Centella asiatica Tissue Culture-Derived Extracellular Vesicles: A Multifaceted Approach to Skincare Applications

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Abstract

Background: *Centella asiatica* has long been used as a medicinal herb in traditional Asian medicine. Its wound healing, skin improvement, and neuroprotective properties have been widely studied. Extracellular vesicles (EVs) are secreted by cells and contain bioactive components with therapeutic properties.

Objectives: This study aims to characterize EVs isolated from *C. asiatica* tissue culture and investigate their therapeutic properties using *in-vitro* assays.

Methods: EVs were isolated from *C. asiatica* tissue culture and their properties were characterized by cell-free chemical assays which investigated free radical activity, metal chelation, and total polyphenol content. Mouse melanoma, human fibroblast and keratinocyte cell cultures were used to characterize cell viability, intracellular reactive oxygen species (ROS), intracellular melanin and tyrosinase assay, skin integrity and pro-inflammatory gene expression level by quantitative PCR.

Results: *C. asiatica* EVs were found to contain high levels of polyphenol, which contributed to their radical scavenging and metal chelating properties and mitigated hydrogen peroxide-induced intracellular ROS. The EVs were further able to reduce intracellular melanin content and tyrosinase activity, and upregulate skin barrier genes procollagen type I, aquaporin 3, and filaggrin. The inhibitory effect of *C. asiatica* EVs on inflammation was evident in downregulation of expression of pro-inflammatory genes COX2 as well as nitric oxide production.

Conclusions: The beneficial effects of *C. asiatica* EVs warrant further studies as promising formulations in skin care applications.

Keywords: *Centella asiatica*, traditional medicine, extracellular vesicles, antioxidants, cosmetics, anti-inflammatory, skin whitening

Introduction

Centella asiatica, also commonly known as Gotu Kola, is a traditional medicinal herb widely used across Asia [1]. Its broad spectrum of therapeutic properties is attributed to a diverse phytochemical profile, including triterpenoids, flavonoids, and polyphenols, which contribute to the plant's anti-inflammatory, antioxidant, and wound-healing properties, as well as its neuroprotective and cognitive-enhancing effects in animal studies [2-4]. Several key triterpenes, including madecassoside, madecassic acid, asiaticoside, and asiatic acid, are well-studied compounds that play primary roles in the above therapeutic effects [4, 5]. Due to its wide-ranging therapeutic benefits, *C. asiatica* has garnered interest in modern pharmacology for potential applications as a natural treatment in chronic inflammation, neurodegenerative diseases, and skin improvement.

Extracellular vesicles (EVs) are membrane-bound particles of various sizes and compositions released by cells into the extracellular environment, serving as carriers of bioactive molecules such as proteins, lipids, and nucleic acids [6]. EVs are classified based on size, origin, and markers: exosomes (30–150 nm), which originate from the multivesicular bodies in the endosomal pathway; microvesicles (100–1000 nm), which bud directly from the plasma membrane; apoptotic bodies (1000-5000 nm), which are formed from membrane blebbing in apoptotic cells [6, 7]. These vesicles play essential roles in intercellular communication, modulating physiological and pathological processes by delivering molecular signals to recipient cells [6]. Due to their stability and biocompatibility, EVs have been explored for applications ranging from drug delivery and diagnostics to regenerative medicine, as well as skin therapies via non-invasive topical applications [7, 8].

Plant-derived EVs share similarities with mammalian EVs. For example, exosome-like particles in plants also form from multivesicular bodies akin to processes in mammalian cells [9]. Recently, plant-derived EVs have attracted considerable attention as an alternative to mammalian EVs due to their comparable therapeutic potential and additional advantages in safety, scalability, cost-effectiveness, and ethical sourcing [9, 10]. Plant-derived RNA, particularly microRNAs (miRNAs), can be found in EVs and play a significant role by influencing gene expression in recipient cells, particularly via cross-kingdom communication, where they can modulate the expression of target genes in other organisms, including humans [11]. Recent studies have shown that exosome-like EVs from plants, including *C. asiatica,* promote skin improvement and wound healing more effectively than conventional plant extracts [12]. For instance, *C. asiatica* exosome-like EVs can upregulate the COLA1 collagen gene and downregulate the MMP1 matrix metalloproteinase gene, and inhibit melanogenesis genes [13]. Furthermore, *C. asiatica* exosome-like EVs can be absorbed into human live cancer cell culture within 24 hours and exhibit anticancer effects by promoting apoptosis of cancerous cells [14].

