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Formulation of açaí (E. oleracea Mart.) pulp and seeds extracts by Co-precipitation in Supercritical Antisolvent (SAS) technology.

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Abstract. *Açaí (Euterpe oleracea Mart.) is a black-purple berry, typically found in Amazon Rainforest, and a natural phytochemical source, which shows a high content of polyphenols and flavonoids, with remarkable properties as an antioxidant, anti-inflammatory, antimicrobial, and natural dye. The precipitation and encapsulation of Açaí extracts with biopolymers by Supercritical Anti-Solvent (SAS) process were investigated and proposed as primary formulation to protect the active compounds from early degradation and its application in pharmaceutical, cosmetic, and alimentary products. The extractives were obtained from pulp and seeds by Pressurized Microwave assisted Energy (PMAE)(300W and 1.5bar) using acidified ethanol/water (1:1 v/v) as a solvent. The extracts were characterized in terms of total polyphenols content (TPC). The SAS process was carried out by semi-continuous batch at constant conditions (40ºC, 100bar). Particle morphology was studied by SEM, TGA and FTIR. The best results were obtained when ethanol and PVP were applied. The process also allow the particle micronization and TPC value increment.*

Keywords: *natural product formulation, Antioxidant, Microencapsulation, Supercritical* CO_2.

1. Introduction

Açaí (*Euterpe Oleracea Mart.*) is a black-purple berry typically found in Amazon Rainforest. The fruit and seeds have remarkable properties as an antioxidant due to its high content of polyphenols, flavonoids, and, anthocyanin. Products such as flavonoids and anthocyanin are studied because of their pharmacological and natural dye potential. The seeds are the main by-product obtained during the industrial processes to extract the fruit pulp, and pulp that is considered inappropriate for human consumption also becomes an industrial residue.

The benefits provided by polyphenols, flavonoids, and anthocyanin convert this class of compounds in an interesting study area. These studies usually focus on the relation of antioxidant effect and its bioavailability [1]. As occurs with many other natural compounds, the application of these biochemical compounds may be limited by their low solubility in aqueous systems. Encapsulating the antioxidant extract in a polymeric matrix for its protection is an excellent method to protect it for an earlier degradation, as well to improve its solubility and, as a consequence, its bio-disponibility [2].

The SAS (Supercritical Anti Solvent) encapsulation is a very versatile process for processing various natural and pharmaceutical compounds, with reasonable control of particle properties, and without product degradation or contamination [3]. In this process, the particle is solubilized in an organic solvent by which it has an affinity. Then, with the aid of a pump, this solution is introduced into a precipitator containing high-pressure CO_2. By introducing the solution into the precipitator as a spray, the dissolving droplets mix with CO_2, which acts as an anti-solvent, as it decreases the solubility of the solute relative to the solvent. In this way, the precipitation of the compound occurs due to the high supersaturation achieved [4]. Compared to precipitation techniques using liquid

antisolvents, the SAS technique has the advantage that, due to the favorable properties in the supercritical medium, very high and homogeneous supersaturation can be achieved very quickly, which favours control over fluid properties. In addition, moderate temperatures below 80°C can be applied, so the process becomes suitable for working with sensitive substances such as products of natural origin [3]. The precipitation and encapsulation technical study apply to sensitive compounds. It has been shown, in a previous study, that polyvinylpyrrolidone (PVP) and Pluronic® F127 are viable encapsulating surfactant option when natural products were formulated [5].

Spray-drying and vacuum-drying, among other techniques, are described in the literature as advantageous formulation process for natural compounds. Many of those techniques require to elevate temperature, as in the spray-drying process where it can reach 125°C, corroborating to the degradation of active molecules, even utilizing low pressure [5]. Vacuum-drying (considered beneficial to natural product formulation by allows water as a solvent) promotes non-spherical particle formation and requires temperature which may also result in degradation of interest compound [6]. The temperature required in vacuum-drying is lower than in spray-drying, however, it is still higher than in other types of formulation, such as in supercritical anti-solvent (SAS).

Concerning the encapsulation material, different cyclodextrins have been purposed by many authors. The cyclodextrin inclusion complex [7], provides the specific water solubility and antioxidant activity improvement [8,9]. The amorphous structured material, resulting from the encapsulation of antioxidants compounds in mixture of Eudragit® E and polyvinyl alcohol, supported by hydrogen bonds has shown a favourable improvement of bioavailability [10], similar to lipid-particles charged with antioxidant compounds by an emulsion freezing process [11].

