EM is capable of capturing the authentic subcellular status of EVs at very low temperatures [72]. Liu et al. used TEM and Cyro-EM to observe a nearly spherical morphology in EVs from *Arabidopsis* leaves [42]. In addition to EM-based methods, atomic force microscopy (AFM), as a useful tool with nanoscale resolution, has also been applied by several laboratories for imaging three-dimensional structures of CHMEVs. As an illustration, TEM and AFM were used to characterize the structure of GDEVs and yielded consistent data [73]. Notably, Cyro-EM and AFM are less commonly used than TEM and SEM in the EV field because they are more expensive and more research interests have focused on the overall morphological structure of EVs rather than detailed surface information such as their stiffness and adhesion properties.

### *Size distribution, zeta potential and number*

The size distribution, concentration, and zeta potential of CHMEVs are often determined using dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) [16]. DLS can obtain the distribution curve of the average particle size according to the fluctuation of light scattering in EV samples [74]. Apart from DLS, NTA remains the most widely utilized technique for concurrently determining the quantity of EVs and the size distribution of individual particles. The size distribution and zeta potential values may vary depending on the isolation methods and types of medicinal plants. Despite this, evidence implies that the average diameter of CHMEVs generally falls within the range of 30–400 nm and the zeta potential mostly tends to be negative values [11], suggesting that CHMEVs can exist independently without undergoing aggregation [50]. The separation methods and general characterization of CHMEVs are summarized in Table 2. Currently, several emerging detection technologies such as nano-flow cytometry (nFCM),