Many medicinal plants, including *C. asiatica,* are subject to overharvesting, which threatens biodiversity and ecosystem health. By using tissue culture techniques, EVs can be obtained without putting strain on ecological resources, supporting conservation efforts and ethical sourcing while meeting production demand [15]. In light of the therapeutic potential of *C. asiatica* and the unique

77 properties of EVs as skin-penetrating biomolecular vehicles, this study aims to investigate the *in-vitro*
78 properties of EVs isolated from *C. asiatica* tissue culture.
79
80 **Materials and Methods**
81 Isolation and characterization of *Centella asiatica* extracellular vesicles
82 *C. asiatica* EVs were extracted and isolated by a multi-step filtration and centrifugation process.
83 Briefly, tissue culture *C. asiatica* was homogenized, coarsely filtered to remove large debris, and
84 centrifuged. Pellets were collected and filtered through a 0.45 µm filter, extracted and finally filtered
85 through a 0.45/0.22 µm membrane filter.
86 The size and morphology of the *C. asiatica* EVs were analyzed with nanoparticle tracking analysis
87 (NTA) and transmission electron microscopy (TEM). The NTA was performed with ZetaView® system
88 (Particle Metrix, Germany). A sample for TEM was prepared by dropping 10 µL of the specimen onto a
89 copper grid, followed by rest for 2 minutes and blotted dry with filter paper. Ten µL of uranyl acetate
90 was then applied to the grid and excess liquid was again dried with filter paper, and the process was
91 again repeated. Afterward, the grid was air-dried and subjected to the TEM procedure.
92 Cell culture
93 The B16F10 cell line (mouse melanoma cells, ATCC CRL6475, BCRC60031) was obtained from the
94 Bioresource Collection and Research Center (BCRC), Taiwan. The cells were maintained in Dulbecco's
95 Modified Eagle Medium (DMEM) (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum
96 (FBS) and 1% antibiotics. Human keratinocyte line HaCaT (AddexBio T0020001) was grown in
97 high-glucose DMEM supplemented with 10% FBS, 4 mM/L-glutamine, and 1% antibiotic-antimycotic
98 solution. Human foreskin fibroblast line HS68 (BCRC 60038) was grown in high-glucose DMEM
99 supplemented with 10% FBS, 4 mM/L-glutamine, and 1% antibiotic-antimycotic solution. Human
100 keratinocytes line Detroit 551 (BCRC 60118) was grown in Minimum Essential Medium (MEM)
101 supplemented with 10% FBS, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. All
102 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.
103 Cell viability assay
104 The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) method was performed
105 for B16F10 cells. Briefly, the cells were exposed to various concentrations of *C. asiatica* EVs for 24
106 hours, and the MTT solution was then added to the wells. The insoluble derivative of MTT produced
107 by intracellular dehydrogenase was solubilized with an ethanol-DMSO (1:1) mixture. The absorbance
108 of the wells at 570 nm was read using a microplate reader. For HaCaT and Detroit 551 cells, 1/10
109 volume of alamarBlue® (resazurin) reagent was added directly after 24 hours of EV treatment and
110 incubated for 4 hours at 37°C. The absorbance was then read at 570 nm with 600 nm as a reference.
111 In both assays, cell viability was calculated as the ratio of absorbance of the treated cells to control.
112 Thiazoline-6-sulfonic acid (ABTS) free radical scavenging assay
113 The ABTS free radical assays were conducted as previously outlined [16]. A 7 mM stock solution
114 of ABTS was reacted with 2.45 mM potassium persulfate, and the mixture was left to stand in the dark

115 for at least 6 hours before use. The absorbance at 734 nm was measured immediately after mixing
116 various concentrations of *C. asiatica* EVs (4×10^6, 1×10^7, and 2×10^7 particles/mL) with 1 mL of ABTS
117 solution to detect changes within 10 minutes. Vitamin C (0.9 mg/mL) and BHA (0.9 mg/mL) were used
118 as positive controls.

119 Intracellular reactive oxygen species (ROS) assay

120 The intracellular ROS assay was performed using an established method [17]. B16F10 cells were
121 cultured in 24-well plates and treated with various concentrations of *C. asiatica* EVs (1×10^9, 2.5×10^9,
122 and 5×10^9 particles/mL), Trolox® (0.05 mg/mL, positive control), or none (negative control) for 24
123 hours. The cells were then incubated with 24 mM H_2O_2 for 30 minutes to induce oxidative stress. After
124 incubation, 2′, 7′-dichloro-fluorescein diacetate (DCFH-DA) was added to the cells and cultured for 30
125 minutes. After treatment, the cells were washed with PBS and trypsinized with trypsin/EDTA. The
126 fluorescence intensities of DCF were measured at an excitation wavelength of 504 nm and an emission
127 wavelength of 524 nm using a Fluoroskan Ascent fluorescent reader (Thermo Scientific, Vantaa,
128 Finland).

129 Total polyphenol content assay

130 Different concentrations of *C. asiatica* EVs (2×10^7, 4×10^7, 5×10^7 particles/mL) and gallic acid
131 standard were mixed with Folin-Ciocalteu reagent and incubated for 5 minutes. Sodium carbonate
132 solution was added and incubated for 30 minutes. The absorbance at 765 nm was measured using a
133 spectrophotometer and the gallic acid equivalent was calculated with the gallic acid standard curve
134 [18].

