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Consensus

Consensus statement on research and application of Chinese herbal medicine derived extracellular vesicles-like particles (2023 edition)

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ABSTRACT

To promote the development of extracellular vesicles of herbal medicine especially the establishment of standardization, led by the National Expert Committee on Research and Application of Chinese Herbal Vesicles, research experts in the field of herbal medicine and extracellular vesicles were invited nationwide with the support of the Expert Committee on Research and Application of Chinese Herbal Vesicles, Professional Committee on Extracellular Vesicle Research and Application, Chinese Society of Research Hospitals and the Guangdong Engineering Research Center of Chinese Herbal Vesicles. Based on the collation of relevant literature, we have adopted the Delphi method, the consensus meeting method combined with the nominal group method to form a discussion draft of "Consensus statement on research and application of Chinese herbal medicine derived extracellular vesicles-like particles (2023)". The first draft was discussed in online and offline meetings on October 12, 14, November 2, 2022 and April and May 2023 on the current status of research, nomenclature, isolation methods, quality standards and research applications of extracellular vesicles of Chinese herbal medicines, and 13 consensus opinions were finally formed. At the Third Academic Conference on Research and Application of Chinese Herbal Vesicles, held on May 26, 2023, Kewei Zhao, convenor of the consensus, presented and read the consensus

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to the experts of the Expert Committee on Research and Application of Chinese Herbal Vesicles. The consensus highlights the characteristics and advantages of Chinese medicine, inherits the essence, and keeps the righteousness and innovation, aiming to provide a reference for colleagues engaged in research and application of Chinese herbal vesicles at home and abroad, decode the mystery behind Chinese herbal vesicles together, establish a safe, effective and controllable accurate Chinese herbal vesicle prevention and treatment system, and build a bridge for Chinese medicine to the world.

Contents

1. Introduction

Extracellular vesicles (EVs) are lipid bilayer vesicles of nanoscale size that are released by nearly all cell types, encompassing exosomes, microvesicles, and apoptotic bodies. These EVs are abundantly present in diverse body fluids and cell supernatants, and serve as stable carriers for crucial signaling molecules implicated in cell communication, migration, angiogenesis, and tumor cell growth (Van Niel et al., 2018). Extensive research has substantiated the multifaceted biological activities exhibited by extracellular vesicles derived from mammalian cells, particularly stem cells (Weng et al., 2021). Plant-derived extracellular vesicles (PDEVs) exhibit morphological similarities to mammalian EVs, characterized by their nanovesicle structure. Additionally, they share similar compositions, encompassing lipids, RNA, and proteins. However, PDEVs possess a distinct non-cellular autonomous role owing to the presence of cell walls, facilitating the exchange of biological information across different species. The abundant sources, substantial yield, and evident activity of PDEVs have garnered considerable interest, as evidenced by extensive attention in the literature (Feng et al., 2023; Cong et al., 2022; Dad et al., 2021; Pinedo et al., 2021).

The term "Chinese herbal medicine (CHM)" mentioned in the consensus statement pertains to plant-based CHM. The CHM vesicles encompass two distinct nanostructures found in CHM, namely EVs derived exclusively from plant extracellular fluid, and EV-like particles (EVLP) derived from plant sap.

Since the inaugural Chinese Herbal Medicine Vesicle Research Forum held during the National Extracellular Vesicles Congress in October 2021, significant strides have been achieved in the field of CHM research. However, it is important to acknowledge the existence of certain prevailing challenges. The Expert Committee on Research and Application of Chinese Herbal Vesicles, Professional Committee on Extracellular Vesicle Research and Application, Chinese Society of Research Hospitals have taken the initiative to develop an expert consensus on the research and application of CHM vesicles, with the primary objective of establishing a comprehensive and reliable system that ensures the safety, efficacy, controllability, and precision of CHM vesicles.

This consensus incorporates a synthesis of literature research findings and expert research foundations to establish consensus recommendations regarding the exploration of CHM vesicles and their clinical application. When applying this consensus, it is imperative to thoroughly consider the current circumstances surrounding the CHM being investigated. This consensus will undergo regular updates and revisions in response to the ongoing advancements in CHM vesicle research, transformations in clinical applications, and the emergence of novel evidence-based evidence.

2. Research status and existing problems of CHM vesicles

Plant-derived nanovesicles have garnered significant interest in the scientific community since the initial identification of polyvesicles in carrot cells in 1967 (Halperin, & Jensen, 1967) and the subsequent isolation of vesicles possessing exosome-like membrane structure from sunflower seeds in 2009 (An et al., 2007). The attention is primarily driven by the numerous advantages offered by the nanovesicles, including their abundant sources and extensive potential for applications in the fields of biomedical research and nanotechnology (Cong et al., 2022). Numerous homogenized plants and juices (Bokka et al., 2020; Wu et al., 2019; Bruno et al., 2021; Pocsfalvi et al., 2018; Perut et al., 2021) exhibit membrane-enclosed structures resembling mammalian EVs in both morphology and molecular characteristics, thereby displaying potential biological activities, notably anti-inflammatory (Ju et al., 2013), anticancer (Xiao et al., 2022; Boccia et al., 2022), and antioxidant activities (Zhang et al., 2022).

