Formulation, Development and Assessment of Novel Phyto-Elastosomes Loaded with Devil's Claw Extract

Pascal Ntemi
Rhodes University
Roderick B Walker
Rhodes University
Sandile Khamanga (s.khamanga@ru.ac.za)
Rhodes University https://orcid.org/0000-0003-2374-8460

Research

Keywords: Arthritis, Devil's claw extract, harpagoside, phytoelastosomes, ethanol, elastic

Posted Date: July 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-631706/v1

Abstract

Background: Management of arthritis requires frequent administration of medications at high doses that may lead to unwanted side effects and diminished patient adherence to the therapy. Devil's claw extract, a herbal medicine from the Kalahari sands possess similar therapeutic efficacy with less side effects as the commercialized NSAIDs. The objectives of this study were to formulate, develop and assess novel phyto-elastosomes loaded with Devil's claw extract in order to combat the toxicity levels associated with Devil's claw and enhance penetration of harpagoside to intended targeted site.

Methods: Screening studies were undertaken to determine the ideal amount of Tween® 80, cholesterol, ethanol, diacetyl phosphate and the pH of the hydration medium necessary to produce stable Devil's claw-loaded phyto-elastosomes. Parameters monitored were particle size, polydispersity index, zeta potential, entrapment efficiency and deformability index.

Results: The use of 20 % v/v ethanol was sufficient to produce novel phyto-elastosomes capable of deforming with minimal size alterations. Hydration of thin films in acidic solution produced phyto-elastosomal dispersions with high entrapment efficiency. The presence of cholesterol impeded harpagoside entrapment and increased cholesterol content affected the stability of vesicles by causing agglomeration. Conversely, increasing Tween® 80 concentration promoted harpagoside entrapment. Diacetyl phosphate promoted the stability of vesicle through charge induction.

Conclusions: Development of Devil's claw loaded phyto-elastosomes is useful in ensuring harpagoside reach the target site of action in arthritis-affected patients. Incorporation of these elastic vesicles in transdermal dosage forms may significantly improve the management of arthritis in the near future.

Background

Devil's claw extract (DC) obtained from plants originating from the Kalahari sands in Namibia exhibit similar therapeutic efficacy and less side effects as the commercialized medicines such as diacerhein [1], rofecoxib [2] and phenylbutazone [3] in the management of osteoarthritis and rheumatoid-arthritis. The herbal extract is recommended by the European Scientific Cooperative on Phytotherapy (ESCOP) for the symptomatic treatment of degenerative rheumatoid-arthritis and painful osteoarthritis. The therapeutic effectiveness of the extract is mainly influenced by its chief active component, harpagoside [1, 4]. About 1.2-2% w/w of harpagoside is present in DC and daily doses of at least 50 mg of harpagoside are effective for treating arthritis [2, 5]. Therefore, the use of DC for symptomatic treatment of arthritis require high doses and long dose durations, of at least 54 weeks [6, 7]. Oral administration presents some obstacles such as decreased patient compliance to the therapy due to the bitterness of the extract [8, 9] and mild gastrointestinal upset [10], consequently the transdermal route seems to be the ideal route of administration. Sustained release formulations may be useful as they increase the dosing interval and thus limit drug toxicity and enhance patient adherence [11].

In recent decades, there has been a keen interest in the development of novel drug delivery system to overcome the challenges associated with the use of conventional dosage forms. Vesicle-based carrier system that consist of a concentric bilayer made-up of amphiphilic molecules that include an aqueous compartment [12, 13] have been found to improve the bioavailability of the encapsulated API [13–15] whilst ensuring the therapeutic activity of the payload is achieved in a controlled manner over an extended period of time [13, 16]. Moreover, in circumstances where the vesicle-based carriers have been made elastic by adding edge activators such as ethanol to the formulation to develop novel elastic vesicular system (NEVS), they have been found to exhibit superior permeation properties, enhanced solubility and stability whilst decreasing skin irritation and toxicity compared to conventional vesicular systems [13, 17, 18]. NEVS include transfersomes [13, 18], ethosomes [19, 20], invasomes [17, 18] and elastic niosomes [21]. Therefore, the objective of this work was to formulate, develop and evaluate NEVS capable of entrapping harpagoside whilst maintaining the stability of the vesicles. The incorporation of phyto-constituents in elastic vesicles results in a vesicular system we termed as phyto-elastosomes. Phyto-elastosomes are ultra-deformable bilayered-vesicles loaded with phyto-constituents and are capable of penetrating through pores smaller than their hydrodynamic size without significant alteration to the original dimension of the carrier.

Materials And Methods

Materials

DC powder was purchased from Hunan Nutramax Incorporation (Changsha, China). Tween® 80 was selected as the surfactant of choice for the development of bilayered-vesicles as it has a HLB value of 15 [22] and a double bond in the alkyl chain [23] which is ideal for the encapsulation of hydrophilic compounds like phyto-constituents. Tween® 80 was donated by Aspen® Pharmacare (Port Elizabeth, South Africa). Cholesterol (CHOL) was purchased from Sigma Aldrich® (St. Louis, USA) and was used to ensure membrane rigidity and excellent physical stability of the vesicles. CHOL is an essential component when forming bilayer vesicles using surfactants with HLB > 6 [23, 24]. Diacetyl phosphate (DCP) was purchased from Sigma Aldrich® (St. Louis, USA) and was used as a charge inducer to enhance particle stability. Phosphate buffered saline (PBS) was used as the hydration medium. PBS was made using analytical grade reagents *viz*. sodium chloride, potassium chloride, potassium dihydrogen phosphate and sodium hydrogen phosphate purchased from Minema® Chemicals (Johannesburg, South Africa). HPLC grade water used as the solvent. Hydrochloric acid (32 % v/v) was purchased from univAR® (Modderfontein, South Africa) and was used to adjust the pH of the PBS. HPLC grade ethanol purchased from Sigma Aldrich® (St. Louis, USA) was added to the hydration medium to impart flexibility characteristics to vesicles.