135 Metal chelating assay

136 The metal chelating activity of the extracellular vesicles was performed with potassium
137 ferricyanide ($K_3[Fe(CN)_6]$) assay [19]. Different concentrations of *C. asiatica* EVs (2×10^7, 5×10^7, 1×10^8
138 particles/mL) were mixed with 0.2 mM PBS and 1% (W/V) $K_3[Fe(CN)_6]$, with 2 mg/mL EDTA as the
139 positive control and deionized water as the blank control. The mixtures were heated at 50°C for 20
140 minutes. After cooling to room temperature, 10% (W/V) TCA was added, and the mixtures were
141 centrifuged at 12,000 rpm and 4°C for 5 minutes. The supernatants were transferred to a 96-well plate,
142 followed by the addition of deionized water and 1% (W/V) $FeCl_3$. The plates were incubated in the
143 dark for 30 minutes. Absorbance was measured at OD 700 nm to assess metal chelating activity.

144 Intracellular melanin content assay

145 The intracellular melanin content was measured as described previously [20]. B16F10 cells were
146 treated with α-Melanocyte-stimulating hormone (α-MSH, 100 nM) for 24 h to induce melanin
147 production. Following this, the cells were treated with different concentrations of *C. asiatica* EVs
148 (1×10^9, 2.5×10^9, and 5×10^9 particles/mL), or the positive control, arbutin (0.54 mg/mL), for an
149 additional 24 hours. Post-treatment, cell pellets containing a known number of cells were solubilized
150 in 1 N NaOH at 60°C for 60 minutes. The melanin content was then assayed at 405 nm.

151 Intracellular tyrosinase activity assay

152 The intracellular tyrosinase activity was determined as described in previous research [21].

153 B16F10 cells were treated with α-Melanocyte-stimulating hormone (α-MSH, 100 nM) for 24 hours to
154 induce melanin production. Following this, the cells were treated with different concentrations of *C.*
155 *asiatica* EVs (1×10^9, 2.5×10^9, and 5×10^9 particles/mL), or the positive control, arbutin (0.54 mg/mL),
156 for an additional 24 hours. After treatments, the cell extracts (100 μL) were mixed with freshly
157 prepared L-DOPA solution (0.1% in PBS) and incubated at 37°C. The absorbance at 490 nm was then
158 measured to assess the tyrosinase activity.

159 Type I procollagen (PIP) ELISA assay

160 Hs68 cells were seeded at 1×10^5 cells per well in 12 well plates and were cultured in 10% FBS
161 DMEM medium at 37°C with 5% CO_2 for 24 hours. After three washes with PBS, several different
162 concentrations ($1x10^7$, $1x10^8$, $1x10^9$) of *C. asiatica* EVs and 5 ng/mL TGF-β1 (AbCam Cat. #ab50036, as
163 positive control) were added and cultured for 24 hours. The level of type I procollagen in cell culture
164 supernatants was determined using a commercial PIP EIA Kit (TAKARA Cat. # MK101) according to the
165 manufacturer's instruction (2 step procedure).

166 mRNA expression of aquaporin-3 (AQP3) and filaggrin (FLG) genes

167 HaCat cells were dispensed into a 96-well plate and cultured for 24 hours under cell culture
168 conditions. Various concentrations of *C. asiatica* EVs and negative control (purified water only) were
169 added to the cells and cultured for 24 hours. Real-time PCR was performed to confirm the expression
170 of aquaporin-3 (AQP3) and filaggrin (FLG) at the gene level, and the test sequence is as follows. For
171 RNA isolation and cDNA synthesis, SuperPrep TM cell lysis & RT Kit for qPCR (TOYOBO, Cat. SCQ-101)
172 was used. To compare and analyze gene expression, the cDNA synthesized above was used as a
173 template, and Real-time PCR analysis was performed using Thunderbird TM SYBR qPCR Mix (TOYOBO,
174 Cat. QPS-201). The gene expression levels of the sample were quantified by normalization with
175 GAPDH using the $2^{-\Delta\Delta Ct}$ method.

176 mRNA expression of pro-inflammatory genes after UVB induction

177 HaCat cells ($1x10^4$/well for 96-well) or Hs68 cells ($5x10^4$/well for 24-well) were cultured for 24
178 hours and exposed to UVB at 90mJ dose. Various concentrations of *C. asiatica* EVs and negative
179 control (purified water only) were added to the cells and cultured for 4 hours. Real-time PCR was
180 performed to confirm the expression of cyclooxygenase-2 (COX) gene as above and the gene
181 expression levels were quantified by normalization with GAPDH using the $2^{-\Delta\Delta Ct}$ method.