In recent years, there has been a surge in research investigating the vesicles derived from various Chinese herbs, including ginseng (Cao et al., 2019), ginger (Zhang et al., 2016), garlic (Sundaram et al., 2022), *Aloe saponaria* (Aiton) Haw. (Kim, & Park, 2022), turmeric (Liu et al., 2022), mulberry bark (Sriwastva et al., 2022), *Aster yomena* callus (Kim et al., 2022), *Asparagus cochinchinensis* (Lour.) Merr. (Tiandong in Chinese) (Zhang et al., 2021), and *Drynariae Rhizoma* (Gusuibu in Chinese) (Cao et al., 2022). The exploration of CHM vesicles within the context of contemporary advancements in medicine and nanotechnology holds promising potential for the exploration of novel approaches to drug discovery and application. CHM vesicles, which are derived from natural plants, possess multi-component and multi-functional characteristics. They exhibit high absorbability by organisms and can be employed as both biological therapeutic agents and drug delivery carriers. These vesicles effectively address the issue of inadequate bioavailability of numerous active ingredients. Moreover, they offer a more cost-effective alternative to traditional Chinese medicine (TCM) monomers and decoctions, while ensuring enhanced biosecurity. Additionally, CHM vesicles can serve as a crucial tool for drug discovery through their ability to bind to proteins, RNA, and bioactive substances.

Research has indicated that CHM vesicles possess the potential to elicit unfavorable immune responses and regulatory effects, which are contingent upon their size and heterogeneity. Furthermore, the utilization of CHM vesicles may give rise to concerns regarding biosafety and toxicity due to the unidentified bioactive constituents they contain. To address the aforementioned limitations associated with CHM vesicles, it is imperative to optimize the separation technique to acquire stable nanovesicles. Furthermore, a comprehensive assessment of pertinent aspects including morphological attributes, quantification, and active constituents is warranted. Moreover, the mechanism underlying vesicle secretion in CHM remains elusive, necessitating further investigation.

Consensus opinion 1

The integration of tradition with contemporary advancements is imperative. CHM possesses abundant resources, and its potential can be maximized through the exploration of extracellular vesicles. The utilization of CHM vesicles holds promise for the development of novel therapeutic drugs, necessitating collaborative efforts among researchers to address challenges and propel the modernization, standardization, and precision of traditional Chinese medicine.

Consensus opinion 2

Prior to utilizing CHM vesicles as therapeutic agents and drug carriers, it is imperative to conduct a comprehensive safety assessment, as this serves as a fundamental and essential consideration. The immunogenicity observed *in vivo* can be attributed to the fact that CHM vesicles are not of human origin. Furthermore, the method of administration plays a crucial role in ensuring safety. It is widely accepted that oral administration of CHM vesicles is a relatively secure approach, supported by extensive clinical practice over an extended period of time. Further investigation and research are necessary to establish the safety and immunogenicity of CHM vesicles for non-gastrointestinal administration, as current evidence remains insufficient.

3. Significance of developing expert consensus on research and application of CHM vesicles

The unique cognitive advantages and practical experience of CHM, combined with the EVs of modern medicine, is a complementary approach that preserves the essence of Chinese medicine while promoting appropriate innovation. The amalgamation represents the modernization and precision of TCM. The experts diligently acquire knowledge from positive experiences and practices across various domains to establish the consensus. They then apply the content of this consensus to the entirety of the TCM inheritance, innovation, and development process. The approach facilitates enhanced exchanges and collaboration among researchers in the field, thereby bolstering the capacity and proficiency of TCM governance. Collectively, these efforts contribute to the advancement of high-quality TCM development, while also providing valuable insights and resources to expedite the construction of a healthy China and promote economic and social development of superior quality.

Numerous researchers have extensively investigated the realm of EVs pertaining to CHM, yielding numerous commendable scholarly accomplishments. However, certain pivotal controversies and issues persist within this domain. For instance, the nomenclature of CHM vesicles exhibits heterogeneity, while the extraction methods employed for CHM vesicles, as well as efforts to enhance their yield and purity, investigate the potential mechanisms of secretion, establish quality standards, design functional research experiments, facilitate transformation, and ensure safety in clinical applications, among other considerations, remain areas of exploration. To build consensus, answer questions, and resolve disputes requires not only the wisdom and experience of experts, but also the clarification of problems, the concentration of efforts, and the cooperation of exploration. In order to promote the establishment of norms and standardization of the naming, extraction, quality control and application of CHM vesicles, promote the better and faster development of CHM vesicles research and application, attract and guide relevant researchers to enter the field of CHM vesicles research, consensus building is the first step and the foundation of future research.

