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Assessment of anti-cancer activity of human umbilical cord tissue-derived Eugenol primed mesenchymal stem cells-extracellular vesicles

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ABSTRACT

Colorectal cancer (CRC) is on the rise, with existing treatment modules showing little therapeutic success. Earlier studies have shown that the alteration of the tumor microenvironment was made possible by the secretory factors of mesenchymal stem cells (MSCs) which include extracellular vesicles (EVs), certain chemokines, cytokines, and other metabolites. MSC-EVs tend to mimic their parental cells, which possess characteristics like pro-tumor and anti-tumor effects. As conventional cancer treatments have shown adverse effects like anticancer drug resistance, the need for the use of natural products is a recent topic of interest. Eugenol, an active component of S. aromaticum, is known for its antioxidant, anti-inflammatory, and anti-cancer properties. This study focuses on the priming of Eugenol with the human umbilical cord tissue (hUCT) MSC derived EVs as an alternate therapeutic option to treat colorectal cancer, followed by EVs characterization. Finally, the anti-cancer activity of the formulated EVs was assessed by *in vitro studies* on colon cancer cells. The *in vitro* anticancer assay showed a significant reduction in the proliferation of cells when treated with Eugenol primed EVs. Thus, the anti-cancer potential of the Eugenol primed EVs was validated, which can be looked upon as a suitable alternative to a cell-free therapeutic option for treating CRC.

1. INTRODUCTION

Among the various prevailing cancers, lung, breast, and prostate cancers are the most widespread, followed by colorectal cancer (CRC). CRC incidence and increased mortality are mostly prevalent in countries where a western lifestyle is being adopted [1]. The growth in the inner lining of the colon and rectum, known as polyps, being benign initially, can eventually lead to the formation of CRC over the course of time. The polyps that could turn into cancer include adenomatous polyps and sessile-serrated polyps [2]. The important risk factor for tumorigenesis is considered to be chronic inflammation of the colon or rectum lining. Even though numerous treatment strategies are in use to fight CRC, the complete eradication of this life-threatening disease is still not available. It is because conventional treatment techniques like surgery, radiation, chemotherapy, etc. show limitations like poor solubility of the drug, resistance to anti-cancer drugs, non-specific targeting, thus affecting normal cells in an adverse way, and low retention effectiveness [3]. To date, colon cancer treatment remains unsatisfactory, even though many screening techniques have emerged.

The above-mentioned interventions can be minimized, thus eliminating the adverse effects caused by them, by using nanotechnology as a modality for cancer treatment [4]. Nanotechnology plays an important role in enhanced anticancer drug delivery mechanisms. Also, cell-based and cell-free immunotherapies offer various possibilities for cancer treatment. The hUCT is a promising source of MSCs because of its ease of collection, as it is painless and accompanied by a faster rate of self-renewal [5]. MSCs can be used to target cancer cells to deliver the cytotoxic effects as they have the potential to be home to cancer cells.. On the other hand, MSCs from certain sources could promote tumor growth. But, due to chronic inflammation being the major progression factor for CRC, studies say that hUCT-MSCs are an effective therapy for tumors [6]. Even though the clinical efficacy of MSC based cancer therapy was limited, the therapy showed safe results [7]. This contradiction is eliminated by using the mediators released from MSCs that possess the intrinsic behavior of migration

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to tumor cells, allowing efficient delivery of the drug and thus efficient targeting of the tumor cells [6]. Extra-cellular vehicles (EVs) include exosomes, microvesicles, and apoptotic bodies, which are cell secreted nanoparticles that are made of a bilateral lipid membrane structure [6]. Evidence shows that the EVs derived have the ability to inhibit the advancement of cancer cells through the delivery of their cargo compounds. The efficacy of the EVs is improved by engineering them in terms of loading them with therapeutic or bioengineered components like chemotherapeutic drugs, nucleic acid components, and priming them with certain phytochemicals that exhibit anti-cancer effects [8].

In the last few years, many studies have shown and proved the efficacy of EVs in the intercellular communication regulation of CRC and target cells. It has been studied that in CRC, the recipient's cell function and phenotype are regulated by EVs through the transfer of cancer associated cargos, thus altering the tumor microenvironment [8]. Experiments have shown that the EVs loaded with miRNAs have curbed the multiplication of colorectal cancer cells and have also shown pro-angiogenetic activity. Also, anti-cancer properties were seen in tumor derived EVs which were confined to HSP and MHC [9].

