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Characterization and metabolomic analysis of Plant-derived Extracellular Vesicles (PdEVs) isolated from indigenous medicinal plants

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ABSTRACT

PdEVs (Plant-derived Extracellular Vesicles) are nano-sized, membranous vesicles released by plant cells for defense mechanisms. They contain nucleic acids, proteins, lipids, and other secondary metabolites. In the current study, we characterized PdEVs derived from medicinal plants and identified the metabolites present in the PdEVs. We isolated PdEVs using the PROSPR method from the apoplastic fluid of four different medicinal plants: *Azadirachta indica, Murraya koenigii, Ocimum tenuiflorum,* and *Coleus amboinicus*. The isolated PdEVs were characterized morphologically by HR-TEM, and functional group characterization was performed using FTIR. Furthermore, metabolomic analysis was performed using GC-MS for the PdEVs from *Azadirachta indica, Murraya koenigii, Ocimum tenuiflorum*, and *Coleus amboinicus*. We found the identified metabolites to possess anti-cancer, anti-microbial, and anti-fungal activities, substantiating the potential use of PdEVs as a potential source for therapeutic applications.

1. INTRODUCTION

Cell communication is essential for organismal development, and both plant and animal cells have evolved numerous ways to communicate. Over the past two decades, the role of Extracellular Vesicles (EVs) in inter-cellular communication has been established and we are witnessing a surge in extracellular vesicle research. These EVs transport bioactive molecules such as metabolites, microRNAs (miRNAs), lipids, and proteins, and they play important roles in intercellular communication [1].

Research on exosomes, a type of EV, has focused on mammalian cell cultures. While mammalian exosomes are routinely recovered from diverse bodily fluids, including saliva, blood, semen, milk, cerebrospinal fluid, urine, and amniotic fluid, the isolation of plant-derived EVs has been constrained with limited exploration in model species like *Arabidopsis thaliana* [2]. Even though the release of vesicles by plant cells was reported before mammalian discoveries, exploration of the plant kingdom for exosome research has been notably lacking. In addition, our understanding of how cargo, specifically secondary metabolites, are encapsulated into Plant-derived Extracellular Vesicles (PdEVs) remains limited. The investigation of the therapeutic potential of PdEVs is still in its early stages, facing challenges due to the absence of standardized isolation procedures and a comprehensive understanding of the biological roles of PdEVs [3].

Unlike traditional plant extracts, PdEVs have been investigated for their potential to mitigate immunogenic responses, making them promising candidates for therapeutic applications with fewer side effects [4,5]. EVs isolated from numerous plant species in recent years have been further investigated for medicinal efficacy. Fruits and spices are the most commonly EV-isolated plant categories. Several clinical trials have also begun to use PdEVs, with the first beginning in 2012. However, comprehensive results of clinical trials involving PdEVs have yet to be disclosed, and investigations are still in the early phases. Some examples of PdEVs that are used for a clinical trial are ginger aloe for insulin-related conditions and chronic inflammation in patients (ID no. NCT03493984), which is currently in the preliminary clinical trial phase. Another example of a PdEV source is grape. It was used to treat oral mucositis conditions and clinical trials have been completed (ID no. NCT01668849). One more study investigated the ability of plant exosomes to deliver curcumin to normal and colon cancer tissue (ID no. NCT01294072) to treat colon cancer, where curcumin is loaded in plant exosomes.

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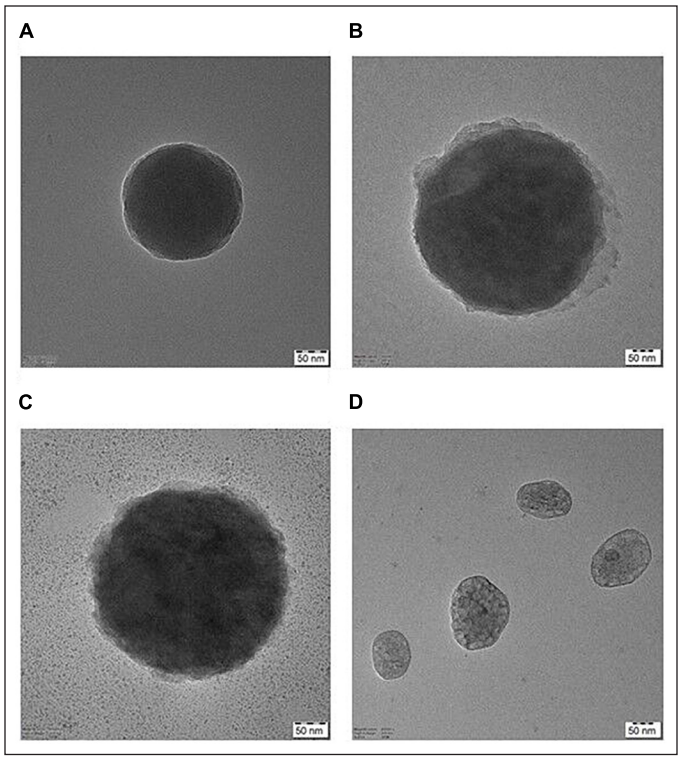

Figure 1: HR-TEM images of PdEVs isolated from **(A)** *Azadirachta indica;* **(B)** *Murraya koenigii*; **(C)** *Ocimum tenuiflorum;* **(D)** *Coleus amboinicus* scale: 50 nm.