Methods

Preparation of novel DC-loaded phyto-elastosomes

The thin film hydration (TFH) technique has been commonly used for the development of bilayered nanovesicles [17,18,21,25] and thus was employed for this study. A schematic representation of the production the vesicles is displayed in Fig.1. In brief, different amounts of Tween® 80, CHOL and DCP were accurately weighed and kept in a round-bottomed flask (RBF) and dissolved with 10 mL chloroform-ethanol (1:1) mixture. The organic solvents were removed using a rotary evaporator under reduced pressure at a rotary speed of 100 rpm and temperature of 75°C resulting in the formation of a thin lipid film at the base of the RBF. The thin film was then hydrated with PBS containing DC at 100 rpm and 75°C leading to the formation of novel DC-loaded phyto-elastosomes. The dispersions were sonicated for 10 minutes in ice-cold Branson 8510 sonicator (Shelton, USA) and centrifuged for 90 minutes using Eppendorf 3154-C centrifuge (Hamburg, Germany) to separate the unbound harpagoside. The phyto-elastosomal dispersions were stored in vials at 4 ± 2°C. Noteworthy, DC-loaded vesicles were prepared using 30 mg of the extract viz. initial drug concentration was 3mg/mL.

The Critical Quality Attributes (CQAs) monitored were particle size (PS) and polydispersity index (PDI) using a Nano-ZS® Zetasizer (Worcestershire, United Kingdom) set in the Photon Correlation Spectroscopy mode and zeta potential (ZP), setting the zetasizer to Laser Doppler Anemometry mode. The entrapment efficiency (%EE) was determined using a validated reversed-phase HPLC method [26] and the deformability index (DI) using the extrusion method.

Screening studies

The elasticity of vesicular membranes is a unique and crucial parameter for deformable vesicles [21, 27]. The elasticity is based on the type and amount of edge activator(s) used in formulations [17–19]. Ethanol, used in high concentrations of > 10 % v/v is considered to be an effective elasticity enhancer for a number of vesicular systems such as ethoniosomes [28], invasomes [18], ethosomes [19] and elastic niosomes [21]. However, at high ethanol concentrations and beyond certain levels, the bilayers of the vesicles become leaky, which may significantly decrease %EE [29]. The ideal concentration of ethanol to be used throughout this study, was investigated using screening studies by varying the amounts of ethanol used in hydration medium by 5%. The formulation composition and manufacture parameters of the batches prepared during screening studies of ethanol is summarized in Table 1. The elasticity of the phyto-elastosomes was determined using the extrusion method [21, 30]. Phyto-elastosomal dispersions were extruded through a 50 nm polycarbonate membrane filter (Millipore, USA) at constant pressure of 1.5 bar for 10 minutes. The DI was then calculated using Eq. 1 [30, 31].

$$DI = j\left(\frac{rv}{rp}\right)^2 \qquad \textbf{Equation 1}$$

Where,

j = the weight of bilayered vesicle dispersions extruded

rv = the size of vesicle after extrusion

rp = the pore size of the filter membrane.

Table 1

Formulation parameters during screening of ethanol

Batch	Tween® 80 (µmole)	CHOL (µmole)	DC (mg/mL)	pH	Ethanol (% v/v)
1	80	20	0	7.0	0
2	80	20	0	7.0	20
3	80	20	3	7.0	0
4	80	20	3	7.0	5
5	80	20	3	7.0	10
6	80	20	3	7.0	15
7	80	20	3	7.0	20
8	80	20	3	7.0	25
9	80	20	3	7.0	30
10	80	20	3	7.0	35
11	80	20	3	7.0	40

The influence of pH on the entrapment of harpagoside was determined by screening the pH of hydration medium. Batches of both phyto-elastosomes and phyto-niosomes prepared by the THF method described above (preparation section) were hydrated using hydration medium of different pH. A summary of formulation parameters used during screening of the pH is shown in Table 2.

Table 2. Formulation parameters during screening of pH of hydration medium

Batch	Tween® 80 (µmole)	CHOL (µmole)	DC (mg/mL)	pH	Ethanol (% v/v)
12	80	20	3	7.0	0
13	80	20	3	7.0	20
14	80	20	3	6.5	0
15	80	20	3	6.5	20
16	80	20	3	6.0	0
17	80	20	3	6.0	20
18	80	20	3	5.5	0
19	80	20	3	5.5	20
20	80	20	3	5.0	0
21	80	20	3	5.0	20

The effect of Tween® 80, CHOL and DCP both the stability and %EE of the vesicles was investigated by developing phyto-elastosomes using different amounts of Tween® 80, CHOL effect and additives. The hydration medium used was a 20 % v/v ethanolic PBS (pH 7.0) solution with a DC concentration of 3

mg/mL. The amounts of excipients used during screening of formulation compositions is summarized in Table 3.