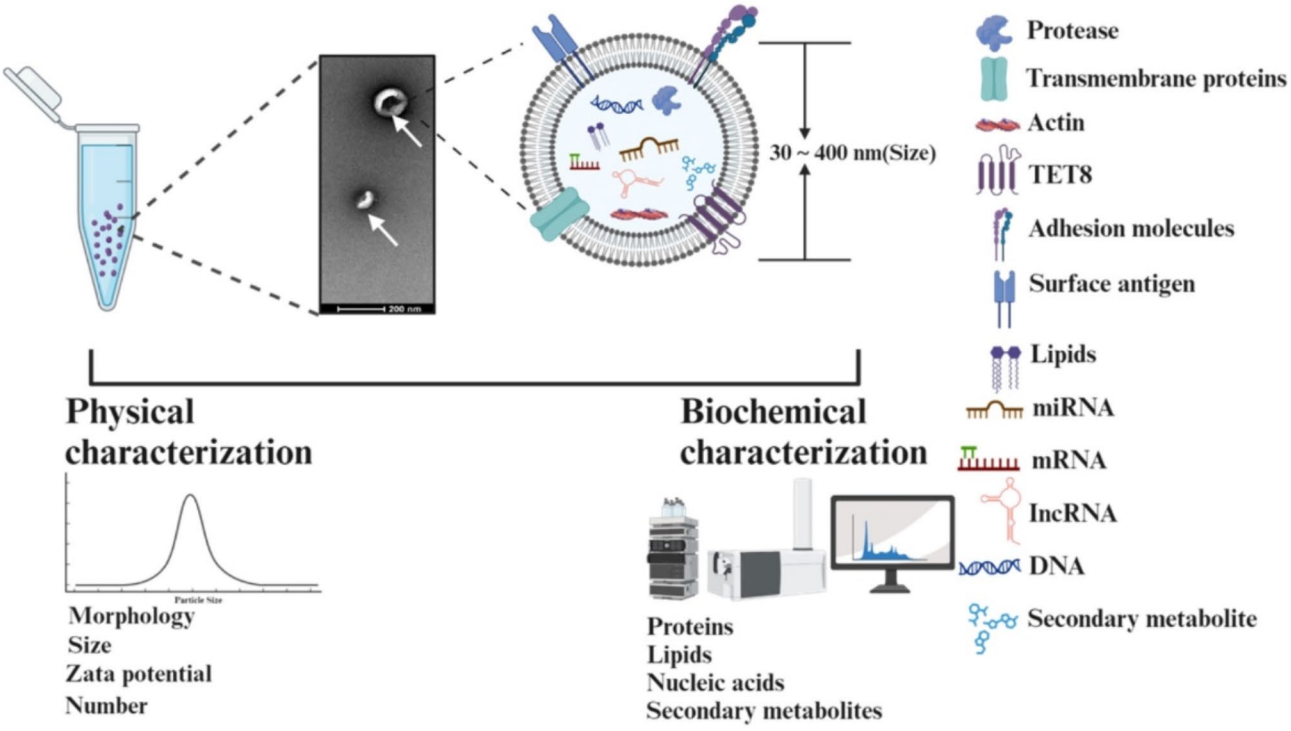


**Fig. 3** Physical and biochemical characterization of CHMEVs

**Table 2** Preparation, general characterization, and therapeutic applications of CHMEVs

| CHMEV Source | Medicine part | Separation and purification method | Morphology | Particle size (nm) | Potential (mV) | Therapeutic applications | Literature |
|---|---|---|---|---|---|---|---|
| Rhizoma Drynariae | Fresh root | DC | Cup-shaped | 75.7 ± 15.8 | −43.2 ± 0.04 | Osteoporosis | [99] |
| *Morinda Officinalis* | Fresh root | DC | Round- or cup-shaped | 61.24 ± 12.74 | \ | Osteoporosis | [76] |
| Yam | Fresh rhizomes | DC | Round-shaped | 168 and 328 | \ | Osteoporosis | [39] |
| Pueraria lobata | Fresh root | DC and ultrafiltration | Round-shaped | 119 to 163 | \ | Osteoporosis | [132] |
| Pueraria lobata | Fresh leaves and stems | DC and ultrafiltration | Round-shaped | 40 to 150 | \ | Macrophages | [117] |
| *Rehmanniae Radix* | Fresh root | DC and DGC | Irregular | Approximately 118 | \ | Acute lung injury | [34] |
| Brucea javanica | Fruits | DC | Cup-shaped | 104.6 ± 29.4 | 9.2 ± 0.1 | Triple-negative breast cancer | [124] |
| Shiitake mushroom | the whole | DC | Sphere-shaped | 120 ± 20 | \ | Acute lung injury | [115] |
| Dandelion | Fresh whole grass | DC and ultrafiltration | Sphere-shaped | Approximately 187 | −6.26 | Wounds | [36] |
| Asparagus cochinchinensis | Fresh root | DC and DGC | Cup-shaped | Approximately 190 | −21 | Hepatocellular carcinoma cells | [49] |
| Houttuynia cordata | Fresh roots, stems and leaves | DC | Round-shaped | 169.5 (root)<br>166.2 (stems and leaves) | −23.04 ± 1.22 (root)<br>−28.73 ± 0.87 (stems and leaves) | Respiratory RNA viruses | [97] |
| Mulberry bark | The tissues outside the vascular cambium | DC and DGC | Cup-shaped | 151.3 ± 45.4 | \ | Colitis | [116] |
| Portulaca oleracea L. | Fresh overground part | DC and DGC | Round-shaped | 180 | −31.4 | Colitis | [148] |
| Panax notoginseng | Fresh root | DC and DGC | Sphere-shaped | Approximately 151.3 | −8 | Ischemia-reperfusion injury | [89] |
| Turmeric | Fresh Rhizomes | DC and DGC | Sphere-shaped | Less than 800 | −15.0 | Colitis | [113] |
| Turmeric | Fresh Rhizomes | DC and DGC | Saucer-shaped or hemispherical with a concave side | 178 | − 21.7 | Colitis | [112] |
| Ginseng | Fresh root | DC and DGC | Sphere-shaped | Less than 344.8 | − 25.4 | Melanoma | [37] |
| Ginseng | Fresh root | DC and DGC | Sphere-shaped | 92.04 ± 4.85 | \ | Senescence | [136] |
| Aster yomena Callus | Fresh root | TFF and cushioned DC | Round-shaped | Approximately 225.2 | \ | Allergic Asthma | [149] |
| Ginger | Fresh root | DC and DGC | Round-shaped | 220–290 | −12 | Colitis | [35] |
| Ginger | Fresh root | DC and DGC | Saucer-shaped | 250 ± 72 | −220 ± 131 | HFD-induced insulin resistance | [150] |
| Ginger | Fresh root | DC and DGC | Sphere-shaped | 386.6 (average) | −24.6 to 29.7 | Alcohol-induced liver damage | [92] |
| Aloe saponaria | Peels | Polyethylene glycol (PEG) and DC | Sphere-shaped | less than 200 | \ | Wound | [128] |
| *Aloe vera* | Fresh leaves | DC and ultrafiltration | Round-shaped | 40–200 | \ | Inflammation | [151] |
| *Aloe vera* | The gel and rind | DC | Saucer-shaped | 138.7 (gel) 220 (rind) | −7.4 (gel) and − 8.9 (rind) | Melanoma | [80] |
| *Dendropanax morbifera* | Fresh leaves and stems | DC and ultrafiltration | Round-shaped | Less than 90 | \ | Melanoma | [135] |
| Garlic | Fresh bulb | DC and DGC | Round-shaped | 200 | \ | Obesity | [152] |
| T-QY305 formula | Herbal decoction | DC | Sphere-shaped | 240.2 ± 6.4 | −7.68 ± 0.8 | Adverse reaction and diarrhea | [144] |
| Rhodiola crenulata | Herbal decoction | DC | Round-shaped | 197.6 | −37 ± 2 | Pulmonary fibrotic | [45] |

Abbreviations: DC, differential centrifugation; DGC, density gradient centrifugation; PEG, Polyethylene glycol; TFF, tangential flow filtration

tunable resistive pulse sensing and Particle Metrix Zeta-View have gained increasing popularity in the MEV multiparameter measurement [46, 75]. They are promising for CHMEV characterization of the size, zeta potential and quantities. In a recent study, nFCM, a high-resolution single-particle platform, has been used to analyze the particle size and quantify the number of EVs from the MO [76].

The purity of CHMEV refers to the number of particles per milligram protein (particles/mg). The Triton X−100 membrane rupture test and bicinchoninic acid (BCA) assay, combined with NTA and nFCM, are often employed to directly or indirectly indicate purity. Additionally, size exclusion combined with high-performance liquid chromatography (HPLC) has been proposed by researchers to mitigate the influence of nucleic acid and protein impurities [11].

### Biochemical characterization

CHMEVs encapsulate numerous biochemical molecules that can influence information transfer and material exchange between cells [50, 77]. Like that of MEVs, the membrane of CHMEVs consists of a phospholipid bilayer. In addition, an increasing number of publications have indicated that CHMEVs contain diverse proteins, lipids, nucleic acids, and other small molecule secondary metabolites that are closely related to their physiological activities. A comprehensive compositional analysis is essential to ensure the quality of CHMEV. The primary technique employed for the biochemical composition analysis of MEVs also applies to CHMEVs. For protein analysis, methods such as BCA, enzyme-linked immunosorbent assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorometric assays can be conducted in CHMEVs. Furthermore, microarray analysis, digital droplet polymerase chain reaction, and next-generation sequencing techniques have been established for the RNA content analysis of CHMEVs [78]. Sulfophosphovanilin assays and total reflection Fourier-transform infrared spectroscopy are also used for lipidomic analysis. In addition to lipidomics, it is also advisable to conduct proteomics and nucleomics analyses to gain a comprehensive understanding of the compositions of CHMEVs. It is noteworthy to mention that ingredients found in CHMEVs have also been detected and quantified using HPLC or LC–tandem mass spectrometry.

In general, the chemical composition profiles of CHMEVs overlap with those previously characterized for MEVs. However, in terms of protein, lipid, and RNA content, CHMEVs vary greatly from MEVs. In this section, we will discuss the composition of CHMEVs concerning proteins, lipids, nucleic acids, and other metabolites.

#### *Proteins*

At present, a limited number of studies have identified various proteins involved in PDEVs; however, these investigations exhibit inconsistencies and lack robust evidence for specific PDEV protein markers. The existing literature indicates that PDEV proteins are commonly composed of cytoplasmic proteins (e.g. actin and proteases) and transmembrane proteins that function as membrane channels and transporters and typically exhibit lower abundance in comparison to MEVs [79]. For example, it has been reported that GDEVs possess lower concentrations of proteins including cytoplasmic proteins such as actin and proteolytic enzymes and membrane channel proteins (including aquaporins and chloride channels) [35]. Moreover, integrins and other adhesion proteins are known to be abundant in MEVs. From scarce sources of proteomic data, some researchers have revealed that three common protein families including heat shock protein 70 (HSP70), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and S-adenosine homocysteine enzymes are common in various PDEVs (e.g., grapefruit, olive, tobacco, and sunflower) [20, 21]. These proteins were also detected in CHMEVs such as aloe-derived EVs and GDEVs [80]. Cao et al. revealed a total of 3,129 proteins in ginseng-derived nanoparticles through mass spectrometry, which were subsequently categorized into three distinct groups according to Gene Ontology: biological processes, cellular compartments, and molecular functions [37]. Raimondo et al. found that approximately 56.7% of proteins from lemon-derived EVs overlapped with mammalian exosomes, regardless of cell origin [81]. Some surface marker proteins found in most EVs can reflect their specificity and work as recognition targets for the separation and purification of EVs. For MEVs, these transmembrane proteins including CD81, CD9, and CD63, etc. are identified as mammalian cell-derived EV markers and are commonly measured [82]. In PDEVs, it was reported that TET8 and peneration1 (PEN1) have been discovered to express in EVs from the model plant *Arabidopsis* [41, 83]. Subsequently, Huang et al. utilized an immunoaffinity method to isolate TET8-positive EVs from the apoplast fluids of the same plant [84]. Although PEN1 and TET8 have been proposed as protein biomarker candidates, a consensus has yet to be reached [41, 85]. The specific protein markers of CHMEVs have not been well-founded and more basic experiments are required for further exploration. Considering the limited number of studies on PDEV and CHMEV proteins, it is very challenging to establish a complete protein database.