4. Nomenclature approach for CHM vesicles

The current nomenclature of CHM vesicles lacks standardization. The nomenclature of CHM vesicles comprises two components: CHM and the vesicles themselves. While certain Chinese medicine names are designated in Latin, English, or Chinese pinyin, various terms are employed to name the vesicles, such as extracellular vesicles, vesicle-like nanoparticles, exosome-like nanoparticles, exosome-like nanovesicles, and decoctosome. For example, turmeric–derived exosome–like nanoparticles (TDNPs) (Sriwastva et al., 2022), garlic exosome-like nanoparticles (GaELNs) (Sundaram et al., 2022), *A. cochinchinensis*-derived exosome-like nanovesicles (ACNVs) (Zhang et al., 2021), *Momordica charantia*-derived extracellular vesicles-like nanovesicles (MCELNs) (Cui et al., 2022), Ginseng-derived extracellular vesicles (GEVs) (Cao et al., 2019), extracellular vesicles from pomegranate juice (PgEVs) (Sánchez-López et al., 2022), decoctosome (Du et al., 2019) and so on. The phenomenon of unclear definition, inconsistent naming, and non-standardization is expected to worsen as related research continues to grow, primarily due to the absence of well-defined biogenetic pathways and rigorous physicochemical characterization. The initial step towards the standardization and internationalization of CHM vesicles would involve the establishment of standardized naming conventions.

The first aspect pertains to CHM. The CHM vesicles derived from CHM. To facilitate the standardization process, these vesicles are referred to by standard Latin names of plants as prescribed in the *Chinese Pharmacopoeia*. To prevent excessive repetition of abbreviations, it is advisable to employ a minimum of two initials. It is imperative to acknowledge that within the context of academic findings, it is advisable to provide extensive and meticulous information, including the source of Chinese herbs, Latin names, Chinese pinyin, and other pertinent details. This practice serves to mitigate potential misunderstandings and negative connotations arising from language and cultural disparities.

The second component pertains to extracellular vesicles. EVs were initially introduced as a collective term for lipid bilayer-obstructing particles derived from cells by György et al. (2011). Subsequently, in 2018, International Society for Extracellular Vesicles (ISEV) (Théry et al., 2018) established the definition of EVs as a comprehensive term encompassing all non-replicating EVLP that are naturally discharged by cells into the extracellular milieu, characterized by a lipid bilayer structure and devoid of functional nuclei. According to *the Minimal Information for Studies of Extracellular Vesicles* 2018 (MISEV 2018) guidelines, particles that fail to meet the established MISEV standards are classified as extracellular particles (EP), based on the specific operational names and size limitations employed in various separation techniques. Non-vesicular extracellular particles (NVEP) are those that lack a lipid bilayer (Cheng & Hill, 2022; Buzas, 2023).

Consensus opinion 3

Based on the MISEV guidelines, the amalgamation of CHM vesicles and the objective of global advancement, the proposed nomenclature is as follows: Chinese Herbal Medicine derived EV-like particles (CHM-EVLP), commonly known as Chinese herbal vesicles. For a specific Chinese herbal medicine, the English designation from the most recent edition of the *Chinese Pharmacopoeia* was employed, resulting in *Astragali Radix*-derived EV-like particles, denoted as AR-EVLP, and *Pogostemon cablin*-derived EV-like particles, abbreviated as PC-EVLP.

Consensus opinion 4

Currently, there is a lack of comprehensive research on the nomenclature of compound vesicles in CHM, primarily due to the disparity between single-flavor pharmaceutical vesicles and compound decocting decoction vesicles. It is imperative to remain attentive to forthcoming research findings, consolidate knowledge regarding the characteristics and functionalities of compound vesicles, and subsequently establish standardized naming conventions.