182 Measurement of nitric oxide production

183 RAW 264.7 macrophage cells were seeded in 10% FBS DMEM medium in 24-well plates at a
184 density of 2×10^5 cells/well and were cultivated at 37°C with 5% CO_2 for 24 hours. Removing the
185 original medium, the cells were treated with $1x10^7$, $1x10^8$, $5x10^8$, $1x10^9$, $2x10^9$, or $4x10^9$ particles/mL
186 of *C. asiatica* EVs or gallic acid (10 and 20 μg/mL, Sigma-Aldrich Cat. #G7384) in serum-free DMEM
187 medium and incubated for 4 hours. After the pre-incubation, the cells were stimulated with 100
188 ng/mL LPS and incubated for 20 hours. The culture supernatants were harvested and centrifuged at
189 7,000 × g for 3 minutes. The samples were mixed with an equal volume (100 μL) of Griess reagent
190 (Sigma-Aldrich Cat. #G4410) and the absorbance was measured at 540 nm after reacting for 10

191 minutes.

192

193 **Results**

194 Isolation and characterization of *C. asiatica* EVs

195 *C. asiatica* EVs were isolated from tissue culture *C. asiatica* by a centrifugation and filtration
196 process. The size distribution of EVs harvested was analyzed with ZetaView® NTA system and showed
197 that the particle has a mean size of 150 nm (Figure 1A). The morphological features shown by the TEM
198 images can be observed as vesicles of approximately 100 nm in size with a lipid bilayer membrane
199 structure (Figure 1B).

200 Cytotoxicity assessment

201 To investigate the effect of *C. asiatica* EVs in cells, an MTT assay was used to evaluate the effect
202 of cell viability. B16F10 melanoma cells were treated with various concentrations of *C. asiatica* EVs
203 and MTT assay was performed. The results showed no significant differences compared to the control
204 group in this test (Figure 2). Thus, high concentrations of up to 5×10^9 particles/mL of *C. asiatica* EVs do
205 not exhibit cytotoxicity in B16F10 cells. In addition, we investigated the cytotoxicity in two human cell
206 lines using the alamarBlue® reagent. No discernable differences in cell viability were detected in either
207 human cell line, confirming that the EVs do not exhibit cytotoxicity in mouse or human cells (Figure
208 S1).

209 Antioxidant activity assessment

210 Here we first assessed the antioxidant activity of *C. asiatica* EVs by using two cell-free assays:
211 ABTS (Thiazoline-6-sulfonic acid) and potassium ferricyanide ($K_3[Fe(CN)_6]$) assays. At the higher
212 concentrations, *C. asiatica* EVs exhibited comparable $ABTS^{+\cdot}$ scavenging capacity to positive controls
213 vitamin C and BHA (Figure S2). The results indicated that the *C. asiatica* EVs can scavenge a significant
214 amount of $ABTS^{+\cdot}$ radical. For the $K_3[Fe(CN)_6]$ metal chelating assay, the results of the reducing power
215 test showed that the reducing capacities of EVs at concentrations of 2×10^6, 5×10^6, and 1×10^7
216 particles/mL were 16.18 ± 0.4%, 45.98 ± 1.64%, and 84.75 ± 0.11%, respectively (Figure S3). The
217 results indicated that the reducing power of *C. asiatica* EVs increases with the particle concentration,
218 exhibiting a dose-dependent effect.

219 A variety of naturally-occurring polyphenol compounds from *C. asiatica* extracts has been
220 studied, which contribute to its antioxidant properties [5]. Here we investigated the total polyphenol
221 content of *C. asiatica* EVs using an established method with the Folin-Ciocalteu reagent. The results
222 showed that the gallic acid equivalent of *C. asiatica* EVs increased with the particle number, indicating
223 that the antioxidant activity of the vesicles may be dose-dependent (Figure 3).

224 To further confirm the antioxidant capacity of *C. asiatica* EVs in a cellular environment,
225 intracellular reactive oxygen species (ROS) levels were measured by ROS scavenging rate using H_2O_2 at
226 20 mM to induce oxidative stress. After treatment, the intracellular ROS clearance was 53.86 ±
227 0.94%, 59.32 ± 1.68% and 66.27 ± 1.10% for $1x10^9$, $2.5x10^9$ and $5x10^9$ (particles/mL) of *C. asiatica*
228 EVs, respectively (Figure 4). The results showed that all concentrations of *C. asiatica* EVs significantly

229 increased ROS scavenging activity compared to the control group (P<0.001), demonstrating effects
230 similar to positive control Trolox.