#### *Lipids*

Similar to MEVs, lipids are the predominant compounds that compose lipid bilayer membrane structures of CHMEVs and protect cargoes from degradation [86]. In

contrast to MEVs, CHMEVs exhibit a high abundance of lipid characteristics primarily encompassing phosphatidic acid (PA), phosphatidylcholines (PCs), phosphatidylethanolamine (PE), digalactosyldiacylglycerol, monogalactosyldiacylglycerol and more [87]. The proportions and specific compositions of lipids depend on the medicinal plant source. For example, the lipidomic analysis suggested that EVs derived from ginseng exhibited a high content of PC (28. 8%), followed by triglycerides (16. 8%), and ceramides (12. 9%) [88], while glucose ceramide (43.55%) and PA (18.12%) were the major lipid components of aloe gel-derived EVs [80]. The distinctive ratio and lipid composition have a crucial impact on membrane stability, targeting site, and therapeutic effects. As an example, lipids, as one of the active constituents of Panax notoginseng-derived EVs, have been shown to assist in alleviating cerebral ischemia/reperfusion (CI/R) injury by altering microglial polarization [89]. Moreover, PA present in GDEVs directly interacts with HBP35 protein in *Porphyromonas gingivalis* to suppress the growth of *P. gingivalis*, thus effectively treating periodontitis [90]. In addition, lipids play a pivotal role in the targeted uptake and in vivo distribution of CHMEVs. PC is able to facilitate the transport of EVs from the intestine to the liver [77]. In contrast, PA in GDEVs tends to be distributed in the gut and is selectively internalized by intestinal stem cells and intestinal macrophages, indicating a strong intestinal targeting [50, 91]. Further research showed that GDEVs migrated from the intestinal tract to the liver via the vascular system and were subsequently absorbed by hepatocytes and Kupffer cells, exhibiting a significant accumulation in the liver. The regulatory process may be mediated by PA lipids in GDEVs [92].

Importantly, lipids in different kinds of CHMEVs perform different physiological functions, including membrane-mediated signal transmission, therapeutic action, and targeting ability, laying a structural basis for the use of CHMEVs as drug carriers. Currently, our understanding of the role of lipid components in CHMEVs is relatively limited. To promote the application of EVs as drug carriers and therapeutic agents, further studies of the structure and biological function of lipids from diverse medicinal plants re recommended.

#### *Nucleic acids*

Several studies have demonstrated that CHMEVs carry diverse nucleic acids, mainly various RNA molecules such as messenger RNA (mRNA), microRNA (miRNA), and non-coding RNA (lncRNA) [87], while DNA is rarely reported. miRNA serves as single-stranded noncoding RNAs with an average length of less than 30 nucleotides that play a crucial role in the regulation of mRNA translation and gene expression [93]. A recent report suggested that a total of 418 miRNAs from 11 fruit and vegetable samples were shown to be highly expressed and it was predicted to be closely associated with pro-inflammatory and anti-cancer activities. Further in vitro experiments preliminarily revealed that miRNAs in edible plant-derived EVs have the potential to regulate human mRNA [94]. Single or a group of miRNAs-loaded CHMEVs have the potential to mediate cross-kingdom interactions [95]. For example, in Panax notoginseng EVs, 40 different miRNAs possessing 20 and 24 nucleotides were predicted to target and regulate the expression of a total of 4010 human genes by binding to the 3'UTR of complementary genes [89]. Growing evidence suggests the promising therapeutic potential of CHMEV-derived miRNAs as a novel class of natural ingredients in various disease models. Deep sequencing reads of GDEVs identified 125 different miRNAs with 15–27 nucleotides [35]. In another similar study, Wang et al. detected 116 miRNAs in GDEVs; among them, 27 highly enriched miRNAs exhibited potential anti-inflammatory effects through cross-kingdom communication to target intestinal Caco2 cells. Further analysis found that osa-miR164d had the capability to regulate the reprogramming macrophage polarization, thereby inhibiting the intestinal inflammatory response [70, 96]. In Houttuynia cordata-derived EVs, miRNAs have been shown to target genes from respiratory RNA viruses and suppress AKT1 and MAPK3 expression, thus alleviating respiratory infection [97]. Rgl-exomiR-7972 derived from fresh Rehmanniae Radix EVs could alleviate lipopolysaccharide-induced acute lung inflammation by targeting the G protein-coupled receptor 161-mediated Hedgehog pathway and repairing gut microbiota dysbiosis [34]. In addition to CHM, miRNA in edible plant-derived EVs such as those from soybeans and apples has also demonstrated potent biological activities [87, 98].

As mentioned above, the nucleic acid components in CHMEVs mainly miRNA harbor excellent biological activities and play a potential therapeutic role in maintaining human health. In light of their therapeutic potential, it would be a promising research direction to further investigate the functions and mechanisms of miRNA in future studies.

#### *Secondary metabolites*

The secondary metabolites (e.g., flavonoids, saponins, and alkaloids) present in CHM have long been recognized as the material basis responsible for its therapeutic effects and have been systematically explored. In addition to the aforementioned cargoes, emerging evidence has found that CHMEVs encompass signature bioactive metabolites from homologous medicinal plants, which may also play a vital role in plant growth and the treatment of human diseases. For instance, naringin found in Drynariae-derived EVs has demonstrated significant

antiosteoporosis activity and is further identified as an active ingredient [99]. Moreover, Zhang et al. detected higher concentrations of ginger active metabolites, namely, 6-gingerol and 6-shogaol in GDEVs using HPLC-MS, which greatly contributed to their anti-inflammatory activities and prevention of inflammatory bowel diseases [35]. Another similar study also demonstrated that GDEVs are abundant in shogaol and specifically target hepatocytes, thereby ameliorating alcohol-induced liver damage [92]. Monotropein, a well-known active substance in MO, exhibits a significant bone protection effect. The characteristic peak of monotropein was also identified using HPLC in MO-derived EVs [76]. In addition, aloe-derived EVs were also found to contain aloe-emodin, a potent anticancer candidate, as well as aloesin and β-sitosterol [80], indicating that aloe-EVs have potential for therapeutic applications. Electrospray ionization scanning analysis revealed that ginsenoside Rg3, an active therapeutic ingredient in ginseng, was highly enriched in ginseng-derived EVs [37]. Woith et al. concluded that secondary metabolites might not be actively packaged but rather become enriched in the lipophilic membrane when they exhibit sufficient lipophilicity [100]. The effects of different secondary metabolites on the biological activities and mechanisms of action of CHMEVs have remained poorly investigated. Therefore, it is advisable to conduct metabolomics analysis to comprehensively identify these metabolites and investigate their role in human diseases.

In summary, comprehensive physical (morphology, size distribution, zeta potential, and number) and biochemical (proteins, lipids, nucleic acids, and secondary metabolites) descriptions contribute to gaining deep insight into the structure and composition of CHMEVs. Different types of components loaded in CHMEVs hold potential biological functions or may play a synergistic role in diseases. The current literature mainly focuses on morphological and lipid analysis. More experiments need to be performed to further understand the compositions of CHMEVs, especially surface proteins, or to establish a corresponding database for a certain medicinal plant, facilitating the consistency and transparency of CHMEV studies.