Meanwhile, increasing resistance to the drug and molecular unpredictability represent the drawbacks of using such drugs [10]. To eliminate these shortcomings and provide a better treatment method, the use of medicinal plants is an alternate approach. Medicinal plants have been used since ancient times for their potential properties to treat various diseases. Its increased effectiveness, minimal cost, and limited complexity have generated a lot of interest. Clove (*Syzygium aromaticum)* oil has phytocomponents including Eugenol and β-caryophyllene; among which Eugenol has biological effects like antioxidant, anticarcinogenic, antibacterial, etc. In addition to that, Eugenol has also been investigated for its therapeutic properties, making it interesting for the purpose of chemoprevention. Eugenol exerts its anticancer activity by inducing of apoptosis and inhibiting the multiplication and migration of cancer cells [11].

In the above context, our study focuses on the integration of MSC-derived EVs with Eugenol to formulate an alternative approach for the treatment of cancer, thus eliminating the existing limitations. Also, our study focused on deriving the MSCs from human umbilical cord tissue (hUCT). As previously studied, to eliminate the usage of xenogeneic products for cell culture, we used lyophilized Human Platelet Lysate (L-HPL) as cell culture media, wherein studies show the increase in shelf-life and stability of HPL in its lyophilized form.

2. MATERIALS AND METHODS

2.1. Materials

Class 10000 clean room facilities were used to conduct the study. For this study, umbilical cord tissue (UCT), LHPL as the cell supplementation medium, hUCT -MSC derived EVs, and Eugenol (Sigma Aldrich, Cat. #E51791) were used as the major components. The cell culture was grown at 37°C in a 5% CO2 humidified environment. The Characterization of EVs was done using TEM analysis, NT analysis, FTIR analysis, the DLS method, protein estimation, and Western blotting techniques. For the *in vitro* studies, the cancer cell line HCT 116 was used to perform cytotoxicity assays, scratch assays, colony formation assays, and AO EtBr staining to prove the anti-cancer activity of the primed EVs. In addition to that, HEK293 cells were used to check the cytotoxicity to compare with the cytotoxicity test on colon cancer cells-HCT116, obtained from the National Centre for Biological Sciences (NCBS).

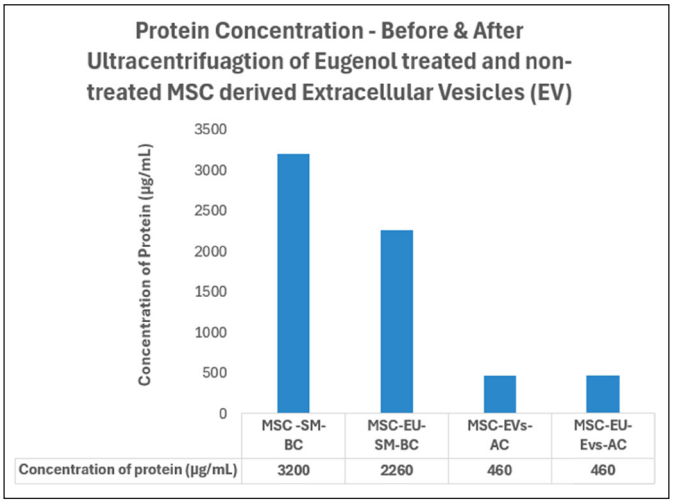

Figure 1: Protein concentration of spent media and EVs observed before and after ultracentrifugation.

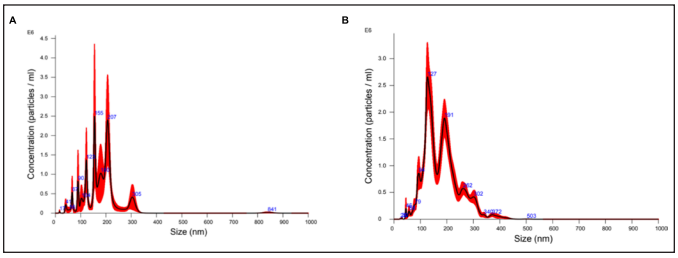

Figure 2: (A) NT analysis of MSC derived EVs. **(B)** NT analysis of Eugenol primed MSC – derived EVs – averaged FTLA concentration.