231 Melanin inhibitory activity assessment

232 In addition to its antioxidant properties, *C. asiatica* has potential skin whitening application
233 through inhibition of melanin synthesis [22]. First, we investigated its inhibition of melanin production
234 in B16F10 melanoma cells treated with α-melanocyte-stimulating hormone (α-MSH). After *C. asiatica*
235 EV treatment, the melanin content in the B16F10 cells was 93.55 ± 1.14%, 90.73 ± 1.17% and 83.69 ±
236 4.31% of control at 1×10^9, 2.5×10^9 and 5×10^9 particles/mL of *C. asiatica* EVs, respectively (Figure 5).
237 For the positive standard arbutin (0.54 mg/mL), the remaining intracellular melanin content was 71.95
238 ± 2.44% of the control. The results indicated that *C. asiatica* EVs could decrease the melanin content in
239 B16F10 melanoma cells. Tyrosinase catalyzes the first two steps of melanin synthesis by hydroxylation
240 of L-tyrosine to L-DOPA [23]. Therefore, to investigate the change in melanin synthesis via tyrosinase
241 activity, we have carried out intracellular tyrosinase activity in B16F10 cells treated with α-MSH. After
242 treatment, the remaining intracellular tyrosinase activity was 92.5 ± 6.77%, 80.2.7 ± 5.13% and 56
243 ± 3.83% for the $1x10^9$, $2.5x10^9$ and $5x10^9$ particles/mL of either *C. asiatica* EVs, respectively (Figure 6).
244 In comparison, the remaining intracellular tyrosinase activity was 66 ± 10.53% of control after the cells
245 were treated with arbutin, a known skin-whitening compound. The results indicated that $5x10^8$
246 particles/mL of *C. asiatica* EVs exhibited stronger inhibitory effect on α-MSH induced tyrosinase
247 activity in B16F10 cells than arbutin, albeit with higher intracellular melanin content than arbutin
248 (Figures 5 and 6).

249 Effect of *C. asiatica* on skin integrity-related genes

250 One of the major component in skin tissue is type I collagen, which is derived from its precursor
251 type I procollagen (PIP) secreted by skin fibroblasts, and lowered expression of PIP is associated with
252 skin UV damage and senescence leading to wrinkle formation [24]. In this experiment Hs68 fibroblast
253 cells were used to investigate the effect of induction of PIP secretion by *C. asiatica* EVs. Results
254 showed increased production of PIP in Hs68 cells in a dose-dependent manner, and induced a similar
255 expression level of PIP to that of positive control TGF-β1 at the highest concentration tested ($1x10^9$
256 particles/mL) (Figure S4). Aquaporin and filaggrin are important in barrier function of skin by
257 regulating water transport and water retention in skin [25]. We used a real-time PCR assay to quantify
258 the expression of mRNA expression of aquaporin-3 (AQP3) and filaggrin (FLG) genes in HaCat
259 keratinocyte cells treated with *C. asiatica* EVs. The results show that the addition of *C. asiatica* EVs to
260 HaCaT cell cultures was found to upregulate the expression levels of AQP3 and FLG genes compared to
261 the control group (Figure S5).

262 Anti-inflammatory effects of of *C. asiatica* EVs

263 One mechanism of the anti-melanogenic effect by *C. asiatica* is the inhibition of UV-induced
264 inflammation by triterpene madecassoside [22]. Here we used the expression levels of
265 pro-inflammatory gene COX2 to gauge the modulation of inflammation by *C. asiatica* EVs. Skin
266 fibroblast Hs68 and keratinocyte HaCaT cells were subjected to UV-induced inflammation and *C.*

267 *asiatica* EVs were then added, and expression levels of COX2 gene were measured. In HaCaT cells, EVs
268 reduced COX2 expression in a dose-dependent manner; however, in Hs68 even though the overall COX
269 expression reduced when the cells were treated with EV, no dose-dependent effect was observed
270 (Figure S6). Furthermore, as nitric oxide (NO) is an inflammatory response messengers secreted by
271 endothelial cells and macrophage, we subjected *C. asiatica* EV-treated RAW 264.7 macrophage culture
272 to lipopolysacchiaride (LPS), which is a TLR4 ligand and induces pro-inflammatory response [26]. The
273 NO production was measured by Griess reagent, which is converted to a pink color after reacting with
274 nitrites. As shown in Figure 7, the amount of nitrite decreases in a dose-dependent manner in cells
275 treated with increasing concentrations of EVs. Taken together, *C. asiatica* EVs were able to reduce
276 inflammatory response in a variety of cell types tested.
277