The present study analyses the use of Supercritical Anti-solvent (SAS) as formulation process for the co-precipitation of açaí (E. oleracea Mart.) pulp and seeds extracts in biopolymers (Pluronic F-127 and PVP). The process aims to promote an formulation of antioxidant obtained from both açaí extracts, improving the water solubility and reducing the early degradation. The effect of the main process parameters (polymer:extract ratio, initial concentration) was studied and products were characterized considering its morphology (size and shape) and composition (total polyphenol content, total anthocyanin content and antioxidant activity).

2. Material and methods

2.1 Extract preparation

Two fractions of açaí by-products (seeds and pulp), obtained from Obidos-PA-Brazil, has been prepared to the extraction process following different protocols. The seeds fraction was milled and dried, and the pulp fraction was lyophilized. The subsequent preparation was to remove the oil content present in both materials [12].

Extracts were obtained by Pressurized Microwave-Assisted Extraction (PMAE) (300W and 1.5bar). Ethanol/water (1:1 v/v) was used as a solvent for the extraction, and citric acid as pH regulator (pH 3). Individually, each raw-material was placed in a pressurizable flask, mixed and homogenized with the prepared solvent. The flask extractor was then introduced into a Circular Energy-Microwave (CEM). CEM was set to constant power (300W), varying the temperature and pressure till 1.5 bar and sample was suddenly cooled introducing the flask into a cold bath. The flask content was transferred into centrifuge-tube. The tube was centrifugated at 5000 rpm for 10 min, and

the supernatant recovered and dried to be used in the preparation of the feed solution, as described in section 2.2.

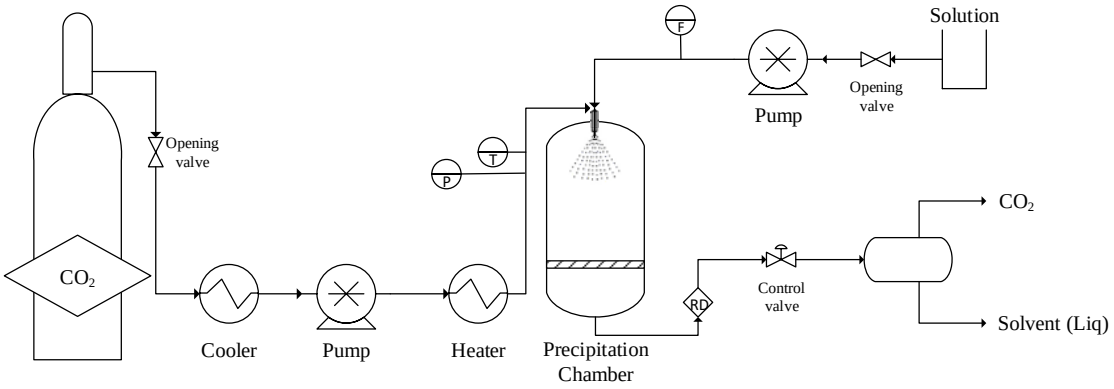
2.2 Preparation of the feed solution

The encapsulation process starts with the preparation of the solution to be encapsulated. Selected extract, polymer, and solvent are mixed at constant stirring. The effect of polymer:extract ratio was studied varying the proportions among both materials ((1:1 m/m), (2:1 m/m), (1:2 m/m), (1:4 m/m)). The effect of the concentration on the formation of the particles was also studied, changing the ratio of total solid (mass) per volume of solvent (0.50 g/mL, 0.33 g/mL, 0.50 g/mL). Furthermore, SAS experiments with the pure polymer and the extract were also performed for comparison purposes.