2.2. Methods

2.2.1. Culturing of UCT-MSCs using LHPL as a culture medium

The study started with the collection of human umbilical cord tissue (hUCT) and culturing the MSCs by an explant method. The study protocol was approved by the Institutional Ethics Committee (Pre-Clinical Studies), Department of Stem Cell Biology, Acadicell Innovations International Pvt. Ltd, Tamil Nadu, India with approval number IEC/ C-P/18/2021, umbilical cord samples were collected from SLIMS, Pondicherry, the cord tissue was cut into smaller segments longitudinally, and the blood vessels were removed. The platelet unit collected by apheresis was subjected to subsequent freeze and thaw cycles at −80°C, to induce platelet lysis for the release of growth factors, and the preparation of LHPL was done. These

Table 1: Results of the scratch assay – rate of proliferation (%).

Sample	0th hour (mm)	24th hour (mm)	Proliferation rate (%)
Control	0.81	0.203	74
Media + Drug	0.99	0.372	62
MSC derived EVs	1.50	0.643	57
Eugenol primed MSC derived EVs	0.71	0.49	31

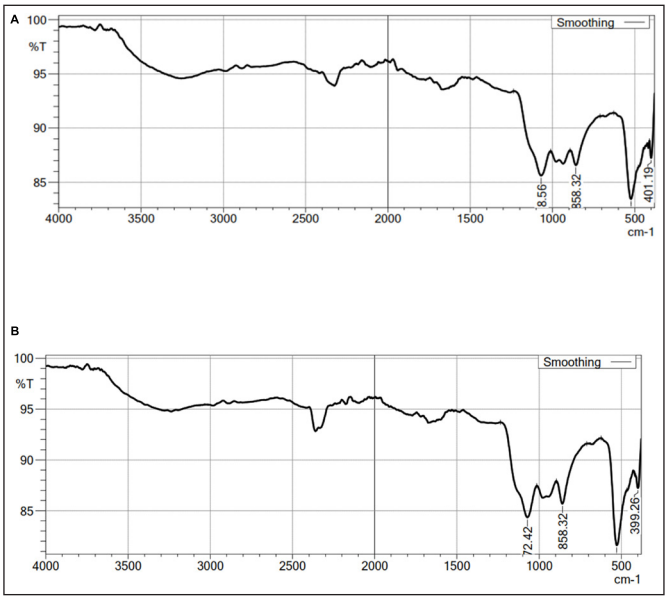

Figure 3: (A) FT-IR spectrum of MSC derived EVs. **(B)** FT-IR spectrum of Eugenol primed MSC derived EVs.

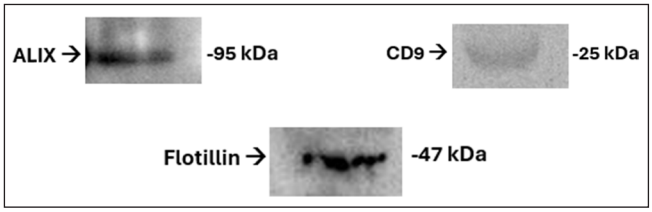

Figure 4: Representative western blots of proteins: ALIX, CD9, and flotillin.

segments were cultured using basal media supplemented with LHPL as culture medium. The explant was incubated in 5% CO2, at 37°C. Media change was done once every 3 days until the emergent of the MSC from the tissue. The MSCs were passaged after removing the segmented cord tissue.