## Stability of CHMEVs

The oral route is one of the most preferred approaches for drug delivery due to its convenience, cost-effectiveness, and high patient compliance [101]. The stability of CHMEVs in the gastrointestinal (GI) tract is a crucial prerequisite for oral administration [102]. CHMEVs are emerging as promising candidates for oral nanomedicine, owing to their resistance to the degrading GI conditions. They can be effectively absorbed through the GI system, exhibiting superior stability in this environment. For instance, EVs derived from mulberry leaves remained unchanged in particle sizes and surface charges during the incubation in different simulated solutions, suggesting their stability in the GI [103]. EVs from grapefruit and lemon juice showed the same pattern, with a high ability of resistance to gastric and intestinal digestion, both in a simulated environment and in mouse models [104]. Remarkably, research has demonstrated that the size and surface charge of some EVs were pH-dependent. Specifically, GDEVs remained stable in size and surface charge in neutral and alkaline pH environments [19]. However, when exposed to acidic solutions, these parameters undergo a slight increase. It remains uncertain whether the size and/or surface charge of EVs have any significant impact on their bioavailability, biosafety, and pharmacodynamics. Owing to their good stability and adjustable size, CHMEVs have been widely utilized as drug delivery vehicles.

## Biological activities of CHMEVs

CHM is a complex group of compounds that include not only active small molecular metabolites such as alkaloids, flavonoids, and polysaccharides but also large molecules such as proteins, nucleic acids, and lipids [105]. It shows a wide range of therapeutic effects on human diseases and represents a natural treasure trove for novel drug discovery. The majority of current research on CHM focuses on its dried form or processed products. However, many fresh CHMs, as a primitive medicinal form, have stronger anti-inflammatory, antioxidant, and anticancer activities than their dried products and are widely used in clinical or folk, such as dandelion [106], Dendrobium [107], and Portulaca oleracea L [108]. It is well-known that the discovery of artemisinin was inspired by the record in the Chinese ancient book stating that juice obtained by wringing fresh *Artemisia annua* plant was consumed for treating malaria. However, the superior effects of fresh medicines have not been adequately explained. CHMEVs are mainly isolated from fresh herbal medicines and exhibit similar or stronger bioactive properties to those of the original medicinal plants. Hence, CHMEVs have more potential for drug development than PDEVs derived from fruits or vegetables. Extensive studies of CHMEVs can provide scientific insights into the material basis of the efficacy of fresh medicines. CHMEVs, as novel natural ingredients, have shown therapeutic potential in four aspects: (1) anti-inflammation activity (2) anti-tumor activity (3) regenerative activities (4) anti-aging activity. Figure 4 depicts the detailed mechanisms underlying the pharmacological activities of CHMEVs.

### Anti-inflammatory activity

Inflammation is a biological defensive response to harmful stimuli, such as infection and tissue injury, and

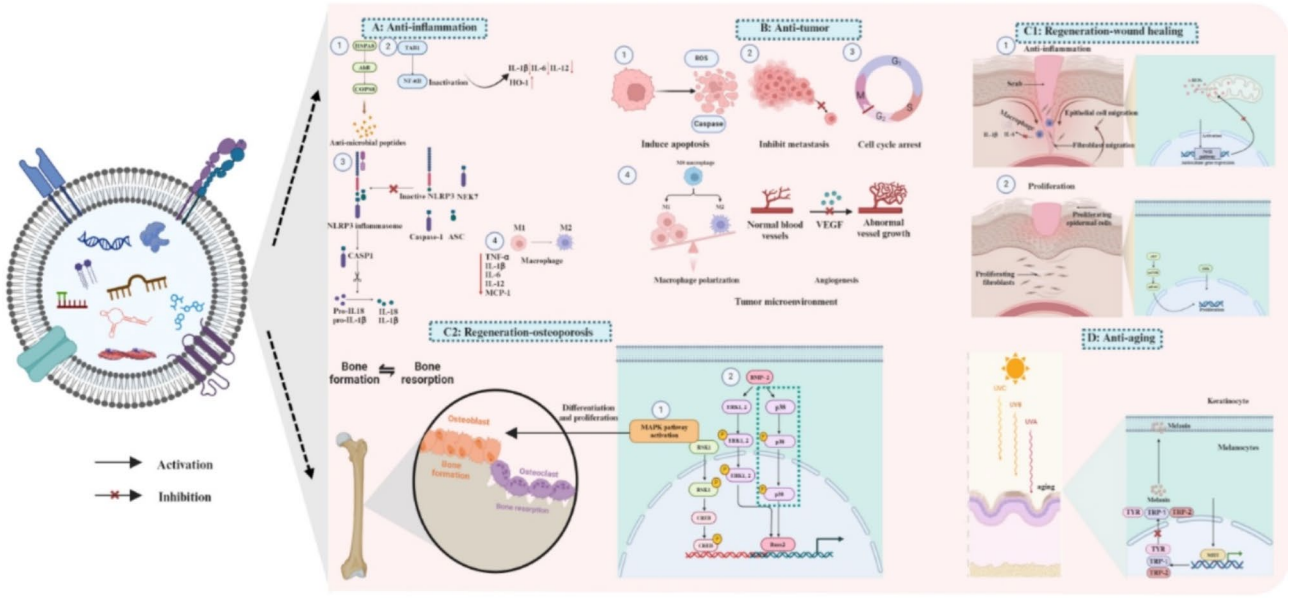


**Fig. 4** Schematic of mechanisms of anti-inflammatory **(A)**, anti-tumor **(B)**, regenerative **(C1-2)**, and anti-aging **(D)** activities of CHMEVs. Take osteogenic activity of yam-derived EVs (YEVs) as an example (Fig. 4C2), YEVs significantly promoted proliferation, differentiation, and biomineralization of osteoblast cells by stimulating BMP-2/p-p38-dependent Runx2 pathway. Specifically, bone morphogenetic protein-2 (BMP-2) that triggers bone formation and differentiation activated p38, which induced the expression of Runx2, an essential transcription factor for the differentiation of osteoblasts and osteogenesis and involved in bone marker gene expression