278 **Discussion**

279 In this study, we have isolated and characterized EVs secreted from *C. asiatica* tissue culture. The
280 EVs have a size of approximately 160 nm and a lipid bilayer morphology similar to that previously
281 reported for exosomes derived from *C. asiatica* and other plants [12, 13]. In a series of *in-vitro*
282 experiments in B16F10 cell culture, we have demonstrated that the EVs are safe for cells and that EVs
283 exhibited notable antioxidant, anti-melanogenic, and anti-inflammatory effects, suggesting potential
284 applications in skin care and therapeutic treatments to combat oxidative stress and
285 hyperpigmentation. Another study also isolated exosome-like EVs from *C. asiatica* but with a smaller
286 average particle size of 63.8 nm [14]. While the EVs from the above study exhibited cytotoxicity in
287 HepG2 human cancer cells, we did not observe any effect on cell viability in mouse melanoma B16F10
288 cells or HaCaT and Detroit 551 human cell lines tested (Figures 2 and S1) [14]. The therapeutic
289 properties of *C. asiatica* have mostly been attributed to the triterpenes and derivatives, including
290 madecassoside, madecassic acid, asiaticoside, and asiatic acid, and these are among the most
291 well-studied compounds in the plant [3-5]. In our study, we observed potent antioxidant effects from
292 *C. asiatica*, although further study is required to determine specifically how contents within EVs
293 (chemicals, proteins, and nucleic acids) can contribute to observed effects. In addition to
294 phytochemicals and proteins, small RNAs and miRNAs in EVs are important modulators of target effect
295 proteins, and RNA binding proteins, such as Argonaute 1, RNA helicases, and Annexins, may be
296 involved in packing RNA into EVs [10, 28] A recent preprint study has provided a comprehensive
297 analysis of the transcriptome human skin keratinocytes after treatment of *C. asiatica* exosomes,
298 revealing 11 novel miRNAs packed within the exosomes [13]. The above study also found that skin
299 integrity and barrier-related genes were upregulated after treatment by *C. asiatica* EVs, which we
300 have also observed in this study with increasing gene expression of PIP1, AQP3, and FLG (Figures S4
301 and S5) [13]. The study also found that *C. asiatica* exosomes induced higher gene expression than *C.*
302 *asiatica* extracts, possibly due to better penetration of skin cells by exosomes and the presence of
303 miRNAs [13]. Thus *C. asiatica* EVs could provide its effect via multitude of effectors such as
304 phytochemicals and miRNAs.

In *C. asiatica,* madecassoside by itself has the ability to inhibit UV-induced melanin synthesis in a keratinocyte and melanocyte co-culture system as well as artificially tanned human skin [22]. Other plant-derived EVs also have anti-melanogenic effect. For example, *Dendropanax morbifera* EVs can inhibit the expression of melanogenesis genes such as MITF, TYR, TRP-1, and TRP-2 [9, 27]. In our study, the EVs appeared to modulate melanogenesis pathways in cell culture, likely through the inhibition of tyrosinase activity, which is a key enzyme in melanin synthesis [23]. The mechanism of anti-melanogenesis by *C. asiatica* include inhibition of inflammation, as observed in previous studies [24]. and in our study, where we have observed that *C. asiatica* can reduce pro-inflammatory gene expression, such as COX2 and decrease the production of NO (Figures 7 and S6). As melanin overproduction contributes to skin conditions like freckles and nevus formation, and age spot development, plant-derived EVs with anti-melanogenic properties represent an economical and natural alternative to conventional treatments, which often come with side effects or limited efficacy [27].

The tissue culture micropropagation technology for *C. asiatica* has been established as a sustainable source of *C. asiatica* [28]. This ensures its conservation in nature while providing a constant yield of raw material, akin to the use of bioreactors in biopharmaceutical drug and vaccine production. Using plant tissue culture as a source for EV production presents several advantages over traditional methods of harvesting from wild plants. First, tissue culture allows for consistent, scalable production of EVs with genetic uniformity, bypassing the variability associated with environmental factors that affect wild plants, such as seasonal changes, soil quality, and climate/geographical conditions [15, 28]. This standardization is particularly important for achieving reproducible results in therapeutic and cosmetic applications, where consistent quality and efficacy are crucial in ensuring the safety and efficacy of the product. Additionally, tissue culture can be optimized to enhance the yield of specific bioactive compounds through controlled environmental conditions and the use of elicitors [15].

In conclusion, this study investigated the antioxidant, anti-melanogenic and anti-inflammatory potential of *C. asiatica*-derived EVs from tissue culture. The advantages of tissue culture over wild plants in terms of sustainability, consistency, and the ability to enhance bioactive compound production further support the use of this approach in developing plant-based EV therapies. Future studies could focus on characterizing bioactive molecules and miRNAs within these EVs, as well as exploring their safety and efficacy in *in-vivo* models. Ultimately, this work paves the way for the sustainable production and utilization of plant-derived EVs in skin care cosmetics and wound healing applications.

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Competing Interests

C.-C. W., C.-H. C., P.-L. K., W.-H. T., L. T.-C. L., C. C., and T.-Y. K. are employees of Schweitzer Biotech Company. W.-Y. Q. and I. P. are consultants employed by Schweitzer Biotech Company. T.-M. C. and S.-S. W. have no competing interests to declare. This study is funded by Schweitzer Biotech Company.