2.2.2. Priming of Eugenol with EVs and collection of EVs

The cultured cells were passaged and subcultured in a T-75 a cell culture flask at cell seeding density of 4000 cells/cm^2. Meanwhile,

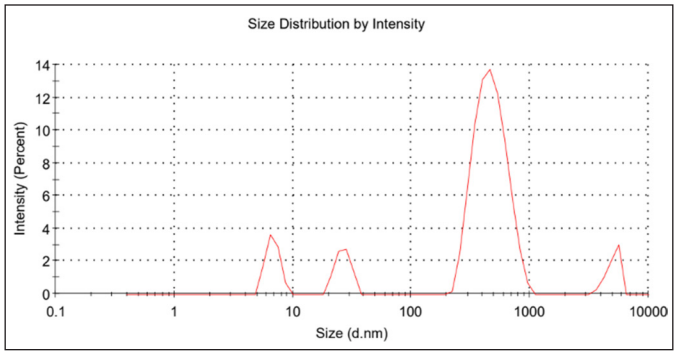

Figure 5: EVs distribution based on size, plotted by intensity.

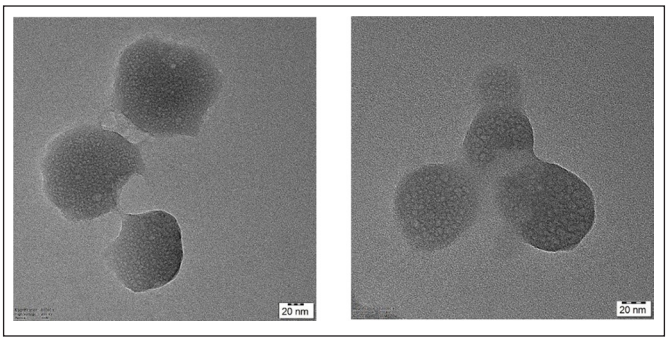

Figure 6: TEM images of MSC derived EVs (left) and MSC derived Eugenol primed MSC EVs (right).

Eugenol was dissolved using an organic solvent. Cells were rinsed with PBS when the confluency reached 70%-80% and preconditioned with Eugenol for 48 h. After preconditioning for 48 h, to obtain EVs, the conditioning medium was collected. The conditioned media was subjected to centrifugation at 300x g for 10 mins and 2000x g for 10 mins for the removal of the cellular debris. Later, the supernatant was centrifuged 10,000x g for 30 mins and the cell-free supernatants were ultracentrifuged at 100,000x g for 70 mins, twice – for collection of cell free supernatant and further washing to proceed with the collection of EVs. As a final step, PBS was used to suspend the EVs, and the collected EVs were further used for analysis.

2.2.3. Characterization of EVs

a) Transmission electron microscopy (TEM) analysis

TEM analysis for Eugenol primed EVs and MSC-EVs was performed using High Resolution TEM (HR-TEM), JEOL Japan. Prior to imaging EVs, the samples were fixed with paraformaldehyde and loaded into a carbon-coated copper grid – 200 mesh. The excess EVs sample was blotted using tissue paper and allowed to dry completely. The grid was placed in the vacuum chamber for image acquisition and evaluation.

b) Nano sight tracking analysis (NTA)

To measure the particle size and concentration, NTA was performed. Eugenol primed EVs and MSC -EVs were diluted in 1 mL PBS and loaded into the sample receiver at the appropriate temperature. Samples

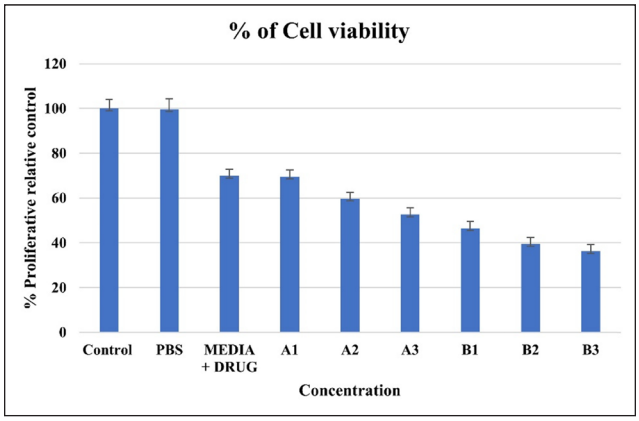

Figure 7: Cytotoxicity assay of HCT 116 cell line with drugs and EVs.