generally occurs in local tissue with the recruitment of leukocytes and plasma proteins [109]. However, dysregulated inflammation is likely to evolve into a range of chronic diseases that affect the normal function of the body [110]. Numerous studies have proven that CHMEVs can attenuate various inflammatory responses by regulating related inflammatory cytokines and signaling pathways. For instance, GDEVs are specifically taken up by intestinal epithelial cells and macrophages, regulate the levels of proinflammatory (TNF-α, IL-6, and IL-1β) and anti-inflammatory cytokines (IL-10 and IL-22), thereby enhancing intestinal repair and alleviating dextran sulfate sodium(DSS)-induced colitis [35]. Research has demonstrated that EVs are abundant in a variety of microRNAs, including miR156a-5p, miR166a-3p, and miR168a-5p. These microRNAs have the potential to be absorbed by mammalian intestinal epithelial cells, suggesting that EV-carried RNAs may have effects on the gastrointestinal tract [111]. A related study conducted by Teng et al. confirmed that microRNAs in GDEVs promoted *Lactobacillus rhamnosus* GG-mediated inhibition effect on colitis by influencing the composition of the gut microbiota and increasing the expression of IL-22 through activation of the aryl hydrocarbon receptor (AhR) pathway [77]. Another similar report indicated that osa-miR164d, encapsulated in GDEVs, directly acted on the 3'-UTR of Table 1 to reprogram macrophages through the downregulation of nuclear factor-kappa B (NF-κB) expression, thus alleviating colitis-related symptoms [96]. Turmeric-derived nanovesicles (TNVs) were also demonstrated to restore damaged intestinal barriers and accelerate colitis remission. After oral administration, TNVs could target inflamed colon sites, downregulate proinflammatory cytokines (TNF-α, IL-6, and IL-1β), promote the expression of heme oxygenase-1 (HO-1), and alter gut microbiota abundance, which might be partially attributed to inactivation of NF-κB pathway [112, 113]. Further analysis showed that the lipids in GDEVs could effectively block NLRP3 inflammasome assembly, thereby suppressing the downstream pathways (caspase 1 autocleavage, IL-1β, and IL-18 secretion, and pyroptotic cell death) of the NLRP3 inflammasome pathway [114]. Shiitake mushroom-derived EVs were found to have a similar inhibitory effect on the inflammasome in acute liver injury [115]. A recent study revealed that mulberry bark-derived EVs activated Hsp70 member 8 (HSPA8)-mediated AhR anti-inflammatory pathway to induce constitutive photomorphogenic homolog subunit 8 (COPS8). COPS8 directly acted downstream of the AhR pathway to stimulate the production of antimicrobial peptides, thus exerting protective effects against colitis [116]. Additionally, it has been found that Pueraria lobata-derived EVs (PLDEs) also exerted an anti-inflammatory effect by promoting the polarization of M1 macrophages toward M2 and downregulating the expression of proinflammatory genes (IL-6, IL-1β, TNF-α, and MCP-1) [117]. In a subsequent study, the same research team further proposed that PLDEs may modulate the phenotypic state

of macrophages and maintain intestinal flora homeostasis to alleviate DSS-induced colitis and associated lung inflammation [38]. In summary, recent research has indicated that the inflammatory activity of CHMEVs is likely attributed to one or more mechanisms of action (e.g., AHR pathway activation; ii NF-κB pathway inactivation; iii inflammasome assembly inhibition; IV macrophage polarization modulation), as indicated in Fig. 4A.

#### Anti-tumor activity

Cancer represents a significant global health challenge, with substantial rates of morbidity and mortality. Over the past few decades, scientists have been working to develop diagnostic and therapeutic methods with few side effects, low cost, and significant effectiveness, but the treatment of cancer remains a huge challenge to public health. Natural compounds derived from CHM are often used to treat various cancers [118]. Accumulating evidence has shown that CHMEVs can restrain the growth and proliferation of tumor cells through distinct mechanisms and are nontoxic to normal cells. Zhang et al. found that *Asparagus cochinchinensis*-derived EVs could significantly inhibit the growth and proliferation of various types of hepatocellular carcinoma cells including Hep G2, Hep 3B, and SMMC-7721 cells, and induce the apoptosis of Hep G2 cells in vivo and in vitro, while showing no apparent toxicity to normal cells. Compared to traditional aspartate extract, the EVs exhibited more pronounced antitumor activity and superior drug-like properties [49]. Chronic intestinal inflammation is known to be a precursor to colorectal cancer (CAC), which is a leading cause of high morbidity and mortality in individuals with ulcerative colitis [119]. GDEVs have been shown to exert significant effects on the prevention and treatment of CAC by suppressing inflammation-induced proliferation of intestinal epithelial cells. This suppression is evidenced by a decrease in the expression of the proliferation marker, cyclin D1 mRNA [35]. Chen et al. indicated that tea flower EVs exhibit remarkable cytotoxicity to breast cancer cells by stimulating the production of excess reactive oxygen species, thus inducing cell cycle arrest and suppressing tumor proliferation, cell migration, and cell invasion. Notably, oral administration is safer than intravenous injection which is likely to stimulate immune responses, alter hemograms, and induce hepatorenal toxicity [120].

CHMEVs not only directly impede tumor cell growth but also potentially impact the tumor microenvironment. Cao et al. found that ginseng-derived EVs were internalized by macrophages mainly via phagocytosis and induced the polarization of tumor-associated macrophages from the tumoricidal M1 phenotype and tumor-supportive M2 phenotype, thereby inhibiting melanoma development. The process largely relied on toll-like receptors and skeletal differentiation antigen 88 (TLR4-MyD88) signaling pathway [37]. Moreover, the same research group further concluded that the integration of ginseng EVs with the programmed cell death protein-1 monoclonal antibody had the ability to alter the cold tumor environment and subsequently triggered a persistent systemic antitumor immunity in tumor models [121]. Follow-up analysis revealed that ginseng EVs could regulate mTOR-T-bet axis-mediated macrophage reprogramming, thus reducing arginase-1 release and improving T-cell exhaustion in tumors [122]. Therefore, EVs derived from ginseng have the potential to function as immunomodulators in cancer immunotherapy. In addition, numerous EVs derived from various fruits and vegetables including garlic, citrus, broccoli, grapefruits, and lemons, have been extensively researched for their potential anticancer properties [14, 17]. *Dendropanax morbifera* EVs exerted an inhibitory effect on cancer-associated fibroblasts in a dose-dependent manner in the tumor microenvironment, implying their antimetastatic activity [123]. In a recent study by Yan et al., Brucea javanica EVs were shown to deliver their contained miRNAs to 4T1 cells, effectively inhibiting the growth and metastasis of 4T1 cells by mediating PI3K/Akt/mTOR signaling pathway along with ROS/caspase-mediated apoptosis. In addition, these EVs also suppressed angiogenesis by downregulating the production of vascular endothelial growth factor, which was further verified in breast tumor mouse models [124].

Collectively, the anticancer activity of CHMEVs mainly involves three mechanisms of action: tumor-selective apoptosis induction, cell cycle arrest, and tumor microenvironment regulation. However, further exploration is required to understand the mechanisms by which CHMEVs specifically act on cancer cells and regulate related genes. Figure 4B illustrates the mechanisms involved in cancer.