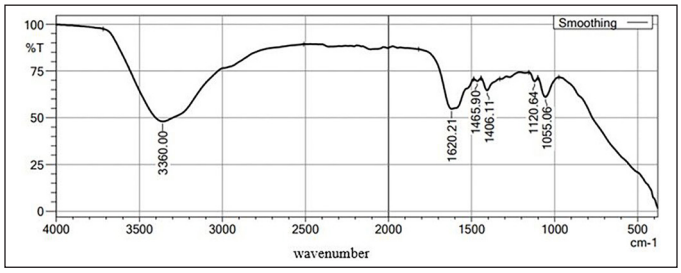

Figure 2: FTIR spectra of PdEVs isolated from *Azadirachta indica.* The peaks in the spectrarange from 1672 to 1400 cm^{-1} represent proteins, and the peaks range from 1267 to1066 cm^{-1} represent nucleic acids.

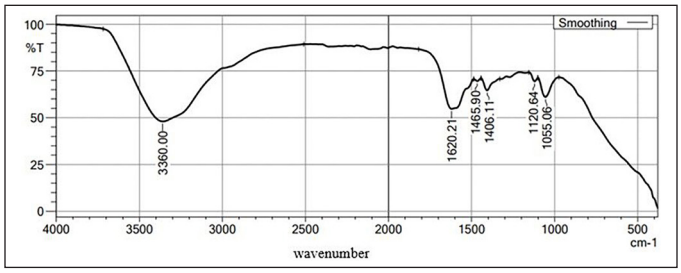

Figure 3: FTIR spectra of PdEVs isolated from *Murraya koenigii.* The peaks in the spectra 2972–2844 cm^{-1} represent lipids, and the peaks in 1672–1400 cm^{-1} represent amides.

Table 1: Functional group identification based on the wavenumber obtained from FTIR analysis of PdEVs derived from four different medicinal plants.

Wavenumber	Functional Group
***Coleus amboinicus* EVs**	
3358.07	Aliphatic secondary amine
1629.85	Organic nitrates
1411.89	Organic sulfates
1058.92	Primary amine, CN stretch
***Azadirachta indica* EVs**	
3360.00	Aliphatic secondary amine
1620.21	Primary amine, NH bend
1465.90	Methyl C-H bend
1406.11	Phenol or tertiary alcohol, OH
1120.64	Cyclic ethers
1055.06	Primary amine, CN stretch
***Murraya koenigii* EVs**	
3329.14	Aliphatic primary amine, NH stretch
2935.66	Methylene C-H asym. /sym. Stretch
1552.70	Carboxylate (carboxylic acid salt)
1406.11	Organic sulfates
1056.99	Alkyl-substituted ether, C-O stretch
***Ocimum tenuiflorum* EVs**	
2927.94	Methylene C-H asym. /sym. Stretch
1614.42	C=C-C Aromatic ring stretch
1519.91	Aromatic nitro compounds
1463.97	Carbonate ion
1404.18	Phenol or tertiary alcohol, OH bend
1286.52	Organic nitrates
1056.99	Alkyl-substituted ether, C-O stretch

These findings hold importance in plant-based therapeutics by exploring PdEVs as a novel delivery system for bioactive compounds. This research bridges the gap between traditional herbal medicine and modern therapeutic approaches, offering a unique perspective on utilizing plant-derived nanovesicles for human health.

Addressing these knowledge gaps and contributing to the understanding of PdEVs, this study employed the PROSPR method [6] to isolate EV-like vesicles from the apoplastic fluid of four indigenous medicinal herbs. These widely used Indian medicinal plants are known for their therapeutic properties, including anti-cancer, anti-microbial, anti-inflammatory, and anti-fungal properties, making them valuable for potential medical applications [7-10]. We further used instruments like HR-TEM, FTIR, and GC-MS for PdEVs characterization.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Tris-HCl (molecular weight 157.6 g mol^{-1}) (catalog number: MB030), sodium chloride (molecular weight 58.44 g mol^{-1}) (catalog number: MB023), and 2-mercaptoethanol (catalog number: TC152) were purchased from Hi-Media (India). Acetone (catalog number: 15168)

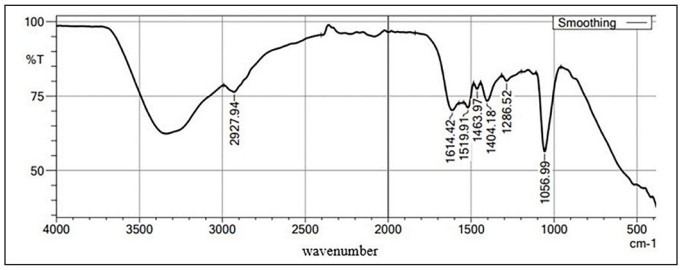

Figure 4: FTIR spectra of PdEVs isolated from *Ocimum tenuiflorum.* The peaks in the spectra 2972–2844 cm^{-1} represent lipids, the peaks in 1672–1400 cm^{-1} represent proteins, and the peaks in 1267–1066 cm^{-1} represent nucleic acids.