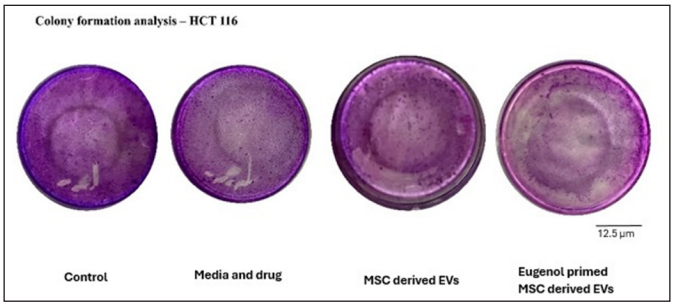

Figure 8: Colony formation analysis between drug treated colonies and EVs treated colonies.

were measured at a size distribution of 2 cycles in 11 positions, with a laser wavelength of 520 nm. ZetaView (Version 8.05.16.SP3) was used to analyze the measurements.

c) FTIR spectroscopy

The EVs sample—Eugenol treated and non-treated, were dissolved in a PBS solution for FTIR analysis. The FTIR spectra of these EVs samples were recorded in the wavenumber between 500-4000 cm^{-1} with a spectral resolution of 4 cm^{-1}.

d) Dynamic light scattering (DLS) analysis

The size distribution of EVs was measured by DLS at the 633 nm wavelength of a solid state He-Ne laser. The intensity of the scattered light was measured at 173°. The measurements were taken at 25°C. For the data processing and analysis, Zetasizer software version 7.13 was used.

e) Western blotting

The marker expression was studied using Zelle Biotech's Western Blotting Kit. The membrane is incubated with the diluted primary antibodies – Anti-rabbit IgG, HRP-linked antibody, and anti-biotin to detect the biotinylated protein markers in 5% w/v Bovine Serum Albumin (BSA), Tris Buffered Saline (TBS) with Tween at 4°C with gentle shaking overnight. 20 µL of collected and purified exosome samples were loaded onto SDS-page and then electrotransfered to a nitrocellulose membrane. Post transfer, the membrane is washed with TBS and incubated in blocking buffer at room temperature for 1 h. For the detection of proteins, the membrane bound HRP is washed, and the substrate is incubated with the membrane and exposed to X-ray film.

f) Protein estimation – microplate BCA method

Eugenol EVs and MSC-EVs samples were incubated with RIPA buffer overnight to isolate the proteins from EVS. A microplate BCA protein kit was used to estimate the concentration of proteins. The manufacturer protocol was followed for assaying the sample. The samples were suspended in double distilled water prior to measurement. The readings were taken using a microplate reader, and the protein estimation graph was plotted.

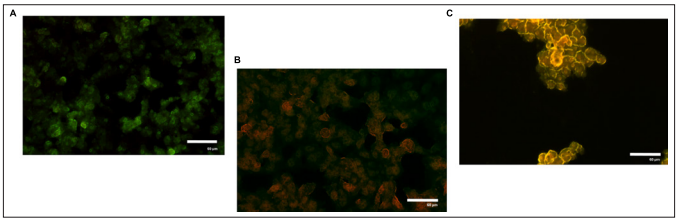

Figure 9: AO EtBr staining of HCT 116 cell line; **(A)** – Control, **(B)**- Cells with MSC -EVs, **(C)** – Cells with Eugenol primed MSC - EVs.

2.2.4. Invitro analysis using the colon cancer cell line – HCT 116

a) Cytotoxicity assay

The MTT assay was done to analyze the effectiveness of EVs on HCT 116 cells. In a 96 well plate, each well had a seeding density of 5000 cells. After 24 h cells were treated with varying concentrations of Eugenol primed EVs: 1, 5, and 10 µL and incubated for 48 h. After the incubation period, MTT reagent was added and kept in the incubator for 4 h. Finally, DMSO was added and incubated in the dark for 30 mins. Then the reading was measured at 570 nm.

b) Scratch assay

The scratch assay was done to measure the migratory properties of the cells. i.e., the rate of cell proliferation when a scratch (wound) is created by using a pipette tip. This test was performed to test and assess cell proliferation and migration after treating the cells in different cases. The HCT 116 cells were grown in a 12-well culture plate. Observations of the plate were taken at the 0^{th} and 24^{th} hour and the distance of the scratch was analyzed using ImageJ software.