2.3 SAS Preparation

The general scheme of the equipment used for SAS experiments can be observed in Figure 1The precipitator consists of a metallic jacketed cylinder that is continuously heated by the refluxing water of a thermostatic bath at 40 °C. It has two flanges, the upper one with three main holes through which the barometer and the safety valve are connected, in the second one, the entrance of the CO_2 and the solution, and in the third one, the flow, temperature, and pressure meters. In the flow flange, the outlet of CO_2 and solvent is placed. In the CO_2 line, after the opening valve, CO_2 is liquefied and pumped by the piston pump with a flow rate 2kg/h. Before the chamber CO_2 is again heated to 40ºC and introduced into the precipitator while measuring temperature and flow. The second inlet line drives the solution for encapsulation, from the baker to the precipitator, helped by a chromatography pump (model 305 Gilson) and the flow is controlled at 2 mL/min. In the precipitator, the inlet-lines have a concentric entrance, which produces a spray effect when the solution and CO_2 go inside simultaneously. This leads to the

149 solubilisation of the solvent and the precipitation of particles from solution onto the filter
150 placed at the bottom of the recipient. The process is carried out by semi-continuous batch,
151 constant temperature (40ºC), and pressure (100 bar). A rupture disc set at 210 bars, an
152 outlet GO-type valve and a flask that separates the CO2 from the residual solvent are
153 placed at the exit of the precipitator [13].
154
155 (Figure1)



156
157 Figure 1- Complete SAS flow diagram. (P- pressure controlling; T- temperature
158 controlling; F-flow controlling; and RD-Rupture disc)
159
160 *2.4 Product characterization*
161 2.4.1 Total polyphenol content (TPC)
162 For TPC analysis a capped test tube was used, adding 40µL of the extract, 3 mL
163 of ultrapure water and 200 µL of ciocalteau reagent. It is also necessary to prepare a
164 control sample using 40µL of extraction solvent, 3 mL of ultrapure water and 200 µL of
165 folin-ciocalteau reagent. Tubes were closed and homogenized at 40°C for 5 minutes.
166 After this period 600µL of Na_2CO_3 (20% v/v) solution was added, tubes were vigorously
167 stirred, and kept in hot-water-bath at 40°C for 30 minutes. Finally, the samples were

analysed by spectrophotometer (λ= 765 nm). Tests were performed in duplicate and the average of TPC concentration is given in Gallic acid equivalent per 100 g of dry material. [14]

2.4.2 Total anthocyanin content (TAC)

The TAC analyses were performed with the aid of a spectrophotometry. Samples were diluted (1:4) in a potassium hydroxide buffer (0.025M KCl) at pH 1.0 and buffered with acetate trihydrate buffer ($CH_3CO_2Na.3H_2O$ 0.4 M) at pH 4.5. Samples were diluted in both buffers solution, at pH 1.0 and pH 4.5, and each dilution was measured at 520 nm and 700 nm. Tests were performed in duplicate and the average of concentration of anthocyanins in each sample is given in g of cyanidin equivalent per 100 g of dry material. [15]

2.4.3 Sample morphology

The study of particle morphology was carried by Scanning Electron Microscopy (SEM) analysis (FLEX SEM 1000 Hitachi), and Fourier transform infrared spectroscopy (FTIR) analysis (Bruker ALPHA FT-IR spectrometry) with a single sampling module of platinum ATR diffraction.

3. Results and Discussions

Table 1 reports the experimental conditions applied in all the experiments performed, as well as the total polyphenol content of the particles produced. The higher TPCs were observed when ethanol was applied as solvent and PVP as a carrier. The assays performed using acetone as solvent produced a sample with a plasticized aspect; in

addition, precipitation was observed in the initial solution. It was also determined that applying moderate heat (30ºC) helped in the homogenization of encapsulation solution.

Different seeds-extract to PVP ratios of 2:1, 1:1, 1:2, and 1:4 were processed. Pulp extract and PVP were successfully processed on ratios of 2:1, and 1:1. The encapsulated material resulting from the processes utilizing the ratio of 2:1, 1:1, 1:2, had shown powder aspect. However, when a ratio of 1:4 was applied the material obtained from the processing had irregular and pelletized aspect. Exploratory experiments performed with ratios above 1:4 resulted in plasticized material (results not shown). All experiments performed with Pluronic F127 were unsuccessful.