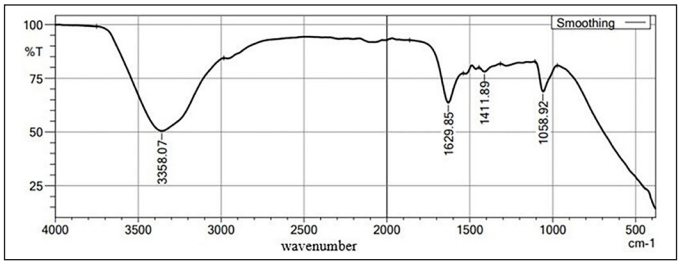

Figure 5: FTlR spectra of PdEVs isolated from *Coleus amboinicus.* The peaks in the spectra range from 1672–1400 cm^{-1} representing amides.

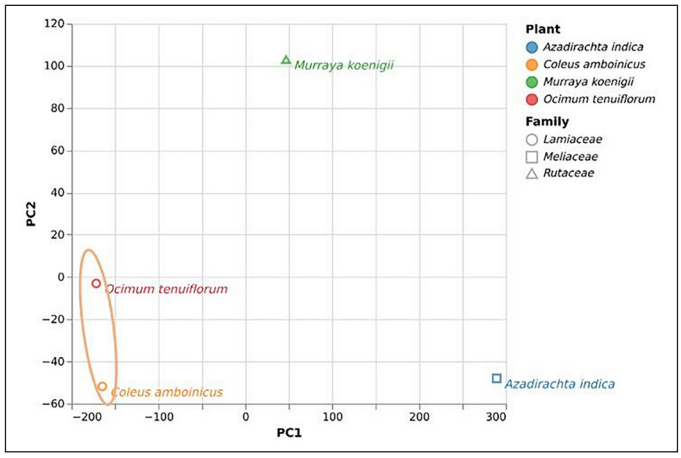

Figure 6: The Principal Component Analysis of the FTIR spectra of PdEVs was obtained from *Azadirachta indica, Murraya koenigii, Ocimum tenuiflorum,* and *Coleus amboinicus*. *Ocimum tenuiflorum* and *Coleus amboinicus* belonging to the family Lamiaceae were grouped, whereas the *Azadirachta indica* which belongs to Meliaceae and *Murraya koenigii* belonging to Rutaceae were segregated.

was purchased from SRL. Methanol was purchased from RANKEM (catalog number: RANKM0170). All of the aforementioned chemicals and reagents were utilized to make the buffers or used as they are for PdEVs isolation and characterization.

2.2. Collection of Plant Leaves

Azadirachta indica (neem), *Murraya koenigii* (curry leaves), *Ocimum tenuiflorum* (holy basil), and *Coleus amboinicus* (Mexican mint) were acquired from Tamil Nadu, India. *Azadirachta indica* was collected from the village Thailavaram, Chengalpattu district, Tamil Nadu at 12°49′39.6″N 80°02′43.1″E. *Murraya koenigii* was collected from Maraimalai Nagar, Chengalpattu district, Tamil Nadu at 12°47′14.2″N 80°01′48.7″E. *Coleus amboinicus* was collected from Madhavaram, Chennai, Tamil Nadu at 13°08′59.1″N 80°14′36.8″E. *Ocimum tenuiflorum* was collected from Guduvanchery, Chengalpattu district, Tamil Nadu at 12°51′15.1″N 80°03′09.4″E. The collected plants were washed thoroughly in distilled water to remove all the impurities, and the leaves were collected from these plants. The plants *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* were authenticated (shown in supplementary data) by Dr. Sivaram, Associate Professor in the PG and Research Department of Botany at Arignar Anna Government Arts College, Villupuram, Tamil Nadu, India.

Table 2: Metabolomic identification in the PdEVs of four different plants based on their retention number obtained from the GCMS analysis.

Plant Scientific Name	Retention Time (Minutes)	A/H (Area/ Height)	Compound	Molecular Formula
Murraya koenigii· *Coleus amboinicus*	29.886	36.01	Tetracosamethyl-cyclodod ecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$
Murraya koenigii· *Ocimum tenuiflorum*	18.766	29.24	1H-Purin-6-amine, [(2-fluorophenyl) methyl]-	$C_{12}H_{10}FN_5$
Murraya koenigii· *Ocimum tenuiflorum*· *Coleus amboinicus*	20.671	21.5	Tetrapenta contane	$C_{54}H1_{10}$
Murraya koenigii· *Ocimum tenuiflorum*	23.062	18.29	Hexacontane	$C_{60}H_{122}$
Murraya koenigii· *Ocimum tenuiflorum*· *Coleus amboinicus*	28.676	14.35	Eicosyl isopro-pyl ether	$C_{23}H_{48}O$
Ocimum tenuiflorum· *Coleus amboinicus*	24.657	11.16	Octadecane	$C_{18}H38$

2.3. Isolation of Apoplastic Fluid From Plant Leaves

Vacuum infiltration buffer (VIB) was prepared with 50 mM Tris-HCl (pH 7.5), 0.6% NaCl, and 0.1% 2-mercaptoethanol for 200 ml [2]. The collected leaves of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* were soaked in the VIB for 48–60 hours. The VIB penetrated the leaf pores and infiltrated the apoplastic space, while the apoplastic fluid was emancipated. To get rid of excess fluids, the leaves were then blotted using blotting paper [11]. The leaves were wrapped around a 1 mL microtip with the help of parafilm. A second parafilm was wrapped around it and placed at