Table 3
Formulation parameters during screening of Tween® 80, CHOL and DCP

Batch	Tween® 80 µmole	CHOL µmole	DCP µmole
22	90	10	0
23	60	40	0
24	25	75	0
25	10	90	0
26	90	10	1
27	90	10	5
28	90	10	10
29	180	20	0
30	120	80	0
31	50	150	0
32	20	180	0
33	180	20	2
34	180	20	10
35	180	20	20

Stability studies

Stability studies of bilayered-vesicles were undertaken by storing DC-loaded phyto-elastosomal dispersions at room temperature (22°C) and in a refrigerator (4 ± 2°C) for 8 weeks. The dispersions were then analysed to determine vesicle aggregation, drug leakage and ZP using Transmission Electron Microscope (TEM), HPLC and Dynamic Light Scattering (DLS) respectively.

Topographical studies

The shape and surface morphology of phyto-elastosomes was studied using TEM. A drop of aqueous phyto-elastosomal suspension was placed onto a copper grid with a carbon film for approximately 30 seconds after which excess liquid was removed using Whatman® 110mm diameter filter paper

(Maidstone, England). The grid was placed onto a carbon film and allowed to dry at a room temperature of 22°C overnight. The sample was then visualized using a Zeiss Libra® 120 TEM (Munich, Germany).

The Energy Dispersive X-Ray Scanning Electron Microscope (EDX-SEM) was used to determine the surface elemental compositions of raw DC and that of the novel DC-loaded phyto-elastosomes. Approximately 1 mg of sample was dusted onto a graphite plate and irradiated at an accelerated voltage of 20 kV using SEM. Liquid nitrogen was used to vaporize the sample. Elemental analysis was undertaken using a Vega® SEM (Vega LMU, Tuscan, Czechoslovakia Republic). The surface elements present in the sample were determined by measuring the number of x-rays emitted by the sample versus the energy of the rays.

Results

Screening studies of ethanol

Novel phyto-elastosomal vesicles had smaller PS and lower ZP than the phyto-niosomal vesicles. This phenomenon may be attributed to phase formation within the vesicles by the hydrocarbon chains of ethanol [32, 33], modification of the net charge of the vesicles due to steric stabilization [34] and reduction of electrostatic repulsive forces on the vesicular surface [35, 36]. Phyto-elastosomes prepared with ethanol concentration ≥ 25 % v/v exhibited vesicle aggregation due to salting out effect of non-surfactants in the ethanolic solution [37, 38] leading to physically unstable vesicles. Ethanol concentration of 15 % v/v and 20 % v/v produced vesicles that exhibited targeted mean PS, revealed no sedimentation or layer separation and had a good DI indicating their great ability to squeeze through small pores. Ethanol also enhanced the solubility of DC in the vesicles leading to an increased %EE [39]. A summary of results from batches produced during screening of ethanol is displayed in Table 4.

Table 4
Analysis of batches manufactured during screening of ethanol

Batch	Ethanol (% v/v)	Initial PS (nm)	PS after 24 hours (nm)	PS after extrusion (nm)	ZP (mV)	%EE	DI
1*	0	348.1	348.8	52.5	-28.1	-	0.33
2*	20	273.9	273.8	265.1	-24.5	-	13.76
3	0	312.3	312.9	81.8	-27.6	28.42	0.80
4	5	297.0	296.4	245.2	-26.3	30.05	8.17
5	10	283.2	282.6	250.3	-25.4	33.67	9.77
6	15	266.5	266.8	258.4	-24.6	37.73	11.75
7	20	249.4	252.5	248.5	-24.1	39.99	12.59
8	25	235.3	395.3	234.8	-22.6	46.51	11.47
9	30	219.4	430.4	218.5	-22.2	48.48	9.93
10	35	202.2	522.2	201.6	-21.9	54.82	8.45
11	40	187.5	650.5	187.1	-21.8	57.14	7.28

Screening studies of the pH of hydration medium

Screening studies showed that the pH of the hydration medium had a significant impact on the %EE and physical stability of the phyto-elastosomes. Harpagoside entrapment increased as the pH of the medium used decreased from 7.0 to 5.5. This is attributed to the enhanced solubility of DC in acidic medium due to ionisation of the ester functional groups in the iridoid glycosides of the DC [40]. Vesicle aggregation and drug leakage were observed in batches prepared with hydration medium pH 5.0 was used. The ZP values decreased as the acidity of the medium were increased. Vesicles were homogeneously distributed in all batches as indicated by PDI. The analytical results of the phyto-elastosomes produced during pH screening studies are displayed in Table 5.

Table 5
Analysis of batches manufactured during screening of pH of the medium

Batch	Initial PS (nm)	PS after 24 hours (nm)	PDI	ZP (mv)	%EE
12	317.8	318.9	0.428	-27.2	27.20
13	247.3	248.6	0.456	-24.1	40.53
14	329.1	330.2	0.573	-25.5	32.67
15	252.3	253.8	0.512	-22.3	46.72
16	301.9	303.1	0.432	-23.8	37.87
17	274.3	276.8	0.353	-20.4	54.29
18	268.1	290.2	0.529	-17.8	46.22
19	307.3	355.4	0.517	-15.7	64.51
20	348.2	863.6	0.489	-14.9	35.32
21	313.6	1272.1	0.454	-12.5	50.16