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Figures

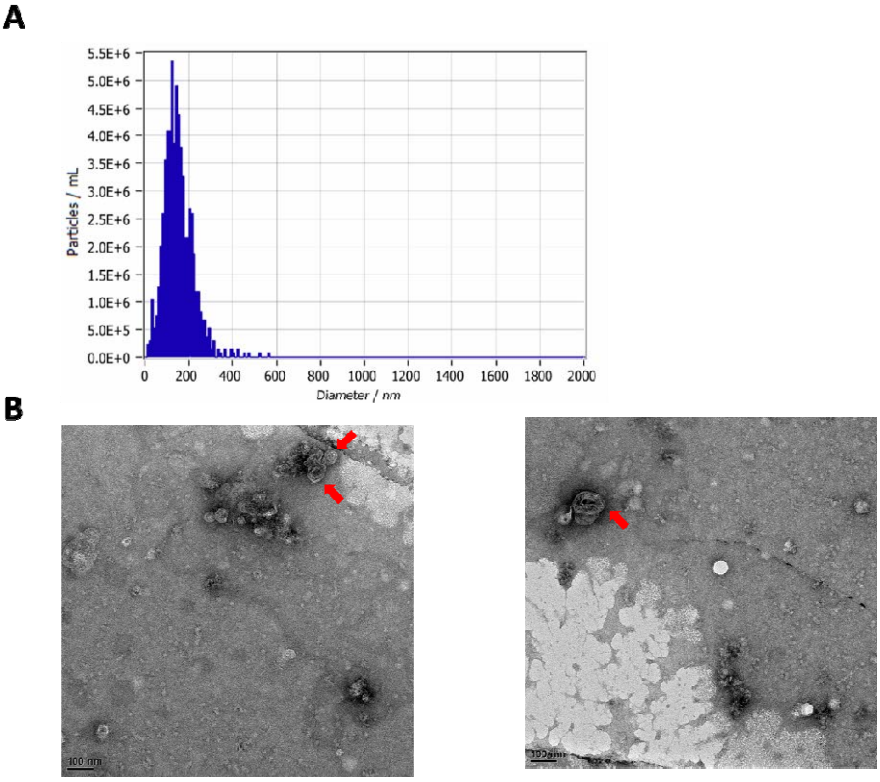

Fig. 1 Size and morphology characterization of *C. asiatica*-derived EVs. A. Nanoparticle tracking analysis (NTA) of size and concentration distribution of EVs by using the ZetaView® system. B. Representative TEM images of purified *C. asiatica* EVs with red arrows showing vesicles with lipid bilayer membrane structure. Scale bar: 100 nm

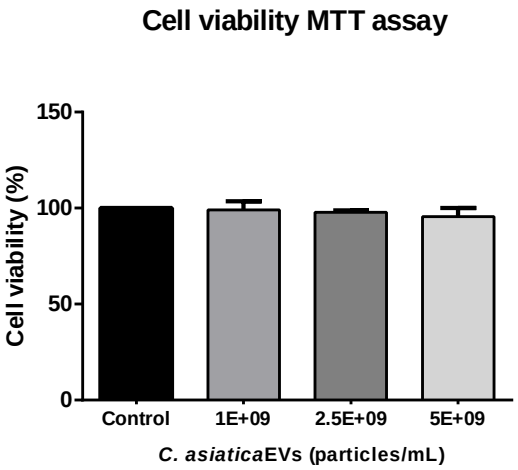

Fig. 2 Cell viability assay. B16F10 cells were treated with different concentrations of *C. asiatica* EVs. After 24h treatment, cell viability was measured by the MTT assay method. Data are presented as mean ± SD (n=3). Statistical results were calculated by one-way ANOVA with Dunnett's multiple comparisons test compared to the control group.

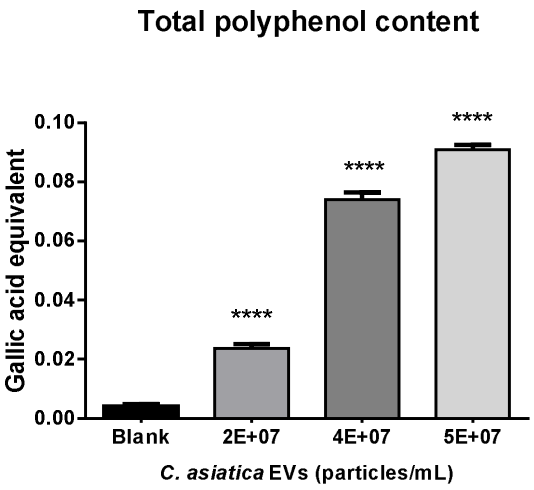

Fig. 3 Total polyphenol content. The total polyphenol content of *C. asiatica* EVs using the Folin-Ciocalteu method. Data are presented as mean ± SD (n=3). Statistical results were calculated by one-way ANOVA with Dunnett's multiple comparisons test compared to the control group). ****P<0.0001