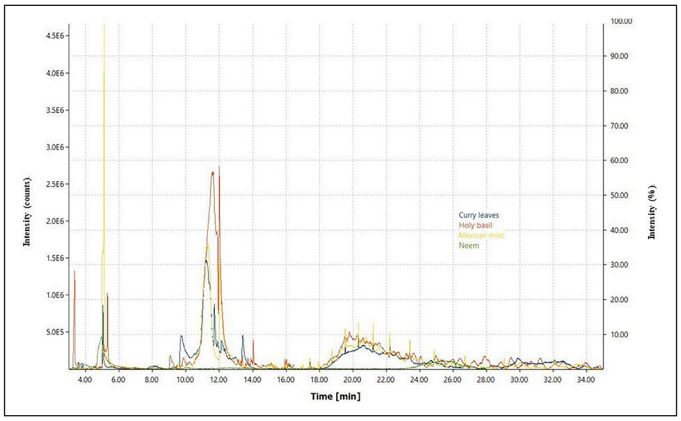

Figure 7: Overlaid GCMS chromatogram of metabolites present in PdEVs derived from *Azadirachta indica, Murraya koenigii, Ocimum tenuiflorum,* and *Coleus amboinicus.*

Table 3: Identification of distinctive metabolites present in PdEVs of *Azadirachta indica* based on GCMS analysis.

Retention Time (Minutes)	A/H (Area/ Height)	Compound	Molecular Formula
9.138	4.53	Cyclohexasiloxane, dodecamethyl	$C_{12}H_{36}O_6Si_6$
18.345	4.25	Hexaborane(10)	B_6H_{10}
22.295	6.22	Lysergic acid, TMS derivative	$C_{19}H_{24}N_2O_2Si$
21.575	5.30	4H-thieno[2,3-D] azonin-4-one, 5,6,7,8,9,10-hexahydro-8-methyl-	$C_{11}H_{15}NOS$
25.505	13.69	Benzoic acid, 4-[[(trimethylsilyl)oxy] methyl]-, trimethylsilyl ester	$C_{14}H_{24}O_3Si_2$

the top of the 15-mL falcon tube. The setup was centrifuged at 2,500 × *g* for 10 min at 4°C, followed by 2,320 × *g* for 5 min at 4°C. The supernatant (apoplastic fluid) was transferred to a fresh tube and stored at –20°C until use [12].

2.4. Isolation of PdEVs From the Apoplastic Fluids

The Protein Organic Solvent Precipitation (PROSPR) method was performed to isolate PdEVs. The proteins located within the cells, apart from the PdEVs, are precipitated by adding four volumes of ice-cold acetone at 4◦C overnight. The PdEVs can be obtained from the supernatant after separation. The isolated apoplastic fluids of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* were centrifuged at 300 x g at 4°C for 10 min; 2,000 x g at 4°C for 10 min; and 6,500 x g at 4°C for 10 min to remove cell debris and other large vesicles. The supernatant was taken in a fresh tube and then filtered using a 0.22 µm filter, which will eliminate the particles above 220 nm. The filtered fluid was mixed well with ice-cold acetone in a ratio of 1:4 and kept for overnight precipitation of PdEVs at –20°C. The mixture sample was centrifuged at 5,000 rpm at 4°C for 3 min. The supernatant was aliquoted in 2 mL tubes and then subjected to vacuum concentration at 30°C using the vacuum concentrator (Concentrator Plus, Eppendorf). The concentrated sample containing PdEVs was stored at –20°C until use [13,14].

Table 4: Identification of distinctive metabolites present in PdEVs of *Murraya koenigii* obtained from the GCMS analysis.

Retention Time (Minutes)	A/H (Area/ Height)	Compound	Molecular Formula
3.155	2.92	2-Hexanone, 4-methyl-	$C_7H_{14}O$
8.051	13.97	Pentanal, 2-methyl-	$C_6H_{12}O$
6.175	3.71	5-Hydroxy-4,4-dimethoxy-4,5,6,7-tetrahydrobenzofurazan	$C_8H_{12}N_2O_4$
8.419	6.06	Brefeldin A	$C_{16}H_{24}O_4$
21.538	4.47	Squalene	$C_{30}H_{50}$
22.520	8.32	1,7-Heptanediol, 2TMS derivative	$C_{13}H_{32}O_2Si_2$
23.670	3.90	Pimelic acid, di(2-chloro-phenyl) ester	$C_{19}H_{18}C_{12}O_4$
23.725	2.70	Methyl Z-11-tetradecenoate	$C_{15}H_{28}O_2$
23.965	7.48	Cyclotridecanone	$C_{13}H_{24}O$
24.035	7.17	Cycloundecanecarboxylic acid, 5-nitro-2,11-dioxo-, methyl ester	$C_{13}H_{19}NO_6$

Table 5: Identification of distinctive metabolites present in PdEVs of *Ocimum tenuiflorum* based on GCMS analysis.