c) Colony formation assay

The assay was performed with HCT 116 cell line. About 500 cells/well were seeded in six well plates and grown overnight. 24 h later, the plates were treated in different cases. After 48 h of incubation, the medium was removed and allowed to incubate for 10 days. After which, the colonies were fixed and stained with formalin and 1% crystal violet, respectively. The number of colonies was estimated, against which the graphs were plotted.

d) AO EtBr staining

The procedure used acridine orange/ethidium bromide (AO/EB) staining on HCT 116 cell lines to identify cancer cell apoptosis. Cells were added to a 96-well plate to a final concentration, and the wells were divided for different cases and cultured until the cells reached confluency of 60 to 70%. A 150 µL dual fluorescent staining solution was pipetted into each well and kept at ambient temperature for 10 mins. After the incubation period, the cells were visualized under an inverted fluorescent phase contrast microscope.

3. RESULTS AND DISCUSSION

We characterized the isolated EVs in accordance with the following guidelines: According to the "International Society for Extracellular Vesicles" (ISEV), the characterization of EVs should include protein estimation, Western blotting for surface marker expression, mass spectrometry, etc. In addition to these tests, single particle characterization by imaging via electron microscopy and evaluation of particle size by NTA or DLS must be performed [12].

3.1. Characterization of EVs Using Total Protein Estimation by BCA Method

Protein estimation was performed before and after the ultracentrifugation step for phytochemical treated vs. non-treated MSC derived EVs.

From the results as depicted in Figure 1, we found that the protein concentration before centrifugation was higher in both cases of MSC – Spent medium (MSC-SM) and MSC – EU- SM (MSC derived Eugenol treated Spent medium) when compared to MSC – EV and MSC – EV – EU (Eugenol primed MSC derived Extracellular Vesicles). Intra comparison with MSC-SM before the ultracentrifugation step showed a higher concentration in the non-Eugenol treated Spent medium than in the Eugenol treated Spent medium. Also, intra comparison with MSC derived EVs with and without the effect of Eugenol showed similar concentrations of proteins.

3.2. Characterization of EVs Using Nanoparticle Tracking Analysis (NTA)

To estimate the concentration and size of EVs, NTA was carried out using Zeta View. The analysis was made for Eugenol primed MSC-EVs and MSC -EVs.

With the results of the NT analysis as depicted in Figure 2, the size of the MSC derived EVs was found to be 184 +/– 10.7 nm, the size of the Eugenol primed MSC derived EVs was found to be 179.8 +/– 2.7, nm and the concentration was found to be 2.52e+08 +/– 1.35e+07 particles/ml.

3.3. Characterization of EVs Using Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is used to detect the characterization of molecular structure. It was also performed to confirm the heterogeneous population of the EVs group [13]. In this study, for the purpose of characterization, we measured the FTIR spectroscopy of MSC -EVs and MSC -Eugenol- EVs.

With respect to Figure 3A, the peaks observed were in the range of 400-1068 cm^{-1}. Also, similar peaks were observed in the case of MSC derived Eugenol primed EVs – Figure 3B, wherein the range was between 399-1072 cm^{-1}. For the 1st case – MSC derived EVs, the percentage of transmittance observed was around 83%-87 % and in the case of Eugenol primed MSC derived EVs it was between 81%-87 %.

3.4. Characterization of EVs Using Western Blotting

The protein presence-based EV characterization is confirmed using Western blotting. Proteins that are involved in EVs biogenesis include annexins, lipid raft proteins, and transmembrane proteins including CD9, CD63, etc. (CD – Cluster of Differentiation) [14]. The organization of cargo into EVs requires a specific accessory protein associated with the endosomal sorting complex that is essential for transportation, like ALIX [15]. The process of EVs biogenesis also includes flotillin, caveolins, etc. The release of EVs through the induction of epidermal growth.

ALIX, CD9, and flotillin factor is initiated by the engagement of flotillin proteins [16]. Among these many markers, our EVs sample tested positive for markers like CD9, ALIX, and flotillin. From Figure 4, it is observed that the ALIX protein was blotted at 95kDa; Figure 4 – CD9 marker was at 25kDa and flotillin was observed at 47kDa.

3.5. Characterization of EVs Using Dynamic Light Scattering (DLS) Analysis

The range of EVs distribution [Figure 5] ranges from 5 nm to 1000nm. Most populations of the EVs were found in the nanometer range of 100-1000.