5. Separation method for CHM vesicles

5.1. Initial treatment of CHM

CHM encompasses a diverse array of varieties, exhibiting extensive geographical distribution and abundant resources. Chinese herbs are categorized into distinct parts, including roots, stems, leaves, flowers, barks, fruits, seeds, and dried ground components. In contemporary literature studies, the predominant treatment approaches for fresh Chinese herbs involve juicing and wall breaking techniques, exemplified by the employment of ginger-derived nanoparticles, garlic exosome-like nanoparticles (Sundaram et al., 2022), and Ginseng-derived nanoparticles. The method of juicing and breaking the wall of CHM is easy to operate, high efficiency and large yield, but the breaking of the wall could cause the cell membrane to break, the cell contents to release, and the membrane structure to rearrange. The second was Apoplastic Washing Fluid (AWF). Huang et al. introduced a method for separating *Arabidopsis* exosomes (Huang, Wang, Cai, & Jin, 2021). The cells obtained by vacuum osmosis had complete structure and less pollution of cellular contents, but the operation was relatively complicated, time-consuming and low yield. Some researchers have compared the wall-breaking and osmotic centrifugation methods, and found that *Arabidopsis* EVs obtained by vacuum osmosis method had smaller particle size, lower potential, smaller density and higher lipid film thickness (Liu et al., 2020). Zhao et al. suggested employing cellulase and pectinase enzymes to facilitate the digestion and disruption of cell walls to obtain the EVs derived from *Morindae Officinalis Radix* (Bajitian in Chinese) (Zhao et al., 2023).

The research team of Professor Chengyu Jiang extracted a nanoparticle substance with heat-stable exosome-like membrane structure from plant decoction called "Decoctosome", and its initial treatment of Chinese herbs such as dandelion and *Andrographis* can be used as a reference method for the preparation of extracellular vesicles of Chinese herbal decoction (Du et al., 2019).

Consensus opinion 5

There exists a diverse array of Chinese herbs, whereby the majority of fresh Chinese herbs can be obtained through the extraction of vesicles via juicing. Specifically, those fresh Chinese herbs possessing a higher juice content can be directly squeezed or juiced. It is worth noting that certain Chinese herbs abundant in juice also contain a considerable amount of fiber components. Consequently, the act of breaking down the cell wall and juicing these herbs results in the formation of a substantial quantity of flocculent fiber complex. The complex poses challenges during the centrifugation/filtration process, making extrusion a more appropriate technique for juice collection. Dry products (exclusively dried and unprocessed) and Chinese herbs with low juice content can be pulverized and extracted by incorporating a suitable quantity of pre-cooled phosphate buffer. Fresh leaves can be subjected to percolation and collection of extracapsular lotion to obtain vesicles. However, fruits, seeds, and other Chinese herbs that contain higher levels of pectin components are not suitable for juicing. In such cases, it is advisable to initiate enzymatic digestion of the cell wall as an initial treatment.

5.2. Differential ultra-high speed centrifugation

Currently, the aforementioned crude treatment in conjunction with the differential ultra-high speed centrifugal technique is extensively employed for vesicle separation. However, there exists variation in the centrifugal parameters employed by different research groups, and a standardized optimal centrifugation protocol has yet to be established. Moreover, the centrifugal method is accompanied by notable drawbacks, such as the propensity for vesicle aggregation, suboptimal recovery rates, potential compromise of vesicle integrity due to elevated centrifugal velocities, and the requirement for substantial ionization equipment.

Sucrose density gradient centrifugation (Gao et al., 2022) and iodine glycol gradient separation (Liu et al., 2022) demonstrated distinct and discernible bands at densities surpassing those of mammalian-derived EVs. However, density-based separations possess certain limitations, such as the potential for separating particles with similar densities but differing molecular compositions or originating from distinct cellular sources, as well as a relatively low recovery rate.

5.3. Ultrafiltration method

Ultrafiltration, employing ultrafine nanofiltration membranes with varying molecular weight cutoff values, is utilized for the separation of EVs from Chinese herbal juice. The method significantly decreases processing time and presents a favorable substitute for the conventional ultracentrifugation approach. Tangential flow filtration (TFF) effectively addresses the issue of filter membrane clogging due to the buildup of larger particles (Haraszti et al., 2018).

5.4. Size exclusion chromatography (SEC)

SEC is a technique utilized to separate molecules based on their size. The method involves the passage of an herbal solution through a stationary phase consisting of porous resin particles. As particles of varying sizes exhibit distinct retention times, SEC enables the separation of molecules based on their size. Notably, the SEC method is advantageous in preserving the natural biological activity of isolated vesicles. Unlike alternative techniques such as ultracentrifugation and filtration, SEC does not compromise the structural integrity of vesicles due to its reliance on passive gravity flow. The optimization of CHM-EVLP' inherent properties can be achieved through the careful selection of an elution buffer possessing physiological osmotic pressure and viscosity, such as PBS. Nonetheless, the obstacle faced in scaling up production lies in the relatively high equipment expenses and the need for supplementary enrichment techniques (You, Kang, & Rhee, 2021).

5.5. Immunoaffinity capture method

Researchers have effectively created the initial immunomagnetic beads that specifically target plant-derived EVs membrane proteins through the immobilization of antibodies on solid substrates, enabling the separation of exosomes (He et al., 2021). The substantial surface area and uniform procedure of this approach facilitate high capture efficiency and sensitivity, while also accommodating a considerable starting sample size, thereby permitting customization for specific applications through scaling up or down. However, it is imperative to take into account that the utilization of non-neutral pH and non-physiologically eluting buffers, employed for the purpose of segregating vesicles from antibodies, may potentially result in irreversible alterations to the biological functionality of the gathered EVs. Furthermore, this methodology also presents challenges such as a diminished output and elevated expenses associated with antibody bases, thereby suggesting that the implementation of chemical antibodies may be a prospective avenue for improvement. Nevertheless, the identification of the distinctive protein present in CHM-EVLP remains ambiguous, and a scarcity of commercially available antibodies further compounds this issue.