Retention Time (Minutes)	A/H (Area/ Height)	Compound	Molecular Formula
16.105	2.91	Heptadecanoic acid	$C_{17}H_{34}O_2$
7.962	3.95	Thonzylamine	$C_{16}H_{22}N_4O$
28.432	4.47	Valeric acid, 2-cyano-4-methyl-, ethyl ester	$C_9H_{15}NO_2$
28.339	5.58	1-Bromoeicosane	$C_{20}H_{41}Br$
16.185	4.08	Phthalic acid, 4-methylpent-2-yl undecyl ester	$C_{25}H_{40}O_4$
4.329	2.85	Propyl hexanoate	$C_9H_{18}O_2$
5.674	3.81	Furan, 2,5-diethyltetrahydro-	$C_8H_{16}O$
6.129	2.49	1,3-Cyclopentanedione, 4-hydroxy-2-methyl-	$C_6H_8O_3$
6.195	1.53	N-2- chloroethyl-N, N-dimethyl ammonium chloride	$C_4H_{10}ClN$
7.295	3.84	N-(4-bromophenyl)-1-piperidinecarbothioamide	$C_{12}H_{15}BrN_2S$

2.5. HR-TEM Analysis

High Resolution-Transmission Electron Microscope (HR-TEM) was used to analyze the morphology of PdEVs [15]. The sample was added to the copper grid and stored in the hybridizer overnight at 37°C. The PdEVs samples of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* have been viewed using the

Table 6: Distinctive metabolites identification that are present in PdEVs of *Coleus amboinicus* based on GCMS analysis.

Retention Time (Minutes)	A/H (Area/ Height)	Compound	Molecular Formula
3.723	2.05	Cyclohexane, 2-(dimethylhydrazono)-3-[4-hexenyl]-1-aci-nitro-, (E,E)-	$C_{14}H_{25}N_3O_2$
4.210	5.06	1-Carboethoxypiperazine-4-thiocarboxylic acid 2-[1-[2-pyridyl 1-oxide]hydrazide	$C_{15}H_{21}N_5O_3S$
4.462	6.05	1,3-Oxathiane, 5-isopropyl-2-methyl-	$C_8H_{16}OS$
4.652	3.79	Tetrahydrofuran-2-one, 3-[1-fluoroethyl]-5-[[2-hydroxypropyl] benzeneethyl	$C_{17}H_{23}FO_3$
4.729	2.77	Oxetane, 2-(1,1-dimethylethyl)-3-methyl-	$C_8H_{16}O$
5.518	2.92	Methyl 4,5,7,8,10,11,13,14,16,17, 19,20-D12 docosanoate	$C_{23}H_{34}D_{12}O_2$
16.139	4.76	Decanoic acid	$C_{10}H_{20}O_2$
6.175	2.63	3-Nonanol	$C_9H_{20}O$
15.115	5.71	Pluchidiol	$C_{13}H_{20}O_2$
6.574	6.17	3-Cyclohexylpropionamide	$C_9H_{17}NO$
27.380	2.99	Digitoxin	$C_{41}H_{64}O_{13}$

JEM-2100Plus Transmission Electron Microscope (Jeol, Japan), and the approximate diameter of the vesicles was measured using ImageJ software [16].

2.6. FTIR and Principal Component Analysis

The FTIR (Fourier transform infrared) spectrometer was used to identify the functional groups and distinguish the PdEVs isolated from different plants [17]. 5 µL of each plant's PdEVs samples were loaded on the glass slides and stored overnight in the hybridizer at 37°C. The PdEVs samples of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* were triplicated and analyzed using a SHIMADZU, IRTRACER 100 FTIR spectrometer. Distinct peaks originating from specific functional groups located on the surface of the PdEVs were detected at precise spectral wavelengths. These peaks were identified by comparing the spectrum acquired from standard FTIR reference data [18]. Further, from the obtained FTIR data, Principal Component Analysis was performed using PCA Jupyter Notebook [19].

2.7. Metabolomic Extraction

The metabolomic extraction of PdEVs isolated from *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* was performed to analyze the metabolites present in PdEVs. 250 µL of PdEVs from these four medicinal plants were subjected to sonication for 10 min using a water bath sonicator to lyse the membrane of the PdEVs. To extract metabolites, methanol was added in a ratio of 1:3 to the processed PdEVs samples, followed by centrifugation at 14,000 rpm for 15 min at 4°C [20,21]. Without disrupting the pellet, the aqueous solutions were aspirated into a fresh tube and subjected to vacuum concentration at 30°C. The concentrated metabolic samples were subjected to GC/MS analysis [21].

2.8. GC-MS Analysis

The gas chromatography-mass spectrometry (GC-MS) analysis helps in profiling the metabolites present in the samples [22]. GC-MS analysis of metabolites extracted from PdEVs samples was implemented using SHIMADZU and QP2010 PLUS gas chromatography-mass spectrometry. The GC-MS program for analyzing these samples is as follows: The column oven temperature was initially kept at 40°C for 2 min, followed by a gradual increase in temperature of about 10°C / minute, and the final column oven temperature was fixed at 300°C for 16 min. The injector temperature was set at 280°C in split mode. The source ion temperature was 250°C, and the interface temperature was 260°C. The m/z range was fixed at 50–850 to measure the spectrum [21]. Based on the peaks obtained in the chromatogram, and compared with the NIST (National Institute of Standards and Technology) library, the metabolomic compounds present in the PdEVs were identified. A Venn diagram has been plotted using Rstudio based on the number of common and distinctive metabolites present in the PdEVs of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus.*

3. RESULTS

3.1. HR-TEM Analysis

The isolated PdEVs of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* were viewed under HR-TEM at the scales of 20 nm, 50 nm, 100 nm, and 200 nm [Figure 1]. Using the ImageJ software, the diameter of the PdEVs was calculated [16]. The mean diameters of the PdEVs of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* were calculated as ˜196 nm, ˜400 nm, ˜298 nm, and ˜143 nm, respectively.