3.6. Characterization of EVs Using Transmission Electron Microscopy (TEM) Imaging

The measurements of EVs [Figure 6] using TEM analysis are similar to those obtained in NT analysis and DLS analysis. Here, the measurements ranged from 100 to 200 nm. The EVs that are observed due to the negative contrast agent (in the image), and other similar EVs have been considered for other experiments in this study.

3.7. Invitro Analysis Using Colon Cancer Cell line – HCT 116

3.7.1. Cytotoxicity assay

MTT was done to identify the inhibitory effects of the drug - Eugenol and EVs in HCT 116 and HEK293 cells. The cells were exposed to varying concentrations of MSC-EVs (A1-A3) in comparison to drug-B1-B3 (Eugenol primed MSC derived EVs). When compared to drugs, the proliferative index of EVs treated cells was identified to be lower in a dose-dependent [Figure 7]. Statistical analysis was performed to confirm the role of EVs which influence the cell viability rate in HCT116 and HEK293. The IC50 value of EVs was calculated and represented in a graph. In our study, we found that the proliferation of HCT116 cells was drastically reduced by EVs.

3.7.2. Scratch assay

The concentration of MSC-EVs and Eugenol primed MSC - EVs was 5 µL based on the proliferative effect, and the same concentration was used in the scratch assay. The different cases and samples used in the assay, along with the proliferation rate (%), are given in the table below. The rate of proliferation in terms of scratch length was observed at the 0th and 24th h.The proliferation rate (%) is calculated using:

$$\textit{Proliferation rate (\%)} = \frac{\textit{(Initial Scratch length measurement – Scratch length measurement at } 24^{th} \textit{ hour)}}{\textit{Initial Scratch length measurement}} * 100$$

The results as depicted in Table 1 suggest a positive effect of decreased development of colon cancer cells in case the of Eugenol primed MSC derived EVs with the least proliferation rate. This rate is lower than that of the MSC derived EVs, which shows the effectiveness of the drug priming with EVs.

3.7.3. Colony formation assay

The long-term effects of EVs were noted in HCT116 cells. The cells were treated with drugs and EVs and incubated for 10 days. The determined drug dosage was given, and a lesser number of colonies were identified in EVs treated compared to the drug. Both drug-treated and EVs treated was compared to untreated HCT116 cells. A marked reduction in the number of colonies in treated samples was noted [Figure 8]. This finding clearly shows that EVs have a greater effect on HCT116cells. Therefore, EVs are potently inhibiting the cell proliferation and colony formation in colon cancer cells.

3.7.4. AO EtBr staining

After the application of AO EtBr stain to MSC - EVs and Eugenol primed MSC -EVs, the apoptotic cells were stained in a yellow – orangish red color. The resultant images are depicted above in Figure 9, and we observed the following: In the control sample, we observed live colon cancer cells. In the MSC-EVs treated group of cells, a mixed population of late apoptotic and necrotic stages of cells was found. Whereas in the Eugenol MSC-EVs treated cells, most of the cell population was in the necrotic stage.