5.6. Polymer precipitation method

The highly hydrophilic polymer interacts with the water molecules around the vesicles, creating a hydrophobic microenvironment that precipitates it. Among various hydrophilic polymers, polyethylene glycol (PEG) is a non-toxic polymer with good biocompatibility (a common excipient used in pharmaceutical products), which has the ability to reshape the water solubility of surrounding materials and has been widely used. The utilization of polyethylene glycol for the precipitation of CHM-EVLP presents a promising approach for the extensive separation. The polymer precipitation method offers several advantages, including minimal equipment requirements, compatibility with large sample sizes, and high efficiency. Nonetheless, the extended processing time and the necessity for intricate compound removal steps may impede downstream analysis and quantitative outcomes. Furthermore, the purity of the CHM-EVLP obtained through this method is comparatively lower than that achieved by alternative strategies, suggesting the potential for optimization (Kocholata et al., 2022).

5.7. Other methods

The present study employed combined electrophoretic technique with 300 kDa cut-off dialysis bag, named ELD, to successfully isolate extracellular vesicles derived from lemon juice, yielding vesicles with comparable size and quantity as those obtained through the standard method of ultracentrifugation (Yang et al., 2020). This technique offers the advantage of time efficiency and does not necessitate the use of specialized equipment, rendering it feasible for implementation in any conventional biological laboratory. Furthermore, a recent investigation demonstrated the efficacy of a rapid capillary-channel polymer (C-CP) fiber spin-off tip method in isolating EVs from a diverse range of fruits and vegetables, including the Chinese herb ginger (Jackson, Mata, & Marcus, 2023).

Consensus opinion 6

Density gradient centrifugation is a recommended technique for the separation of high purity EVs. TFF and SEC methods are recommended for the separation of CHM-EVLP. It is advisable to conduct confirmatory experiments in institutions, taking into consideration various factors such as clinical applications, scientific research objectives, equipment availability, cost-effectiveness, and the specific characteristics of CHM. The considerations will aid in the selection of the most suitable method for separation and extraction. Scholars in the particular domain are strongly encouraged to collaborate and persistently investigate and enhance novel research methodologies by building upon established technical approaches. Additionally, they should strive to optimize innovative techniques for separating vesicles and consistently enhance the efficacy of separating CHM.

6. Quality control of CHM-EVLP

In the investigation and implementation of CHM-EVLP, it is imperative to give due attention not only to the techniques of separation and purification, as well as stability, but also to exercise comprehensive quality control measures, which is crucial to mitigate the variability between different batches arising from the inherent heterogeneity of vesicles.

6.1. Morphology

CHM vesicles typically have diameters ranging from 30 to 400 nm, and their observation can be facilitated through various microscopy techniques such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), and cryo-electron microscopy (Cryo-EM). Each of the instruments possesses distinct advantages and disadvantages. Among them, TEM is widely utilized due to its ease of operation and efficiency. However, it is important to note that the dehydration and fixation processes involved in sample preparation for TEM may potentially lead to deformation of the extracellular lipid network. On the other hand, SEM allows for dynamic observation but exhibits relatively lower resolution and requires specific sample types. AFM exhibits exceptional resolution; however, it is accompanied by sluggish scanning speed, elevated costs, and limited image size. Conversely, cryo-EM enables the depiction of vesicles in their authentic state. Nevertheless, the freezing process induces heightened fragility in the sample, rendering deliberate selection of the desired field of view unattainable.

6.2. Particle size, potential, concentration and yield

The utilization of Dynamic Light Scattering (DLS) enabled the measurement of temporal fluctuations in light intensity, thereby providing insights into the particle size and potential of CHM-EVLP. Nanoparticle Tracking Analysis (NTA) was employed to determine the size and concentration of vesicles through the analysis of light scattering and Brownian motion. Nanoflow cytometry (nFCM) detection emerged as a viable method for assessing particle size and concentration. Laser Transmission Spectroscopy (LTS) was utilized to examine the scattering spectra of incident light at various frequencies, thereby facilitating the analysis of molecular vibration and rotation information for particle size determination (Feng et al., 2023).

6.3. Purity

The Bicinchoninic Acid Assay (BCA) is utilized to determine the concentration of protein in CHM-EVLP, while NTA or nFCM techniques are employed to measure particles concentration, which is subsequently converted into the number of particles per milligram of vesicles protein (particles/mg) (Zhao et al., 2023).