#### Regenerative activities

Current research indicates that CHMEVs have significant regenerative potential, particularly in two key areas: skin regeneration for wound healing and bone regeneration for osteoporosis. Wound healing is a sophisticated process involving hemostasis, inflammatory responses, cell proliferation and migration, and tissue remodeling (Fig. 4C1) [125]. Different types of CHMEVs have been confirmed to exert beneficial effects on the wound-healing process. Tan et al. conducted a study to investigate the therapeutic effect of dandelion-derived EVs on wound infection caused by *Staphylococcus aureus* (*S. aureus*). Their findings suggested that the EVs neutralized hemolysis triggered by *S. aureus* exotoxins via specific binding to exotoxins. In order to ensure the exposure of the EVs at local lesions, hydrogel materials were used as

carriers to load these EVs. Further research demonstrated that dandelion-derived EVs-laden hydrogel could also effectively promote cell proliferation and migration and inhibit the inflammatory response induced by exotoxin, thereby improving wound healing [36]. Angiogenesis plays a crucial role in the normal transport of nutrients during wound healing. Ginseng EVs have been shown to not only enhance cell proliferation by regulating ERK and AKT/mTOR pathway and elevate the expression of wound healing-related genes, but also promote angiogenesis, one of the crucial steps for wound healing. Meanwhile, ginseng EVs also promoted the elimination of inflammation in the later stages of wound healing [126]. Moreover, Aloe EVs could effectively activate the antioxidant defense mechanisms to promote the wound-healing process via the Nrf2 activation in HaCaT and fibroblasts [127]. Likewise, Kim et al. discovered that Aloe EVs significantly reduced the expression of proinflammatory (IL-6 and IL-1β) in RAW264.7 macrophages while causing no apparent harm to other cell types. Furthermore, the EVs also promoted angiogenesis in human umbilical vein endothelial cells [128]. Wheat-derived EVs could promote the proliferation and migration of endothelial cells, epithelial cells, and fibroblasts. Additionally, they have been found to increase mRNA levels of type I collagen, leading to an increase in collagen production, promote angiogenesis in endothelial cells, and facilitate wound healing, suggesting skin regeneration activity of wheat-derived EVs [129].

Osteoporosis is a systemic skeletal disease characterized by decreased bone mass, deterioration of the microarchitecture of bone tissue, and increased bone fragility [130]. Osteoblast growth and bone metabolism are key factors for bone regeneration in osteoporosis [131]. CHMEVs have been confirmed to exert regulatory effects on osteoporosis by mediating distinct pathological and physiological processes. In a recent study, Cao et al. discovered that MO-derived EVs (MOEVs) were shown to target bone tissue in postmenopausal osteoporosis mouse model and did not cause any damage to other organs. Further analysis suggested that these EVs could activate the mitogen-activated protein kinase (MAPK) signaling pathway to promote bone formation by stimulating osteoblast differentiation and proliferation, thereby exerting an antiosteoporotic effect [76]. Another study revealed that yam-derived EVs promoted the proliferation, differentiation, and mineralization of osteoblasts; upregulated the expression of bone differentiation markers (OPN, ALP, and COL-I), and promoted bone regeneration in ovariectomy-induced osteoporotic mice. These effects might be ascribed to the activation of the BMP-2/p-p38-dependent Runx2 pathway rather than other MAPK pathways of ERK1/2 and JNK. Interestingly, yam-derived EVs do not contain saponins such as diosgenin and dioscin, which are known to primarily exert osteogenic activity in yams [39]. It has been reported that the intestinal flora and their metabolites play an important role in regulating bone metabolism. Zhang et al. isolated EVs from Pueraria lobata and demonstrated their beneficial effects on differentiation and mineralization of primary human bone mesenchymal stem cells (hBMSCs) via enhancing autophagy mediated by the degradation of trimethylamine-N-oxide (a metabolite of gut microbiota) in ovariectomy-induced osteoporosis [132]. Furthermore, some fruit EVs such as plums [133], and apples [134] also have antiosteoporotic effects. Taken together, these findings suggested that CHMEVs can be used as safe and effective therapeutic agents for wound healing and osteoporosis.

#### Anti-aging activity

CHMEVs are loaded with a variety of bioactive ingredients such as proteins and active metabolites secreted by the original cells. These nutrients can infiltrate cells and stimulate collagen synthesis, thereby sustainably nourishing skin and delaying aging. For instance, EVs extracted from the leaves and rhizomes of *Dendropanax morifera* significantly were observed to decrease melanin production and tyrosinase (TYR) activity in melanoma cells in a dose-dependent manner, achieving skin-whitening effect. Further analysis confirmed that the inhibitory effect was mediated by downregulating the expression of melanogenesis-related genes and proteins such as microphthalmia-associated transcription factor, TYR, tyrosinase-related protein 1 (TRP-1), and TRP-2. Surprisingly, these EVs possessed more potent melanin inhibition than did arbutin, a tyrosinase inhibitor [135]. Similarly, ginseng EVs could effectively alleviate the replicative senescent and senescence-associated pigmented phenotypes of human dermal fibroblasts and ultraviolet B radiation-treated human melanocytes. As the concentration of ginseng EVs increases, there is a gradual down-regulation of senescence-related genes (CDKN1A, CDKN2A, MMP1, and IL-8) and melanogenesis-associated proteins such as TYR, TRP-2, and Ras-related protein 27 [136].

Furthermore, some EVs derived from edible plants including citrus [137], beta vulgaris [138], and strawberry [139], etc., demonstrate outstanding anti-aging properties attributed to their high levels of antioxidants. CHMEVs are expected to serve as alternatives to chemotherapeutic agents in the medical aesthetics and skincare market.

## Drugs loading

Given their high biocompatibility, stability, and safety, CHMEVs are suitable as a novel drug delivery platform, playing a functional synergistic role with loaded drug

molecules, proteins, miRNA, etc. In particular, following engineering modification with hydrophobic agents, these EVs can be endowed with excellent targeting ability, thereby enabling accurate delivery of drugs to specific lesion locations [11]. This targeted approach allows for more effective and efficient treatment outcomes. For instance, Zhang et al. successfully fabricated a nanovector using reassembled ginger-derived lipids. The drug carrier could effectively load the chemotherapy drug doxorubicin (Dox) and be preferentially and selectively internalized by colon cancer cells. Compared with free Dox, the delivery system modified with folic acid on the surface was shown to specifically target Colon-26 tumors in vivo and promote the chemotherapeutic inhibition of tumor cell proliferation. Notably, in comparison to commercially available liposomal-Dox, they also demonstrated a more excellent drug release at acidic pH of the tumor extracellular microenvironment, leading to minimal side effects [140]. Likewise, GDEV carrier surface-engineered with folic acid-displaying arrowtail RNA improved the specific delivery of siRNA to human oral epidermoid carcinoma cells, thus exerting a superior tumor cell inhibition effect [48]. Zeng et al. loaded photosensitizer indocyanine green (ICG) into aloe EVs by coincubation and found that the nanovectors showed great stability after 30 days of loading ICG, with a retention rate of approximately 90%. After treatment with these ICG-loaded EVs, the growth of melanoma was effectively suppressed and there was no significant difference between the freshly prepared carrier and those after 30 days of storage. These findings suggest that CHMEVs have a protective effect on the structure and function of the loaded drugs. Additionally, considering their outstanding transdermal properties, nanovectors are expected to be used as innovative drug delivery vehicles for the effective treatment of skin cancer [80]. EV carrier from edible plants plays a pivotal role in alleviating the adverse effects and drug resistance of antitumor medications. Research has indicated that encapsulating methotrexate (MTX) into grapefruit-derived EVs significantly minimized the toxicity of MTX while enhancing its effectiveness against DSS-induced colitis at lower doses [141]. In another study, after loading Dox onto lemon-derived EVs coated with functional heparin-cRGD on the surface, the delivery system demonstrated potent antiproliferative effects on Dox-resistant ovarian cancer cells [142]. The relevant studies of CHMEVs as drug delivery carriers are shown in Table 3.