3.1 Composition analysis

Composition analysis revealed that the material obtained from the processing of seeds-extracts had the expected total polyphenol content (TPC) corresponding to the encapsulated ratio; however, those materials wherein pulp-extract was applied did not present significant phenolic content besides the presence of typical color produced by anthocyanin. In Table 1, the results obtained from the analyses of the total phenolic content (TPC) is expressed in Gallic acid equivalent (GAE) per gram of precipitate. The original extract was processed with no polymer addition and taken as a reference to compare particle ratio formation. The original extract processed by SAS was enriched in terms of polyphenol content compared to the original sample that was not processed by SAS, due to the selectivity of CO_2. During the precipitation, not all the components in the initial extract were precipitated, as there were still remaining components in the extract that showed affinity to CO_2 (such as residual oil). However, CO_2 has low affinity to polyphenols that are precipitated. So, the obtained products are particles enriched in

polyphenols in relation to the initial extracts. The influence of the initial concentration of the solution was also studied using a constant extract/polymer ratio (1:1) and varying the extract/solvent proportions (2.50 g/L, 3.33 g/L and 5.00g/L). The results were based on TPCs values. The highest TPC value was observed in those particles obtained by the proportion of 3.33 g/L, 211 mg GAE/g particle, while for the other proportions studied (2.50 and 5.00 g/L) the TPC values were 80 and 78 mg GAE/g particle, respectively. In addition, the SAS processing increased the TPC value of processed extract (500 mg GAE/g particle) concerning the original extract (261 mg GAE/g particle), the TPC value found was almost twice higher than initial TPC showing the capacity of SAS acts as purification process for natural extracts.

Similar to that observed with the açaí seeds extract, the pulp product also presented better results when intermediate concentrations were applied. Although it was not possible to recover or perform the relevant probes at the single processed pulp extract, it was possible to compare the results with the original pulp and to observe that the processed material has lower TPC than expected. Tests were also performed to evaluate the total content of total anthocyanins by differential buffer pH in spectrophotometry, and none of the tested materials showed detectable anthocyanin content although all have light-pink coloration, typically induced by the presence of this substance in trace amounts.

Table 1 - Experimental conditions and total polyphenol content of SAS-processed particles.

(Table 1)

Extract (Material)	Polymer	Solvent	Polymer: extract Ratio	Initial solution concentration (g/L)	TPC content mg GAE/g particle	SD
Seeds (unprocessed)	-	-	-	-	261	3
Seeds	-	Ethanol	-	3.33	500	5
Seeds	PVP	Ethanol	1:1	5.00	78	3

Seeds	PVP	Ethanol	1:1	3.33	211	8
Seeds	PVP	Ethanol	1:1	2.50	80	4
Seeds	Pluronic	Acetone	1:1	3.33	132	8
Seeds	PVP	Ethanol	2:1	5.00	142	6
Seeds	PVP	Ethanol	2:1	3.33	187	8
Seeds	PVP	Ethanol	1:2	5.00	54	3
Seeds	PVP	Ethanol	1:2	3.33	72	3
Pulp (unprocessed)	-	-	-	-	108	3
Pulp	-	Ethanol	-	-	-	-
Pulp	PVP	Ethanol	2:1	3.33	7	>1
Pulp	PVP	Ethanol	1:1	5.00	27	1
Pulp	PVP	Ethanol	1:1	3.33	31	2
Pulp	PVP	Ethanol	1:1	2.50	35	>1

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241

242 *3.2 Morphological study*

243 The morphology was studied by Scanning electron microscope (SEM) images.
244 The SAS process has promoted the particle seed-extract micronization. SEM images were
245 used to analyse the differences in the morphology of the particles obtained from the
246 single-processed seed-extract with respect to the original material. Moreover, when PVP
247 was applied as carrier in the encapsulation process, it was possible to produce even
248 smaller particles (Table 2). Pluronic F-127 did not show good properties as a carrier for
249 seed-extract, because all experiments resulted in a plasticized material.

250

251

252 Table 2- Comparative of SEM images of encapsulated material, PVP, seed-extract, and,
253 seed-extracts SAS processed.

254 (Table 2)

255

As observed from TPC analysis, the solvent to solute proportion was considered an important variable once the mass/solvent ratio affected the co-precipitation. The particles presented in Figure 2 have appropriate morphologies, with particles well covered with the polymer. These particles were also the ones with the highest TPC value, 211 mg GAE/g particle.

(Figure2)

Figure 2 – Seed-extract co-precipitated with PVP at (1:1) ratio, 3.33g/L.