3.2. FT-IR and Principal Component Analysis

Based on the spectra obtained from FTIR analysis for PdEVs samples isolated from *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus*, the graph has been plotted between wavelength (x-axis) and percentage transmittance (y-axis). From the acquired FTIR spectra [Figure 2], the peaks obtained in *Azadirachta indica* PdEVs show the presence of amine groups and organic sulfates [Table 1]. The peaks obtained from *Murraya koenigii* PdEVs [Figure 3] establish the existence of amine groups, phenolic groups, and cyclic ethers [Table 1]. The peaks obtained from *Ocimum tenuiflorum* PdEVs [Figure 4] represent amine groups, organic sulfates, alkyl-substituted ethers, and carboxylate groups [Table 1]. In the PdEVs isolated from *Coleus amboinicus*, the peaks obtained from FTIR analysis represent aromatic nitro compounds, carbonate ions, organic nitrates, alkyl-substituted ethers, and OH groups [Figure 5 and Table 1] [18].

The Principal Component Analysis (PCA) was executed with the FTIR data, as shown in Figure 6. Based on the FTIR data, the PdEVs obtained from *Ocimum tenuiflorum* and *Coleus amboinicus*, belonging to the Lamiaceae family, were grouped in the 3rd quadrant. The PdEVs from *Azadirachta indica,* belonging to the Meliaceae family, were segregated in the 4th quadrant. The PdEVs isolated from *Murraya koenigii*, which belongs to the Rutaceae family, were segregated in the 1st quadrant.

3.3. GC-MS Analysis

An overlaid chromatogram was generated using OpenChrom software, illustrating the metabolites present in the PdEVs of *Azadirachta indica*,

Table 7: Biological activities of metabolites present in PdEVs of different medicinal plants.

Plants	Compound	Biological Activity	References
Azadirachta indica	Glycerin	Anti-microbial	[36,42]
Azadirachta indica	1-Trimetthylsiloxy-1-(3,4-di(trimetthylsiloxy) phenyl)-2-(isopropylamino)ethane	No activity reported	
Azadirachta indica	Benzoic acid, 2,6-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	Anti-microbial, anti-fungal	[43]
Azadirachta indica	Lysergic acid, TMS derivative	Hallucinogen, anti-anxiety agent, creativity and performance enhancer, treatment for psychoneuroses and mental illness	[44,45]
Azadirachta indica	Cyclohexasiloxane, dodecamethyl	Antifungal, antiperspirants, antimicrobial, deodorants, and hair/skin care products	[23,24,37]
Murraya koenigii, Coleus amboinicus	Tetracosamethylcyclododecasiloxane	Hepatoprotective, anti-spasmodic, anti-rheumatic	[46]
Murraya koenigii, Ocimum tenuiflorum	1H-Purin-6-amine, [(2-fluorophenyl) methyl]-	Plant hormone, cell division, and growth regulatory factor	[30]
Murraya koenigii	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	Anti-microbial, anti-fungal	[46]
Murraya koenigii, Ocimum tenuiflorum, Coleus amboinicus	Tetrapentacontane	Anti-cancer, anti-diabetic	[39]
Murraya koenigii, Ocimum tenuiflorum, Coleus amboinicus	Eicosyl isopropyl ether	Anti-microbial, anti-fungal	[38]
Murraya koenigii	Propanoic acid	Anti-mycobacterial, anti-microbial, anti-cancer, anti-convulsant, anti-inflammatory, analgesic, anti-parasitic, anti-viral, anti-diabetic, anti-hypertensive	[47]
Ocimum tenuiflorum	Hexadecane	Anti-oxidant, anti-microbial	[48]
Ocimum tenuiflorum	Batilol	Increase WBC count; anti-oxidant activity, prevention from radiation in radiotherapy	[32,33]
Ocimum tenuiflorum	1,54-Dibromo tetrapentacontane	No activity reported	
Ocimum tenuiflorum	2-Methylhexacosane	Hypocholesterolemic, anti-microbial	[32]
Ocimum tenuiflorum	Acetic acid	Anti-bacterial; anti-otitic; anti-salmonella; anti-vaginitic; expectorant; acidulant; fungicide	[48]
Ocimum tenuiflorum	Thonzylamine	Anti-histamine, anti-cholinergic	[40]
Ocimum tenuiflorum	Heptadecanoic acid	No activity reported	
Ocimum tenuiflorum	Hexadecanoic acid	Anti-oxidant, flavoring agent, anti-androgenic, hypocholesterolemic, nematicide, pesticide, lubricant	[48]
Coleus amboinicus	Pluchidiol	Anti-oxidant and anti-inflammatory activity	[49]
Coleus amboinicus	Decanoic acid	Anti-microbial, anti-inflammatory	[50]
Coleus amboinicus	Longipinane, (E)-	Anti-feedant, anti-cancer activity	[41]
Coleus amboinicus	Hexadecanoic acid, ethyl ester	Anti-bacterial, anti-oxidant, hypocholesterolemic, nematicide, pesticide, anti-androgenic, anti-fibrinolytic, lubricant, anti-inflammatory, hepatoprotective, anti-histamine, anti-eczemic, anti-acne, anti-arthritic, helps in treating cardiac diseases, anti-fungal	[49,51,52]
Coleus amboinicus	Ethane, 1,1-diethoxy-	Flavoring agent, aromatic compound	[35]
Coleus amboinicus	Digitoxin	Anti-cancer, heart failure treatment	[34]