In summary, the two primary areas for CHMEV application as a drug carrier are targeted delivery and alleviation of drug resistance. Although CHMEVs are efficiently taken up by cells, the mechanisms underlying their internalization remain incompletely understood. Further research into transport and uptake mechanisms will contribute to enhancing the precision and therapeutic impact of drug treatments.

## Clinical research of CHMEVs

Despite considerable endeavors, only a limited number of CHMEV studies have progressed to the clinical stage. Currently, three clinical trials are underway, but still in their initial stages of development. These included grape-derived EVs (trial number NCT01668849), GDEVs, and aloe-derived EVs (trial number NCT03493984). For instance, grape-derived EVs have been investigated as anti-inflammatory agents to alleviate oral mucositis caused by radiation and chemotherapy, while GDEVs and aloe-derived EVs are applied to mitigate insulin resistance and chronic inflammation in patients diagnosed with polycystic ovary syndrome. Apart from acting as therapeutic agents, the phase I clinical trial of PDEVs (NCT01294072) as a hydrophobic drug delivery system is ongoing to evaluate their ability to carry curcumin to normal and malignant colon tissue [87].

During the clinical transformation of CHMEVs, biosafety is a major issue that researchers must consider. According to current reports, CHMEVs vai oral or injection administration can target the lesion site, showing no obvious damage to organs. In addition, in vitro experiments have revealed that CHMEVs have no significant toxicity to normal cell lines. Hence, CHMEVs possess high biosafety. Nevertheless, due to limited knowledge about the compositions and properties of CHMEVs,

**Table 3** A summary of CHMEVs as a drug delivery platform

| Carrier | Loaded drug | Target object | Advantages | Literature |
|---|---|---|---|---|
| Ginger-derived lipids | Dox | Colon-26 cells | Promote drug-targeted delivery and increase drug release | [140] |
| GDEVs | Arrowtail RNA | Human oral epidermoid carcinoma cells | Enhance tumor-specific targeting and efficacy | [48] |
| Ginger-derived lipids | siRNA-CD98 | RAW 264.7 and colon-26 cells. | Targeted specifically to colon tissues and decrease CD98 expression | [153] |
| Ginger-derived lipids | Dmt1 | Duodenal epithelium | Inhibit Dmt1 mRNA expression | [154] |
| Aloe EVs | ICG | Melanoma cells | Maintain the stability of drug molecules and extend storage time | [80] |
| Grapefruit EVs | Methotrexate | Intestinal macrophages | Minimize the toxicity and promote effectiveness | [141] |
| Lemon EVs | Dox | Ovarian cancer cells | Reverse drug resistance | [142] |

Abbreviations: Dox, Doxorubicin; Dmt1, Divalent metal-ion transporter 1; ICG, Photosensitizer indocyanine green

there is a critical need for more comprehensive efforts, including better-designed experiments, improved preparation and characterization methods, and biodistribution studies. Indeed, there is still a long way to go to realize the clinical transformation of CHMEVs.

## Conclusion and future perspective

CHM has been used in TCM for thousands of years due to its abundant resources, significant therapeutic effects and minimal side effects. Evidence has suggested that EVs sourced from medicinal plants inherit these pharmacological characteristics in the treatment of diseases. Despite the discovery of plant EVs in the 1960s, research surrounding CHMEVs has only recently emerged. Current findings offer a new direction for the future development of CHM, opening up a completely new field. In general, CHMEVs exhibit similar morphology, size, and even in vivo behavior to other types of nanoparticles to a certain extent. Moreover, CHMEVs, as natural products from medicinal plants, offer significant advantages such as high safety, cost-effectiveness, high stability, and source accessibility, rendering them promising candidates for nanomedicine. As crucial mediators for cross-kingdom communication, CHMEVs can cross various physiological barriers and exchange information between cells across species, playing therapeutic and regulatory roles in inflammatory diseases, tumors, wound healing, osteoporosis, etc. Based on their advantages, relevant researchers strongly advocate the utilization of CHMEVs as a novel dosage form and recommend conducting comprehensive quality control according to their active ingredients and contents [11]. Similar to other types of EVs, CHMEVs have also been verified to target specific lesion sites and are readily internalized by host cells. The excellent targeting ability and high biocompatibility are highly consistent with warping (*guijing* in Chinese) characteristics of CHM. Therefore, it will confer modern scientific connotations on TCM if integrating the natural properties of CHMEVs with traditional characteristics of CHM such as four natures, five flavors, and the fluctuations of rise and fall. Furthermore, CHMEVs are readily available from abundant sources and can be efficiently mass-produced at a relatively low cost. As research progresses, the sources of CHMEVs are gradually expanding, providing researchers with more possibilities to select suitable raw materials for gaining nanoparticles according to their specific research objectives. In addition to the most commonly used fresh medicinal plants, CHMEVs can also be obtained from herbal decoctions usually prepared by boiling dry herbs with water. Recently, decoctosome, a lipo-nano particle consisting of 0.5-2.5% of the decoction has been discovered by Jiang et al. Further component analysis revealed that sRNAs loaded in the decoctosome serve as a class of active substances with a greater anti-pulmonary fibrosis effect than the decoction [45, 143]. In addition, self-assembled nanostructures present in the Chinese medicine formula QY305 have been proven to function as its pharmacodynamic material basis for mitigating cutaneous adverse reactions and diarrhea, offering modern scientific evidence for compatibility mechanisms of CHM [144].

While current studies have substantiated the therapeutic significance of CHMEV in human diseases, some challenges and limitations remain to be addressed. Firstly, in relevant literature, there is little consensus on the nomenclature method of CHMEVs and the corresponding naming standards are lacking. In general, the nomenclature of CHMEV is composed of CHM and the EVs themselves. Although the name of CHM is often described in Latin, English, or pinyin, EVs are variously named, including extracellular vesicles, vesicle-like nanoparticles, exosome-like nanoparticles, exosome-like nanovesicles, or decoctosome. Thus, it is imperative to establish a standardized naming system for CHMEVs as related research continues to increase. This is the initial step to realize the internationalization and standardization of CHMEVs. Moreover, the study of CHMEVs commenced late. In the laboratory, the extraction and purification methods used mainly refer to those applied in MEVs, which are time-consuming and may not ensure high yield and purity of CHMEVs due to differences in their physical and biochemical properties. CHMEVs require customized isolation and purification methods to meet specific experimental requirements and large-scale production. Another noteworthy issue is that CHM exhibits typical regional characteristics, and its quality and clinical efficacy are susceptible to several factors, such as region, harvesting period, and cultivation conditions. Additionally, the therapeutic effects of various medicinal parts (e.g., root, tuber, leaf, flower, and fruit) may vary significantly, further complicating the investigation of CHMEVs. As a result, to avoid variations in CHMEVs and guarantee experimental consistency and reproducibility, we recommend that more detailed information on the CHM source origins, such as medicinal parts, harvest time, botanical origin, etc., should be well documented and reported in all studies. The third issue that requires attention is the absence of specific surface markers, rendering accurate biological characterization of CHMEV and PDEV challenging. It is difficult to verify that they adhere to the traditional definition of EVs. We propose to initiate a comprehensive lipid and proteomic analysis of CHMEVs to further investigate generic and specific markers, which will assist in obtaining valuable insights into the characteristics of CHMEVs across different plant species. It has been confirmed that CHMEV comprises a diverse array of natural constituents, including lipids, RNAs, proteins, and small-molecule metabolites.