However, the excessive reduction of the mass/solvent ratio caused the pump to malfunction, causing blockages, which impairs the constant flow pumping of suspended material. The resulting malformed content produced by these problems can be observed in figure 3.

(Figure 3)

Figure 3 – Seed-extract co-precipitated with PVP at (1:1) ratio, 5.00 g /L.

The best concentration found was 2.5g/L, at this concentration, a more efficient co-precipitation, particles with better distribution, less exposed areas and small particles sizes were obtained. The solvation ratio changed the final particle size.

(Figure 4)

Figure 4 – Seed-extract co-precipitated with PVP at (1:1) ratio, 2.50 g /L.

When pulp-extract was single processed, without polymer, no particles were obtained, which was attributed to a strong micronization, where the particle could not be retained by the filter (0,45 µm). SEM images (Figure 5) of the product, showed small extract fragments of extract inserted in the polymer. Açaí pulp is a very oily material; 43 % of its dry-weight is oil content. The pulp was treated to remove the oil before the extraction, but small remaining amounts of oil in the Açaí pulp extract can justify the differences observed between both extracts.

(Figure 5)

Figure 5 - Pulp-extracted and PVP processed at (1:1) ratio, (a) 5.00g/L, and (b)3.33g/L.

3.3 Thermostability

The thermostability study was made by the thermogravimetric analysis (TGA). The analysis measured the loss of mass in percentage through the temperature increment. The first thermo-decay, called onset-temperature (T onset), can be taken as a qualitative measurement of thermostability. In this way, to increase the T onset is a relevant advantage of the formulation process. Seed-extract single processed (Figure 6), with no carrier addition, showed a very small loss in T onset concerning the original seed extract, the difference observed was around 2ºC, the process achieved to increase the extract water-solubility. In the ratio of 1:1, the TGA obtained was similar to pure PVP, presenting a small variation in T onset. The material thermostability increased when more polymer was added to the process, as the T onset was higher the following gap formed between baseline and tangent was less evident, being almost imperceptible for the ratio of (1: 2).

(Figure 6)

Figure 6- Thermogravimetritric (TG) curve obtained for seed-extract co-precipitated indifferent proportions with PVP.

The Derivative Thermogravimetry (DTG) is the first derivative curve obtained from TGA analysis and simulation of Differential Scan Calorimetry (DSC). This analytical technique is able to detect the precise inflection point of the TG curve, marking the point of mass changes due to vaporization or degradation. The results provided in Figure 7 showed the existence of a first peak relative to the loss of humidity, followed by two degradation peaks. It is possible to see (figure 7) that all peaks are narrow and simple, denoting that each loss process happened in a unique step. In addition, second peaks are displaced among them denoting the soft variation in the thermostability of obtained material, as previously commented.

(Figure 7)

Figure 7 – Derivative Thermogravimetry (DTG) curve of co-precipitated seed-extract and PVP material.

Meanwhile, Pulp-extract showed a different behaviour when it was processed by the SAS technique. The materials obtained did not present modifications on TGA or peaks displacement in DTG denoting the encapsulation was not performed successfully.

3.4 FTIR

The FTIR analysis of the formulated material can be compared to the source material. In both cases, the formulated material showed characteristic peaks of the source material seeds-extract (Figure 8), and pulp-extract (Figure 9).

(Figure 8)

Figure 8 – FTIR of PVP, original seedes-extract, and encapsulated material: characteristic peaks of (I)seeds-extract and (II)PVP.

(Figure 9)

Figure 9 – FTIR of PVP, original pulp-extract, and encapsulated material: characteristic peaks of (I)pulp-extract and (II)PVP.

4. Conclusion

In this work the encapsulation of active extract obtained by pressure-assisted microwave extraction using as a source of extract the seeds and pulp residues (not suitable for direct human consumption) of the açaí fruit (*Euterpe oleracea* Mart) was studied. The encapsulations were tested with different polymers (PVP and Pluronic F127) and solvents (ethanol and acetone). Tests were also performed with variations of the extract/polymer and solid/liquid ratio present in the starting solution. The best results were obtained when ethanol was applied as the solvent and PVP as the encapsulating polymer, as these conditions yielded higher TPC contents and more regular particle morphologies.