Understanding the role of EVs is a must in the current healthcare scenario, and it has a major impact on our understanding of intracellular and cell-cell communication, tumor and stem cell biology, and other fields [18]. To date, there are many studies and experiments being performed to support the role of EVs in every aspect of cancer formation and prognosis. On the other hand, anti-tumor activity of EVs is also observed for breast cancer, gastric cancer, liver, and lung cancer [19]. Almost every cell releases EVs, and these are in general classified as nanoparticles. These extracellular vesicles (EVs) do participate in both proximal as well as distal cell-cell communications [18]. MSCs have an increasing ability to treat various diseases due to their paracrine effects that are mediated by extracellular vesicles (EVs). Bioactive substances like lipids, proteins, DNA, RNA, and other metabolites that plays a vital role in cellular communication are found in EVs [20] MSC-EVs help inhibit the development of the tumor in a paracrine manner. This is closely associated with the tumor – related miRNAs that are present in the MSC derived EVs [21]. Also, proteins and long non-coding RNA (lnc RNA) in the EVs are attributed to suppressing the proliferation of the cancer cell and thus initiating the apoptosis of the cancer cell [22, 23]. Angiogenesis is the vital factor for tumor establishment, which ensures an adequate supply of oxygen and nutrients to the growing cancer cells [24]. Studies have reported that MSC derived EVs reduce the rate of angiogenesis by downregulating the expression of VEGF and CD31 [25]. On the other hand, EVs have the ability to act as antigen presenters, stimulating the adaptive and innate responses of the immune system [26]. MSCs have different triggering actions in the signaling pathways in terms of blocking and activating. Studies reported that MSCs block the Wnt signaling pathway by modulating the Dickkopf-related protein 1(DKK1) that is released by the malignant cells, downregulating c-Myc and Cyclin D2, and upregulating P21CIP1 and P27KIP1, thus resulting in the suppression of cancer cells [27]. MSC-EVs are also beneficial due to their properties of immunosuppression and immune evasion in the tumor microenvironment (TME) [28]. In accordance with the therapeutic benefits of EVs, we tend to isolate hUCT-MSC -EVs for treating colorectal cancer due to the tumor homing and immunomodulatory properties they exhibit, thus providing new modalities for cancer treatment [29].

We characterized EVs based on ISEV and the results we encountered in NT analysis, wherein the particle size was < 200 nm in accordance with the classification given by Jeppesen and team [30]. The morphology of the EVs was also confirmed by TEM, and the heterogenous population of EVs was confirmed by the varying size distribution in DLS, in accordance with the EVs characterization [31]. The FT-IR spectra result of our study, which ranged between 400-1608 cm^{-1} is in par with the results obtained from the study of osteosarcoma cell derived EVs and other suspended cells [32]. The Western blotting results of our study showed positive markers for CD9, ALIX, and flotillin which are on par with other studies [33]. Furthermore, the BCA protein assay shows the same protein levels for both Eugenol primed EVs vs. non-primed EVs, indicating no change or alteration in the proteins. This finding also matches the lack of significant results in the case of other engineered EVs [34].

The anti-cancer effects of the EVs and Eugenol primed EVs were found using various *in vitro* studies. Although the cancer cell proliferation of EVs has been studied, the contradictory nature of the same, i.e., the use of EVs in cancer therapy, must be studied, and we found that the final concentration of 5 µL of MSC derived EVs is sufficient to kill the cells, which was replicated as a percentage of proliferation rate, cytotoxicity assay, and AO/EtBr staining. This also proved the non-promoting effect of EVs on cancer cell lines [35].

With respect to Eugenol, the addition of this phytochemical to the HCT-116 cell line has been studied, and it was found that the cells showed signs of autophagy and apoptosis, thus inhibiting proliferation [36]. As per the earlier studies, it is seen that the antioxidant effect of stem cells can be promoted by preconditioning the EVs with Eugenol [36]. Our study combined the anti-cancer effects of both MSC derived Eugenol primed EVs and found positive results in the *in vitro* analysis.

4. CONCLUSION

In brief, the overall findings of our study an exhibited anti-cancer effect against HCT 116. The underlying molecular mechanism of the anti-cancer effect of the formulated compound is yet to be studied. Administration of Eugenol primed MSC -EVs is a viable and effective approach to treating CRC, thus eliminating the limitations of conventional cancer therapies. The study showed that the formulated drug is biocompatible and, hence, could be concluded to be a novel therapeutic approach for treating colon cancer.

5. ACKNOWLEDGMENTS

The authors acknowledge SRMIST, Kattankulathur for the TEM facility.

6. AUTHORS' CONTRIBUTIONS

LMG: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft. NG: Investigation, Writing – original draft, Writing – review & editing. AJ: Validation, Investigation, Writing – original draft, Writing – review & editing. RP and PKL: Conceptualization, Methodology, Validation.

7. FUNDING

There is no funding to report.

8. ETHICAL APPROVAL

The study protocol was approved by the Institutional Ethics Committee (Pre-Clinical Studies), Department of Stem Cell Biology, Acadicell Innovations International Pvt. Ltd, Tamil Nadu, India with approval number IEC/ C-P/18/2021, Dated: 27.12.2021.

9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

12. PUBLISHER'S NOTE

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