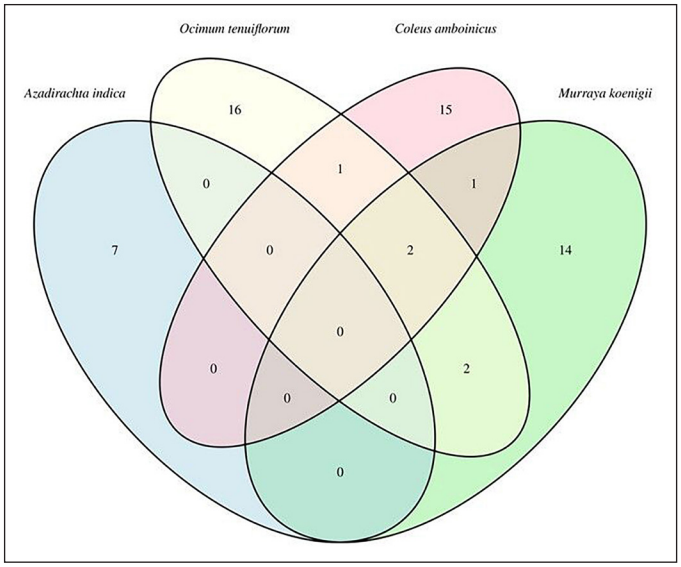

Figure 8: Distribution of metabolites in the PdEVs isolated from *Azadirachta indica, Murraya koenigii, Ocimum tenuiflorum,* and *Coleus amboinicus.* The PdEVs *Azadirachta indica* have 7 distinct metabolites; *Murraya koenigii* has l4 unique metabolites; *Ocimum tenuiflorum* has 16 divergent metabolites; and *Coleus amboinicus* has 15 unique metabolites.

Murraya koenigii, *Ocimum tenuiflorum,* and *Coleus amboinicus*, as depicted in Figure 7.

The metabolites from *Azadirachta indica* commonly have the potential to suppress the activity of microbes. Glycerin is a humectant that helps to moisturize the skin and is used as a sugar alcohol in food products. Lysergic acid is used as a hallucinogen that changes the perception and mood of humans. Cyclohexasiloxane, dodecamethyl [23,24] is the bioactive compound reported previously in the leaf extract of *Toddalia asiatica* (L.) [25] and the antiviral compound in *Nostoc linckia* [26]. This was added as a main ingredient in ointments and skin moisturizers. Benzoic acid is the most typical compound used in the making of perfumes and dyes. It has also been used as an insect repellent. The peaks derived from the GC-MS results and the metabolites within the PdEVs of *Azadirachta indica* were documented in Table 2, with their respective biological activities detailed in Table 7. Table 3 outlines the distinctive metabolites exclusive to *Azadirachta indica* among the four plants.

The most abundant compounds of extracellular vesicles from *Murraya koenigii* are Tetracosamethyl-cyclododecasiloxane, 1H-Purin-6-amine, [(2-fluorophenyl) methyl]-, Octadecane, 3-ethyl-5-(2-ethyl butyl)-, Tetrapentacontane, Eicosyl isopropyl ether, and Propanoic acid. Tetracosamethyl-cyclododecasiloxane has shown antibacterial, antifungal, and antioxidant properties [4,5,7,11,27]**.** 1H-Purin-6-amine, [(2-fluorophenyl) methyl]- as an alkaloid with antitumor and antioxidant activity [28,29]. 1H-Purin-6-amine, [(2-fluorophenyl) methyl]-(CAS), is a cell division and growth regulation factor found in various plant parts and yeast. This compound is known for its antimicrobial and antifungal activities. It is subsequently highlighted as a potent mechanism-based inhibitor of several enzymes like acyl-coenzyme A, cholesterol acyltransferase, monoamine oxidase, heat shock protein 90, cathepsin D, and c-Jun N-terminal kinases. Its derivatives are also known to possess antitubercular, anti-inflammatory, antitumor, amoebic, antiparkinsonian, anthelmintic, antihypertensive, antihyperlipidemic, antiulcer, chemoprotective, and selective CCR3 receptor antagonist activity [30]. Tetrapentacontane shows the antioxidant properties reported in prickly pear pulp extract [31]. Similarly, Table 2 presents the metabolites within the PdEVs of *Murraya koenigii* and their associated biological activities recorded in Table 7. Unique metabolites within *Murraya koenigii* among the four plants are summarized in Table 4.

In *Ocimum tenuiflorum*, some potential metabolites like batilol (alkylglycerol) used as cosmetic stabilizing and skin conditioning agents were observed. It also helps to stimulate erythrocytes and leucopoiesis. It prevents radiation sickness, X-rays and radiotherapy [32,33]. For *Ocimum tenuiflorum*, the metabolites identified in the PdEVs are listed in Table 2, while their respective biological activities are indicated in Table 7. The specific metabolites particular to *Ocimum tenuiflorum* among the four plants are provided in Table 5.

Metabolites from PdEVs of *Coleus amboinicus,* such as digitoxin are used as drugs for cancer and heart failure treatment [34]. Ethane, 1,1-diethoxy, is used as a flavoring agent and an aromatic compound in the food industry [35]. Table 2 outlines the metabolites present in the PdEVs of *Coleus amboinicus*, accompanied by their biological activities described in Table 7. The distinctive metabolites within *Coleus amboinicus* among the four plants are summarized in Table 6.