Screening of additives

Phyto-elastosomal dispersions produced with ≥ 40 µmole of CHOL were highly viscous, non-spherical and exhibited low %EE. Retardation of harpagoside was due to increase in both lipid content of the formulation and hydrodynamic diameter of the vesicles. Although there was a PS decrease due to CHOL addition, CHOL increased both the viscosity [41] and rigidity of the vesicles [42, 43] which may affect the shape of the vesicles [41–43]. Reduction in PS is due to decrease in water uptake across the lipid bilayer [44] and tightening of the bilayers [45, 46] as CHOL concentration increased. The addition of DCP resulted in an increase in the electrostatic repulsive forces between vesicles thereby increasing the ZP and PS due to separation of the lipid bilayers. The interaction between DCP and the oleate fatty acid in Tween® 80 led to an increase in %EE [25]. Increasing the amounts of Tween® 80 led to production of large sized vesicles and with increased repulsive forces due to the presence of anionic fatty acid impurities and adsorption of hydroxyl ions from the hydration medium [47, 48]. As a hydrophilic surfactant, Tween® 80 facilitated an increase in the entrapment of harpagoside. The analysis of phyto-elastosomes produced during screening of formulation compositions is shown in Table 6.

Table 6
Analysis of batches produced upon screening of formulation compositions

Batch	Initial PS (nm)	PS after 24 hours (nm)	PDI	ZP (mV)	%EE
22	247.8	248.7	0.303	-23.8	47.11
23	209.2	211.7	0.528	-23.2	42.88
24	188.1	197.3	0.547	-22.6	24.46
25	174.2	181.5	0.501	-23.2	20.72
26	254.1	255.9	0.513	-26.7	49.28
27	290.2	291.8	1.000	-34.8	49.56
28	351.3	352.6	1.000	-37.7	52.14
29	344.1	346.9	0.389	-25.7	50.64
30	317.4	320.8	0.621	-25.1	46.07
31	266.2	274.1	0.542	-26.7	22.98
32	248.4	259.2	0.535	-25.6	18.75
33	353.0	354.7	0.552	-29.5	52.82
34	400.8	401.2	1.000	-36.5	53.89
35	450.3	451.7	1.000	-39.1	57.20

Stability studies

Stability studies showed that vesicle aggregation and high API leakage in all phyto-elastosomes stored at room temperature (22°C). The aggregation may be due to an increased rate of collision [49] and harpagoside leakage was attributed to chemical degradation of the surfactant at high temperatures that weakened the membrane packing and integrity [50]. Phyto-elastosomes containing DCP and stored at 4°C had minimal bilayer expansion and API leakage. The API leakage for all batches stored at 4°C were within the acceptable limit for loss of API (< 10%) as suggested in the ICH guidelines [51]. Upon sonication for 5 minutes, aggregates of particles in batches containing DCP were dispersed albeit with a conspicuous increase in PS. However the vesicle aggregates in batches where no DCP was included did not disperse. Vesicle aggregates that dispersed on sonication was also observed for batches without DCP that had been stored at 4°C. The results of stability studies are summarized in Table 7.

Table 7
Results for assessment of stability studies

Batch	Temperature °C	Day 0			Week 8		
		PS	ZP	%EE	PS	ZP	%EE
N04	22	297.0	-26.3	30.05	4306.3	-13.8	21.74
	4	297.0	-26.3	30.05	712.2	-21.3	28.17
N07	22	249.4	-24.1	39.99	3014.3	-11.8	28.49
	4	249.4	-24.1	39.99	650.7	-20.1	37.93
N15	22	252.3	-22.3	46.72	2804.8	-11.6	36.52
	4	252.3	-22.3	46.72	689.3	-19.7	43.62
N17	22	274.3	-20.4	54.29	3293.5	-09.1	40.35
	4	274.3	-20.4	54.29	636.5	-16.4	51.76
N30	22	317.4	-25.1	46.07	2608.4	-12.5	35.04
	4	317.4	-25.1	46.07	725.6	-21.1	43.84
N33	22	353.0	-29.5	52.82	604.3	-27.2	52.39
	4	353.0	-29.5	52.82	355.5	-28.2	52.57
N34	22	400.8	-36.5	53.89	541.1	-34.9	53.55
	4	400.8	-36.5	53.89	402.3	-36.1	53.73

Topographical analysis

For most formulation compositions, nano-sized spherical-shaped vesicles were produced. Non-spherical vesicles were observed in batches N24 and N31 that contained ≥ 40 µmole CHOL. The shape and size of phyto-elastosomal vesicles of selected batches observed using TEM are depicted in Fig. 2a-f.

The EDX-SEM analysis showed that the surface of the DC-loaded phyto-elastosomal vesicles was comprised only of carbon and oxygen; thus confirming the incorporation of DC in the inner aqueous layer of the vesicles as other elements that were observed in the EDX-SEM spectrum of the herbal extract *viz.* sodium, silicon, aluminium, chlorine, phosphorus and potassium were absent. Phosphorus was also present on the surface of phyto-elastosomal vesicles containing DCP. The EDX-SEM spectra and chemical content of DC and selected batches of phyto-elastosomes are displayed in Figs. 3–5 and summarized in Tables 8–10 respectively.