Triton X-100 exhibits the ability to induce lysis in membranous vesicle particles, while exerting minimal impact on non-membranous particles. Following incubation and lysis with Triton X-100, the NTA or nFCM techniques are employed to quantify the proportion of membranous particles present in CHM-EVLP. The measurement serves as an indirect indicator of the sample's purity to a certain degree (Zhao et al., 2023).

Nevertheless, the aforementioned two approaches are incapable of assessing the composition and content of impurities. Consequently, certain scholars have suggested employing size exclusion-high-performance liquid chromatography as a purity indicator, as it effectively eliminates the interference caused by nucleic acid and protein impurities (Cong et al., 2022).

Consensus opinion 7

It is advisable to assess the morphology, potential, size, concentration, and particles/protein ratio (particles/μg) of CHM-EVLP. When considering EVs as the primary subject of evaluation, it is essential to conduct a test on the efficiency of Triton X-100 film breaking. It is recommended that the 1 % Triton X-100 film breaking efficiency exceeds 80 %.

Furthermore, researchers should prioritize the assessment of vesicles yield in Chinese herbs, considering the abundant resources and easily accessible sources of these herbs. Yield of CHM-EVLP refers to the quantification of vesicle particles or protein obtained per gram of herbs.

6.4. Contents

Contents of CHM-EVLP are essential to maintain information exchange. It is imperative to comprehend the biogenetic diversity of CHM-EVLP acquired through various species and isolation methods.

Protein: the protein of CHM-EVLP was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and proteomics. The co-expression of proteins, including heat shock protein (HSP70), S-adenosine homocystease, and glyceraldehyde 3 phosphate dehydrogenase, has been investigated in the proteomic intersections of *Arabidopsis thaliana*, olive, tobacco, and sunflower (Cui et al., 2020). However, there are currently no identified protein markers specific to plant and CHM-EVLP.

Nucleic acid: the detection of nucleic acid bands through DNA/RNA electrophoresis, along with the use of RNA sequencing, has provided insights into the presence of diverse functional RNAs and distinct RNA degradation fragments in Arabidopsis-derived EVs (Tran et al., 2022). These findings suggest the potential regulatory impact of these molecules on human genes, as demonstrated in previous research.

Lipids: lipid detection by thin layer liquid chromatography (TLC), and lipid omics screening for characteristic lipid components. For example, *Arabidopsis* leaf tissue has a different lipid composition from EVs, which contains a large amount of sphingomyelin (Liu et al., 2020).

Metabolites: the analysis of the metabolomics of CHM-EVLP can be conducted by high performance liquid chromatography-mass spectrometry (HPLC-MS) and metabolomic investigations. The metabolomics and lipidomics detection of ginger-derived EVLP showed that gingerol, a component, could reverse alcoholic liver injury (Zhang et al., 2016).

Consensus opinion 8

The current protein/lipid/metabolic markers associated with CHM-EVLP exhibit limited diversity, lack uniformity, and remain poorly understood. Consequently, there is an immediate need to establish comprehensive and species-specific markers for CHM-EVLP in medicinal plants. It is recommended that researchers undertake comprehensive omics investigations and perform multi-parameter and multi-index assessments, encompassing lipid, nucleic acid, protein, metabolite analysis, as well as harmful substance analysis of CHM-EVLP. This approach facilitates the identification of CHM-EVLP markers, analysis of their medicinal value, examination of downstream transformations, and targeted drug utilization.

6.5. Signature small molecule compounds

CHM has *Chinese Pharmacopoeia* as a quality control standard. CHM-EVLP are derived from TCM, and the content standard of active ingredients stipulated by the pharmacopoeia can be used as the quality standard of CHM-EVLP (Zhang et al., 2016). Characteristic chromatogram by HPLC and mass spectrometry were used to detect the signature small molecule compounds in vesicles. On the one hand, there was a characteristic spectrum corresponding to the national industry standard, and on the other hand, the content of active ingredients in the national pharmacopoeia was up to the standard.

Consensus opinion 9

In accordance with *Chinese Pharmacopoeia*, it is recommended to conduct quality control of CHM-EVLP based on the new dosage form of Chinese herbal medicine. Additionally, the levels of iconic components present in the CHM-EVLP can serve as an indicator for assessing purity quality control.