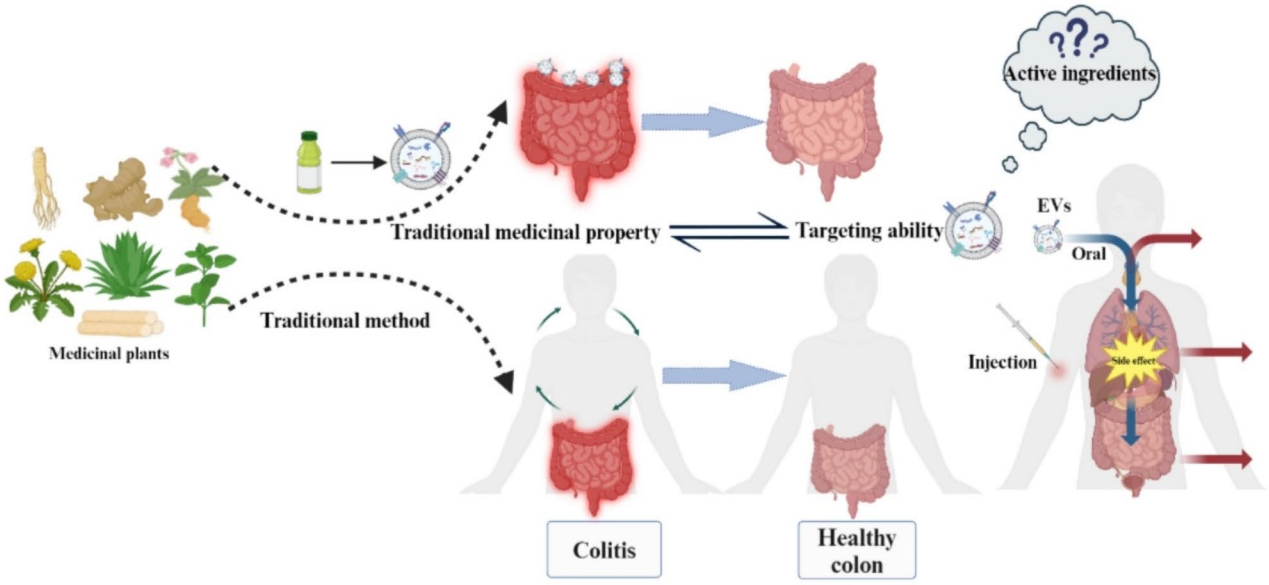


**Fig. 5** Schematic diagram of three key research areas about CHMEVs in the future. **(A)** Investigating the integration of traditional medicinal properties with the targeting capabilities of CHMEVs. **(B)** Identifying what constituents within CHMEVs exert therapeutic effects, and **(C)** Assessing the potential toxicity of CHMEVs through oral or intravenous administration

However, there is a scarcity of evaluations regarding the mechanisms of action of various CHMEVs in specific disease contexts and isolation techniques for specific compounds. Furthermore, it remains uncertain whether these components are potentially toxic when they interact with targeted organs. A comprehensive identification of the constituents within CHMEVs, coupled with the exploration of their underlying regulatory mechanisms, is crucial for gaining a profound understanding of CHMEVs and advancing their standardization. In addition to the abovementioned common challenges and limitations, we propose several considerations and questions on CHMEV research as follows (Fig. 5):

(1) What ingredients constitute the unique superiority of CHMEVs at the molecular level? For instance, while yam EVs do not contain saponins known to mainly promote the osteogenic activity of yam, they can prevent osteoporosis. But GDEVs do the opposite.
(2) Do CHMEVs share a common therapeutic mechanism with their origin plants in the treatment of the same disease?
(3) Will CHMEVs be toxic to the body, and under what conditions will they occur (high dose, low purity, or contamination)?
(4) How is the targeting ability of CHMEVs realized? Is there a potential relationship with the traditional medicinal property of CHM? And if so, how do we connect them?

In conclusion, research on the biogenesis, release, and functional mechanisms of CHMEVs is still in the nascent phase, with numerous unknown areas to be resolved. Certainly, we may encounter some unforeseen challenges in achieving future clinical transformation of CHMEVs. However, these obstacles will be addressed, as has been witnessed in any newly emerging field. We strongly believe that advancements in the purity, characterization, and molecular mechanisms of action of CHMEVs will further unveil the potential value and modern scientific connotation of CHM, opening new avenues for the modernization of TCM. CHMEVs are expected to become an indispensable nanomedicine in the coming year.

**Abbreviations**

| | |
|---|---|
| EVs | Extracellular vesicles |
| CHM | Chinese herbal medicine |
| CHMEVs | Chinese herbal medicine-derived extracellular vesicles |
| MEVs | Mammalian-derived EVs |
| PDEVs | Plant-derived EVs |
| TEM | Transmission electron microscopy |
| MVBs | Multivesicular bodies |
| GDEVs | Ginger-derived EVs |
| ILVs | Intraluminal vesicles |
| PBS | phosphate-buffered saline |
| MO | Morinda officinalis |
| SEC | Size exclusion chromatography |
| DC | Differential centrifugation |
| DGC | Density gradient centrifugation |
| TFF | Tangential flow filtration |
| PEG | Polyethylene glycol |
| EM | Electron microscopy |
| AFM | Force microscopy |
| DLS | Dynamic light scattering |
| NTA | Nanoparticle tracking analysis |
| BCA | Bicinchoninic acid assay |
| nFCM | Nano-flow cytometry |

| | |
|---|---|
| HPLC | High-performance liquid chromatography |
| HSP70 | Heat shock protein 70 |
| PA | Phosphatidic acid |
| PC | Phosphatidylcholines |
| PE | Phosphatidylethanolamine |
| miRNA | microRNA |
| TNVs | Turmeric-derived nanovesicles |
| PLDEs | Pueraria lobata-derived EVs |
| CAC | Colorectal cancer |
| TRP | 1-Tyrosinase-related protein 1 |

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**Author contributions**
ZJ conceived the idea and drafted the manuscript. TS, GL, ZH, MZ revised the manuscript, while MM conceived and substantively revised it. All authors read and approved the final version.

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## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
All authors agree to publish this manuscript.

**Competing interests**
The authors declare that they have no competing interests.

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