Table 8
Chemical compositions of DC

ELEMENT	WEIGHT %	ATOMIC %
Carbon	94.01	94.84
Oxygen	5.41	4.52
Sodium	0.53	0.26
Aluminium	0.14	0.06
Silicon	0.20	0.08
Phosphorus	0.45	0.16
Chlorine	0.16	0.05
Potassium	0.11	0.03
TOTAL	100.99	100

Table 9
Chemical compositions of DC-loaded phyto-elastosomes

ELEMENT	WEIGHT %	ATOMIC %
Carbon	78.45	89.93
Oxygen	17.57	10.07
TOTAL	96.02	100

Table 10
Chemical compositions of DC-loaded phyto-elastosomes containing DCP

ELEMENT	WEIGHT %	ATOMIC %
Carbon	88.92	94.49
Oxygen	6.79	5.41
Phosphate	0.24	0.10
TOTAL	95.95	100

Discussions

Novel phyto-elastosomes loaded with DC were successfully prepared, screened and evaluated. Topographical analysis through EDX-SEM and HPLC analysis conclusively revealed the entrapment of

phyto-constituents, including harpagoside in elastic bilayered-vesicles. The ideal ethanol concentration to guarantee stable vesicles was found to be 20 % v/v. At this concentration, the vesicles produced possessed the best elastic properties, with a deformability index of 12.59; and thus the 20 % v/v ethanol concentration was used in all subsequent batches. The pH of the hydration medium for maximal entrapment of main constituent, harpagoside, in the elastic vesicles was found to be 5.5. Vesicles produced using ≥ 40 µmole cholesterol were the smallest in size among all batches, however they were also non-spherical in shape and had an EE% < 50%. Elastic bilayered-vesicles comprised of 180 µmole Tween® 80, 20 µmole cholesterol and 2 µmole DCP produced the best stable vesicles, possessing a ZP values of -29.5 mV, PDI *viz*. 0.552, PS *viz*. 0.353 nm and minimal particle size alterations even after 8 weeks *viz*. 0.026% upon storage in the refrigerator. These vesicles also showed an acceptable EE% *viz*. 52.8%.

Conclusions

Novel phyto-elastosomes are an ideal candidate for the entrapment of harpagoside contained in DC. The elastic vesicular system showed superior permeability through a membrane and stability as well as better entrapment of the API compared to non-elastic vesicular system. The study also revealed enhanced stability, solubility and entrapment of harpagoside in acidic environment, addition of DCP and Tween® 80. Advanced studies on the optimization of the factors affecting the loading of phyto-constituents in the elastic vesicles, the stability of the vesicles and release of harpagoside from the vesicles to achieve an ideal DC-loaded phyto-elastosomes are expected to be undertaken in the near future. Moreover, the vesicles are expected to decrease skin irritation and toxicity of both the API and transdermal dosage form and studies on the formulation, development and evaluation of these NEVS into transdermal dosage forms such as gels will be undertaken so as to achieve a formulation that may be used in the management of arthritis, especially osteoarthritis as soon as possible.

Abbreviations

NSAIDs: Non-steroidal anti-inflammatory drugs; API: Active Pharmaceutical Ingredients; HPLC: High Performance Liquid Chromatography; HLB: Hydrophilic-Lipophilic Balance; ICH: International Conference of Harmonization.

Declarations

Acknowledgements

The authors would like to acknowledge the Biopharmaceutics Research Group Laboratory (Rhodes University) for their technical help and support.

Authors' contributions

Conceptualization was done by Pascal Ntemi, Roderick Walker and Sandile Khamanga; methodology was undertaken by Pascal Ntemi; formal analysis and investigation were performed by Pascal Ntemi; resources were obtained by Roderick Walker and Sandile Khamanga; data curation was undertaken by Roderick Walker and Sandile Khamanga; writing—original draft preparation was done by Pascal Ntemi; writing—review and editing was performed by Roderick Walker and Sandile Khamanga; supervision was done by Roderick Walker and Sandile Khamanga and funding acquisition was done by Roderick Walker and Sandile Khamanga. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the National Research Fund (NRF) South Africa and the Rhodes University Research Committee (RB Walker and SMM Khamanga).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethic approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

Authors' details

All authors are from Rhodes University, Grahamstown, South Africa. Pascal Ntemi – email address: pntemi90@yahoo.co.uk; Roderick Walker – email address: r.b.walker@ru.ac.za; Sandile Khamanga – email address: s.khamanga@ru.ac.za