A representation of the number of common and distinctive metabolites that are present in the PdEVs of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* has been plotted as a Venn diagram using Rstudio, as depicted in Figure 8. There are 7 metabolites present distinctively in *Azadirachta indica*, 14 metabolites uniquely present in *Murraya koenigii*, 16 metabolites distinctive to *Ocimum tenuiflorum*, and 15 metabolites divergent to *Coleus amboinicus*. There are five metabolites commonly present in the PdEVs of *Murraya koenigii* and *Ocimum tenuiflorum*. In the PdEVs of *Ocimum tenuiflorum* and *Coleus amboinicus*, four metabolites are present in common. Four metabolites were found in the PdEVs of *Murraya koenigii* and *Coleus amboinicus*.

4. DISCUSSION

In our study, the leaves were collected from four different medicinal plants, namely *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus,* and the apoplastic fluid was extracted from them. From the collected apoplastic fluid, the PdEVs were isolated. Gallart-Palau, X., et al. have used the organic solvent precipitation-based method to extract extracellular vesicles from biological fluids and tissues of the central nervous system [13,14]. In this study, we adapted the same method to isolate PdEVs from different medicinal plants. HR-TEM analysis was performed to understand the morphology of the isolated PdEVs from different medicinal plants. We observed the spherical shape, and the size ranges from 140 nm to 400 nm. Dash M. et al. have performed ATR-FTIR analysis to discriminate the exosomes from different cell lines using three different methods [6]. Qualitatively, we study the composition of PdEVs based on the functional groups using ATR-FTIR.

Based on the FTIR spectra, the PCA graph was plotted to discriminate the PdEVs derived from different plant families. The PdEVs extracted from the same plant families, including *Ocimum tenuiflorum* and *Coleus amboinicus,* were grouped in the Lamiaceae family, whereas the PdEVs derived from *Azadirachta indica* belonging to the Meliaceae family and *Murraya koenigii* belonging to the Rutaceae family were segregated separately. Thus, it can be hypothesized that PdEVs cargo and composition differ for each plant family.

The GC-MS chromatogram exhibited distinctive peaks that corresponded to the metabolites present in the PdEVs of *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus*. It was observed that most of the compounds present in the PdEVs possess anti-cancer, anti-microbial, and anti-fungal activities. The compound glycerin was observed to be present in the PdEVs of *Azadirachta indica*. Gilbert et al. have reported that the compound glycerin possesses anti-microbial activity by treating it against *Staphylococcus aureus* [36]. Within the four plant species, a notable presence of the compound cyclohexasiloxane, dodecamethyl, was detected in the PdEVs of *Azadirachta indica*. This compound has been previously reported by Mebude, O. O., and Adeniyi, B. to exhibit antifungal and antimicrobial properties when found in the ethanolic extract of Cola nitida [37]. The compound eicosyl isopropyl ether was identified in the PdEVs of *Murraya koenigii*, *Coleus amboinicus,* and *Ocimum tenuiflorum*. Lykholat et al. identified Eicosyl isopropyl ether in endophytes, which expressed anti-microbial and anti-fungal activities [38]. Tetrapentacontane was identified in the PdEVs of *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus*. Ali et al. identified the compound tetrapentacontane in higher quantity in *Santolina chamaecyparissus*, which is reported to possess anti-cancer and anti-diabetic activity [39]. Among the four plant specimens, the compound Thonzylamine emerged as a distinctive presence within the PdEVs of *Ocimum tenuiflorum*. Previous insights from Zhu, Q., and Tao, C., underscored the compound's potential, suggesting its possession of antihistamine and anticholinergic activities [40]. The compound Longipinane (E)- was observed to be present in *Coleus amboinicus*. Cerda-Garcia-Rojas et al. reported that the compound Longipinane possesses anti-feedant activity by administering it against herbivorous insects and anti-cancer activity by administering it against lung cancer cells [41]. Among the four plants, the compound Digitoxin exhibited a unique presence within the PdEVs of *Coleus amboinicus*. Elbaz, H. A., et al. previously documented that Digitoxin demonstrates anti-cancer activities [34]. These PdEVs contain compounds expressing anti-histamine, anti-oxidant, anti-spasmodic, anti-cholinergic, and analgesic actions. The current study describes the discrimination of PdEVs derived from different plants and the biological activities of metabolites in the PdEVs derived from *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus*.

5. CONCLUSION

PdEVs were isolated from the apoplastic fluid of the plants *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus*. These PdEVs were morphologically analyzed using HR-TEM. Then, the isolated PdEVs were characterized using an FTIR spectrometer, and the PdEVs can be analyzed based on functional groups. The functional groups present in the PdEVs derived from *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* were identified. Based on the FTIR analysis, PCA was performed to discriminate the PdEVs derived from different plant families. Further, the metabolomic content of PdEVs was analyzed using GC-MS, and the biological activities of those metabolites present in PdEVs were reported. In the future, PdEVs derived from *Azadirachta indica*, *Murraya koenigii*, *Ocimum tenuiflorum,* and *Coleus amboinicus* can be studied for their potential anti-cancer, anti-microbial, and anti-fungal activities. These PdEVs can be administered as drugs since they do not trigger any immune reactions and do not need to be engineered for therapeutic applications.

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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

10. ETHICAL APPROVALS

This study does not involve any experiments on animal or human subjects.

11. DATA AVAILABILITY

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

12. 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.

13. PUBLISHER'S NOTE

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