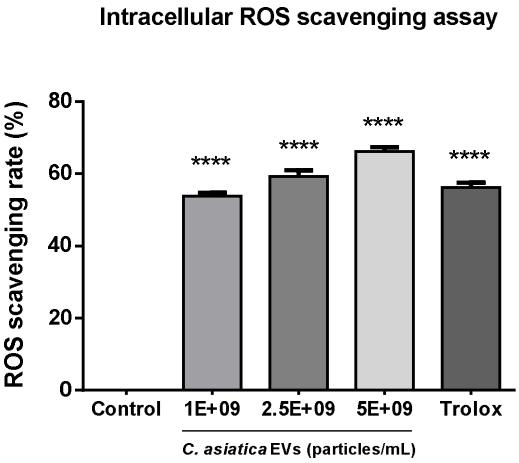

Fig. 4 Intracellular ROS level. B16F10 cells were pretreated with various concentrations of *C. asiatica* EVs, Trolox (0.05 mg/mL, positive control), or left untreated as a control for 24 hours. The cells were then incubated with 24 mM H_2O_2 and DCFH-DA. The fluorescence intensities of DCF were measured to calculate the ROS levels. Data are presented as mean ± SD (n=3). Statistical analysis was calculated by one-way ANOVA with Dunnett's multiple comparisons test, compared to the control group. ****P<0.0001

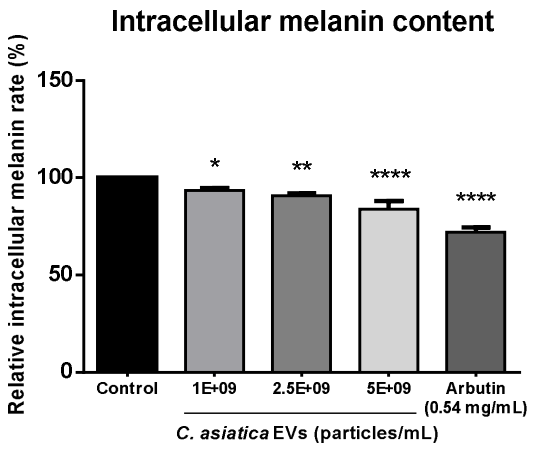

Fig. 5 Intracellular melanin content. Inhibitory effects of different concentrations of *C. asiatica* EVs on melanin content in B16F10 cells. Data are presented as mean ± SD (n=3). Statistical results were calculated by one-way ANOVA with Dunnett's multiple comparisons test compared to the control group). *P<0.05, **P<0.01, ****P<0.0001

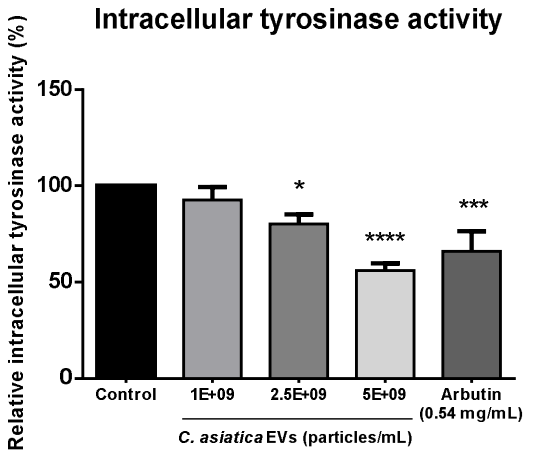

Fig. 6 Intracellular tyrosinase activity. Inhibitory effects of different concentrations of *C. asiatica* EVs on intracellular tyrosinase activity in B16F10 cells. Data are presented as mean ± SD (n=3). Statistical results were calculated by one-way ANOVA with Dunnett's multiple comparisons test (compared to the control group). *P<0.05, ***P<0.001, ****P<0.0001

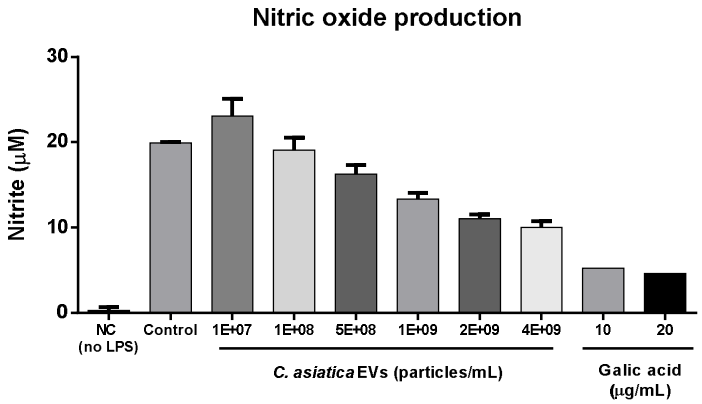

Fig. 7 Nitric oxide (NO) production in RAW 264.7 macrophages. Cells were treated with different concentrations of *C. asiatica* EVs or gallic acid after induction by LPS. The resulting NO produced was detected by the Griess reagent.