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Figures

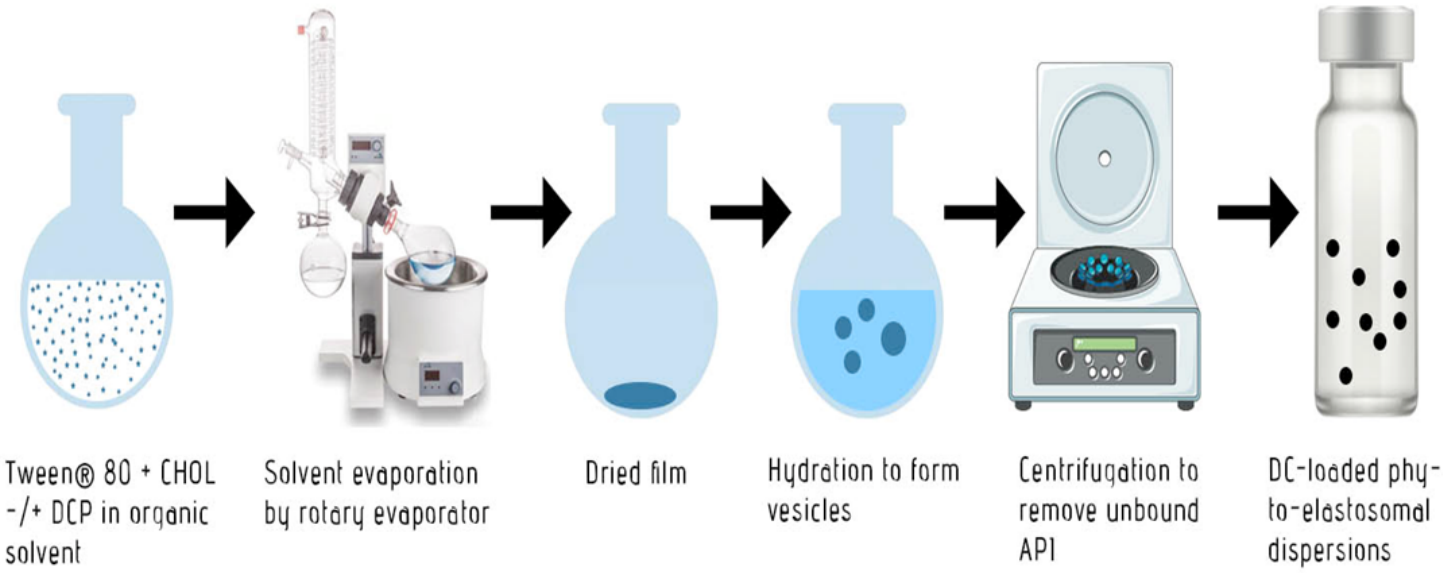
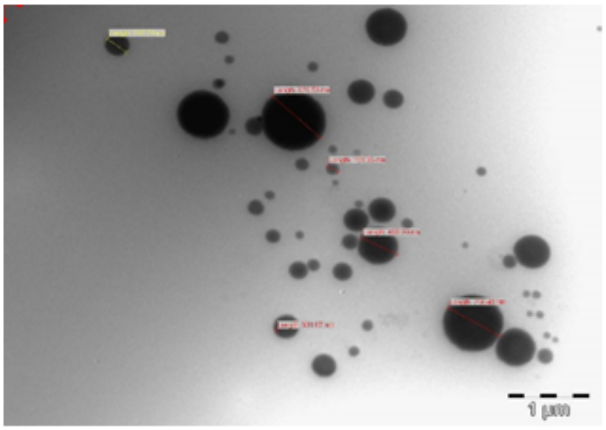


Figure 1

Schematic representation of vesicular system development by thin film hydration method.



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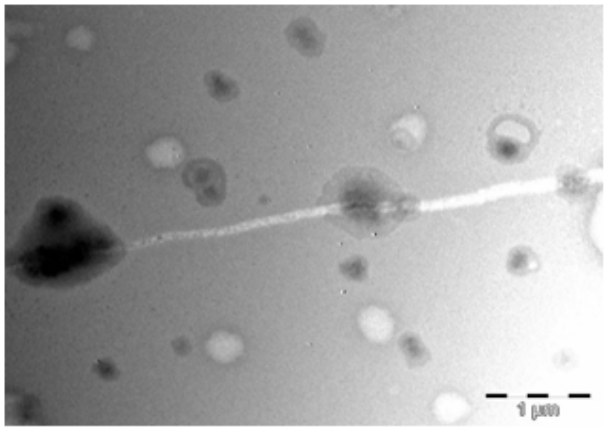

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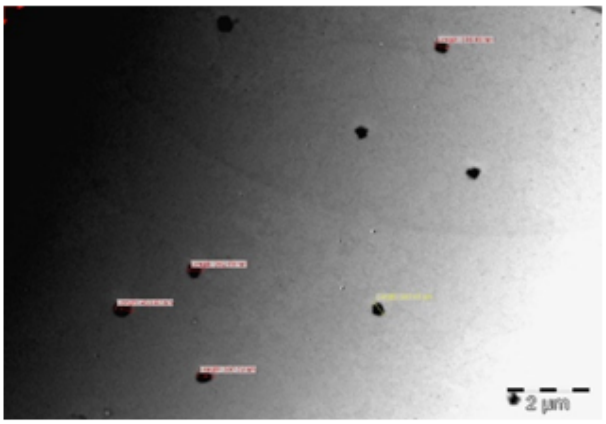

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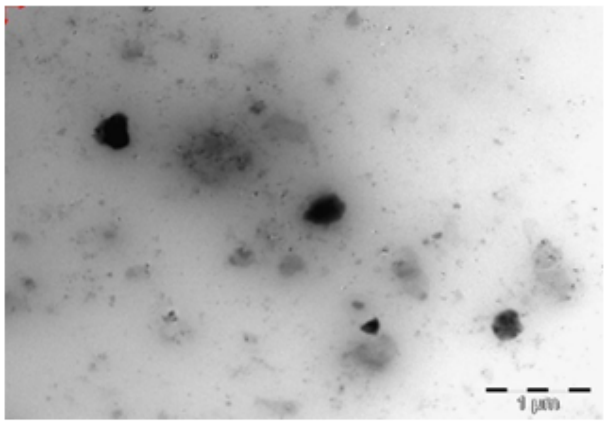

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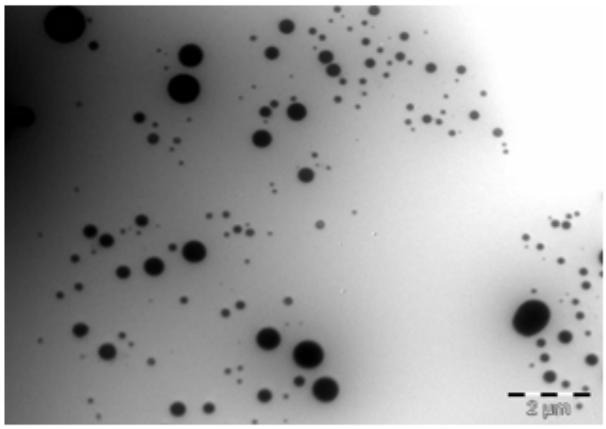