6.6. Biological activity

CHM-EVLP encompass the majority of the active components found in CHM, serving as a concentrated essence of its efficacious constituents. Professor Zhang's research team successfully obtained turmeric-derived exosome-like nanovesicles (TDNPs), which exhibit remarkable anti-inflammatory and anti-oxidant attributes. In the context of oral administration, it has been observed that TDNPs could traverse the upper digestive tract without adverse effects and efficiently accumulating at the site of inflammation in the colon. This accumulation has shown significant efficacy in alleviating ulcerative colitis induced by Dextran Sulfate Sodium Salt (DSS) and expediting the resolution of inflammation by modulating the NF-κB signaling pathway (Liu et al., 2016). Anil Kumar et al. have made the discovery that small RNAs present in ginger-derived nanoparticles (GDNP) possess the ability to influence the composition of the intestinal microbiota, enhance the functionality of the intestinal barrier, and stimulate the expression of interleukin-22 through the activation of the arylhydrocarbon receptor pathway. These effects ultimately contribute to the potentiation of *Lactobacillus rhamnosus* GG-mediated suppression of colitis in mice (Kumar et al., 2022). Professor Cao's team found that EVs-liked ginseng-derived nanoparticles (GDNPs) change macrophage polarization to inhibit melanoma growth (Cao et al., 2019), and can reprogram the tumor microenvironment to enhance the efficacy of immune checkpoint antibodies (Han et al., 2022).

The team of Chengyu Jiang of Peking Union Medical College extracted the exosome containing Hongjingtian-derived small RNA m7 (HJT-sRNA-m7) from a CHM *Rhodiolae Crenulatae Radix* et *Rhizoma* (Hongjingtian in Chinese, HJT), and confirmed its good anti-pulmonary fibrosis effect through *in vivo* and *in vitro* experiments. The study found that HJT-derived EVLP (decoctosome) plays an important role in reducing disease symptoms in mice and further determines the value of CHM-EVLP as new active ingredients. The decoctosome exhibits biological activity, and sRNA is assembled with the lipid components of the decoctosome (Du et al., 2019).

The findings reveal that CHM-EVLP, including polyjuice decoctosome, can be taken by the human body, and the molecules contained in them, such as microRNA (miRNA), can interact with target organs and genes to prevent and treat diseases, and also confirm from the side that CHM-EVLP can be used as a new active ingredient of CHM.

Consensus opinion 10

It is imperative to conduct a series of studies on various Chinese medicines due to their distinct growth periods, varying medicinal efficacy, and differential types and expression levels of biomarkers in the EVs they produce.

The diverse processing techniques employed in CHM underscore its therapeutic efficacy. Consequently, it is imperative to investigate the potential impact of these processing methods on the functionality of EVs released by CHM.

The efficacy of different parts of the same CHM is different to some extent. Analyzing and comparing the content, biological activity, and drug delivery capability of partial vesicles will establish a foundation for the standardization of CHM-EVLP.

7. Application of CHM-EVLP

The study demonstrates that CHM-EVLP exhibit favorable biocompatibility and can be readily obtained in substantial quantities, thereby presenting promising clinical application potential and prospects. Phytodrugs, comprising the primary constituents of TCM, have been extensively investigated for their secondary metabolites, including flavonoids, saponins, and alkaloids. However, the complex nature of TCM, characterized by its multi-component, multi-target, and multi-pathway attributes, has hindered the effective elucidation of the active components and underlying mechanisms of numerous TCMs. MiRNAs derived from plants have been identified in human milk and have the potential to impact various crucial biological pathways in infants (Benmoussa et al., 2016). A study conducted by Professor Jin Hailin's research group at the University of California, Riverside, USA, has demonstrated that host Arabidopsis cell derived EVs can transfer small RNAs (sRNAs) to the fungal pathogen botrytis, resulting in its enrichment and subsequent gene silencing of key pathogenic factors (Liu et al., 2020). Furthermore, ongoing research is being conducted to explore the role of the EVs as active components in TCM.

The utilization of resistance and defense mechanisms. Arabidopsis-derived EVs play a role in the immune response of plants. Following infection by *Pseudomonas*, Arabidopsis releases EVs that are abundant in defense proteins associated with biological stress response (Liu et al., 2020). Cai et al. conducted a comprehensive investigation to confirm the antifungal capabilities of Arabidopsis EVs, demonstrating that EVs enriched with specific proteins accumulated at the infection site to internalize mRNA silencing target genes involved in lysis (Cai et al., 2018).

Consensus opinion 11

In the context of treating internal injuries, Chinese herbal medicine is typically administered orally, while for external injuries, it is commonly applied externally. In order to investigate the effects of Chinese herbal medicine, researchers may opt for a consistent mode of administration, with oral administration being the preferred choice. However, to study the therapeutic mechanisms, various drug delivery methods can be employed, including oral delivery, caudal vein injection, intraperitoneal injection, among others. Simultaneously, it is imperative to conduct a meticulous examination of particular issues in accordance with the prevailing disease scenario, such as the technique involving lateral ventricle injection in Alzheimer's disease.

CHM-EVLP have been extensively employed in various applications such as skin regeneration, immune modulation, anti-inflammatory effects, anti-tumor properties, among others. Ginger nanoparticles can prevent alcohol-induced liver injury and inhibit the pathogenicity of *Plimonas gingivalis* (Zhuang et al., 2015).