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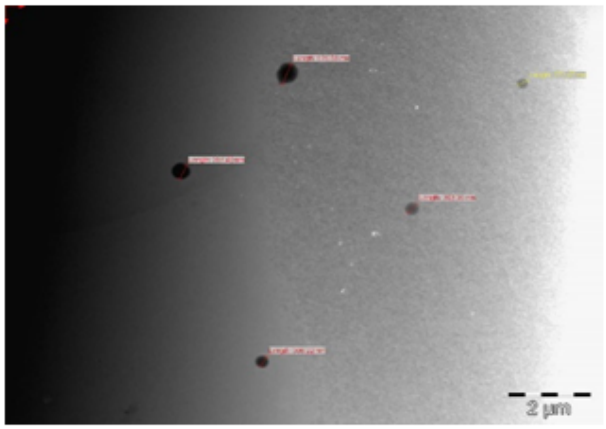

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Figure 2

TEM images of phyto-elastosomes from selected batches.

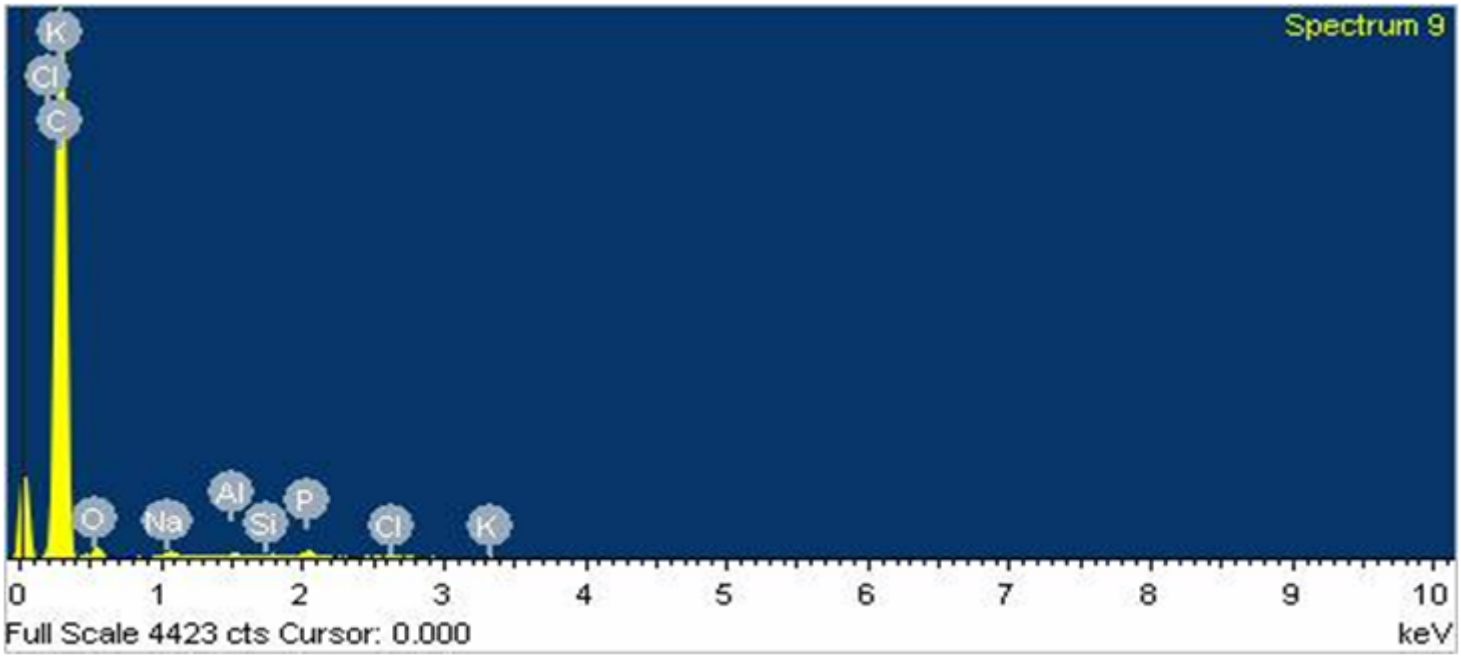

Figure 3

EDX-SEM spectrum of the surface elemental analysis of DC after filtration.

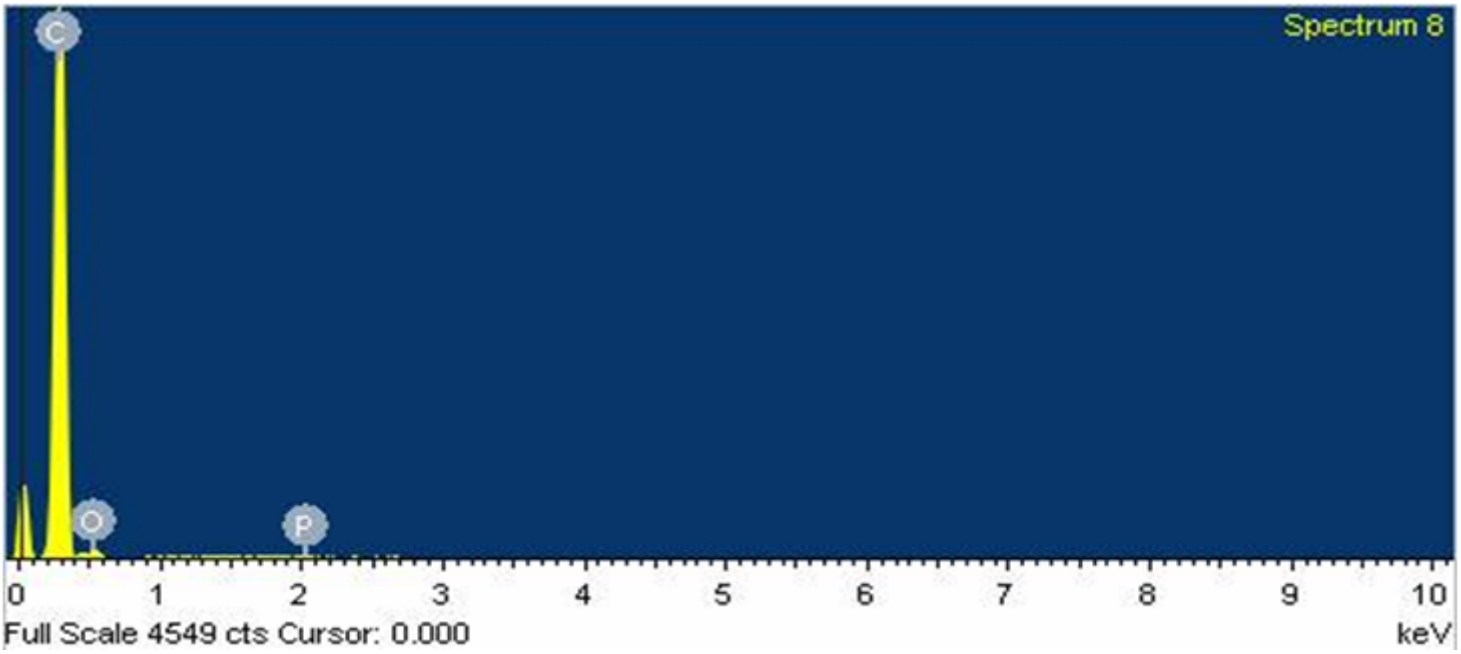

Figure 4

EDX-SEM spectrum of the surface elemental analysis of DC-loaded phyto-elastosomes.

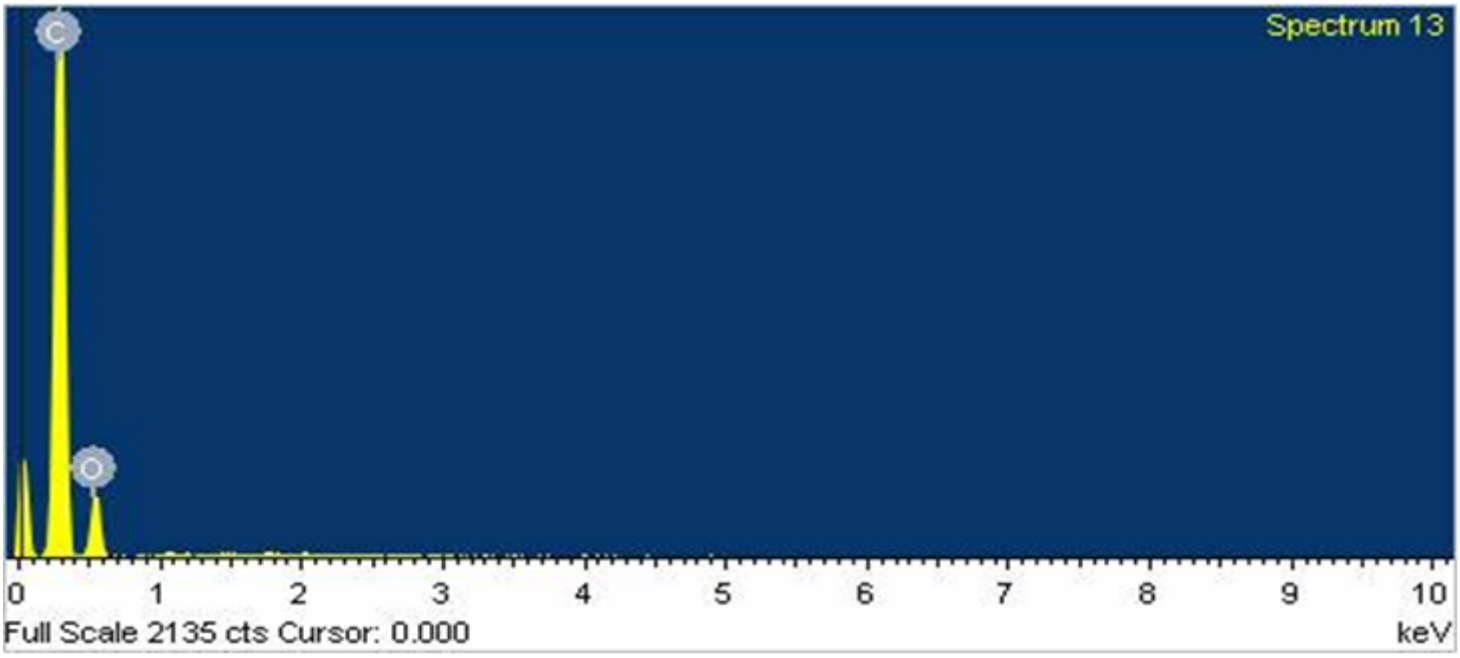

Figure 5

EDX-SEM spectrum of the surface elemental analysis of DC-loaded phyto-elastosomes containing DCP.