CHM-EVLP can be used in clinical medicine (enhancing efficacy and reducing toxicity, targeted therapy), beauty, daily chemical and other fields.

In the realm of medical aesthetics and skincare, CHM-EVLP serve as carriers for potent bioactive compounds, including proteins and active factors secreted by protocells. These vesicles effectively activate the regenerative capabilities of cells, releasing encapsulated nutrients upon skin infiltration. This continuous nourishment and adjustment of the cellular microenvironment enhance cell vitality, stimulate cell migration, and facilitate collagen production and elasticity, thereby promoting skin rejuvenation. Additionally, EVs extracted from leaves and stems of *Dendropanax morbifera* exhibit the ability to diminish melanin production and tyrosinase activity, thereby facilitating skin whitening (Kim et al., 2020).

The utilization of CHM-EVLP in the context of anti-inflammatory and wound healing purposes involves their ability to facilitate tissue repair, stimulate cell proliferation, regulate immune responses, and effectively reach remote injury sites via capillaries. Furthermore, their potential to be synergistically combined with established active drugs enhances therapeutic efficacy, fosters inflammation resolution, and promotes wound healing.

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Ensuring the safety, yield, purity, and standardization of CHM-EVLP is crucial to consider when contemplating their indication, as it serves as a prerequisite for achieving application transformation.

8. Other issues to explore

With the advancement of research in the domain of EVs, it is anticipated that they will serve as a significant conduit for the transportation of drugs, medical beauty products, skincare treatments, and vaccines, thereby functioning as a novel nano delivery system. Research investigations have revealed that ginseng vesicles possess the ability to induce neural differentiation in stem cells through the transportation of miRNA (Xu et al., 2021). Similarly, lemon vesicles, when loaded with doxorubicin, can effectively surmount cancer resistance (Xiao et al., 2022). Additionally, grapefruit vesicles, upon fusion and activation of white cell membranes, exhibit the potential to carry anticancer drugs for tumor eradication (Niu et al., 2021). The exceptional natural targeting ability of CHM is primarily attributed to its traditional warping characteristics. By means of engineering modification, this medicine can be effectively directed towards specific tissues and organs, thereby addressing the targeting challenges that conventional delivery systems struggle to resolve. Nanoscale vesicles, capable of transporting cell surface molecules, exhibit the capacity to surmount biological barriers while offering advantages of safety, cost-effectiveness, and convenient storage.

The expansive landmass, intricate topography, and pronounced variations in climate and soil composition within China offer distinctive ecological circumstances conducive to the proliferation and propagation of diverse medicinal flora, thereby resulting in abundant resources of medicinal materials. Moreover, the regional specificity, multifariousness, safety, and consistency of CHM further augment the intricacy associated with investigating CHM-EVLP.

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Functional subjects preferentially selected authentic medicinal materials. To improve the repeatability of the experiment, select the herbs with specific environment, growth years and stable curative effect. Researchers are encouraged to conduct systematic research on a certain Chinese herbal medicine in different places and different growth years. It will be a great breakthrough in the modernization of Chinese medicine if we can combine the characteristics of CHM-EVLP with the characteristics of Chinese medicine four natures and five flavors the fluctuations of rise and fall, and explain the theory of traditional Chinese medicine with modern research.

9. Summary and outlook

Chinese herbal medicine is the precious wealth accumulated by the Chinese nation in the process of long-term struggle against diseases. The *Outline of the Strategic Plan for the Development of Traditional Chinese Medicine* issued by the State Council pointed out that on the basis of inheriting and carrying forward the advantages and characteristics of TCM, making full use of modern science and technology to promote the modernization and internationalization of TCM to meet the development of the Times and the growing needs of the people for medical care is the responsibility entrusted to us by history. CHM has low immunogenicity, high safety, and can be produced in large quantities at low cost, so that CHM vesicles can prevent and treat diseases or produce coordinated therapeutic effects as drug transport carriers. However, there are also some challenges: development and standardization of vesicles purification methods, screening of specific markers, biological functions, etc. The study and application of Chinese herbal vesicles is a new opportunity for the modernization of Chinese medicine.

The consensus gathers the wisdom of domestic experts and scholars in the fields of CHM and CHM-EVLP, enriches the medical source, absorbs the ancient and seeks the new, fully combines and gives play to the unique advantages of TCM, and hopes to lead the high-quality development of CHM-EVLP research at home and abroad, achieve higher, faster and stronger breakthrough results, and effectively promote the inheritance and innovation of TCM.

The consensus has been developed in accordance with the guidelines outlined in *the Reporting Standards for the Consensus of Experts in the Clinical Application of Traditional Chinese Medicine*.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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