Among the proven extracts, better results were observed for the material extracted from the açai seeds, which is attributed to the higher oil content of pulp extracts. The experiments in which the extract obtained from the seeds and PVP were carried out

yielded viable results for the proportions (1:1), (2:1), and (1:2). No complete coating was achieved in any of the experiments carried out in the SAS study. When the extract to PVP ratio was increased to (1: 4), the resulting material showed particle plasticization.

The pulp extract was processed by SAS with PVP in different proportions, the resulting materials showed no significant differences between each other by mean of TG analysis. Although the precipitated content has shown the typical coloration conferred by the presence of anthocyanin, and the FTIR analysis indicated the presence of both substances (extract and PVP) on it, the materials obtained did not have a substantial total polyphenols content. In addition to the characteristic colour, the FTIR analysis showed co-precipitation of the material. It was not possible to carry out a study to analyse the pulp extract processed without polymer addition, once the material could not be recovered after SAS processing.

The importance of the solid to liquid ratio on the particle formation was demonstrated within the study of seeds extract co-precipitation. This parameter not only affects the distribution of the co-precipitate but also implies the particle size.

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Figure Caption:

Figure 1 – Complete SAS flow diagram

Figure 2 – Seeds-extract co-precipitated with PVP at (1:1) ratio, 3.33 g/L.

Figure 3 – Seeds-extract co-precipitated with PVP at (1:1) ratio, 5 00 g /L.

Figure 4 – Seeds-extract co-precipitated with PVP at (1:1) ratio, 2.50 g /L.

Figure 5 – Pulp-extracted and PVP processed at (1:1) ratio, (a) 5.00 g/L, and (b)3.33 g/L.

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Figure 7 – Derivative Thermogravimetry (DTG) curve of co-precipitated seed-extract and PVP material.

Figure 8 – FTIR of PVP, original seeds-extract, and encapsulated material: characteristic peaks of (I)seeds-extract and (II)PVP.

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474 **Tables:**

475

476 **(Table 1)**

477 Table 1 – Experimental conditions and total polyphenol content of SAS-
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Seeds	PVP	Ethanol	1:1	5.00	78	3
Seeds	PVP	Ethanol	1:1	3.33	211	8
Seeds	PVP	Ethanol	1:1	2.50	80	4
Seeds	Pluronic	Acetone	1:1	3.33	132	8
Seeds	PVP	Ethanol	2:1	5.00	142	6
Seeds	PVP	Ethanol	2:1	3.33	187	8
Seeds	PVP	Ethanol	1:2	5.00	54	3
Seeds	PVP	Ethanol	1:2	3.33	72	3
Pulp (unprocessed)	-	-	-	-	108	3
Pulp	-	Ethanol	-	-	-	-
Pulp	PVP	Ethanol	2:1	3.33	7	>1
Pulp	PVP	Ethanol	1:1	5.00	27	1
Pulp	PVP	Ethanol	1:1	3.33	31	2
Pulp	PVP	Ethanol	1:1	2.50	35	>1

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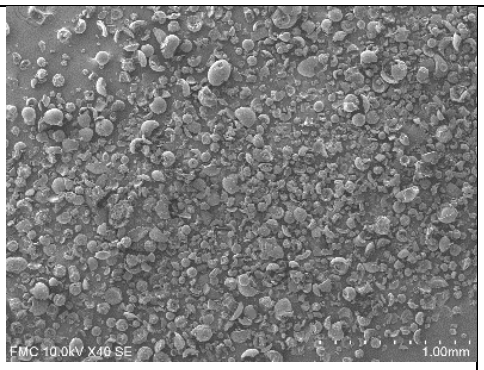
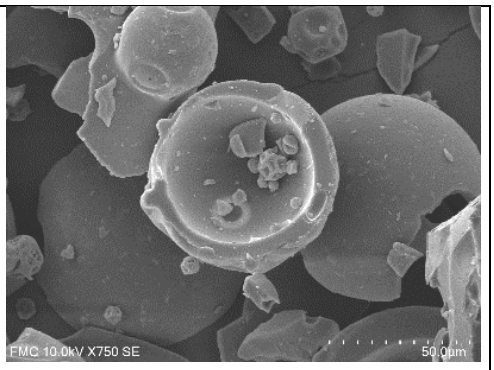
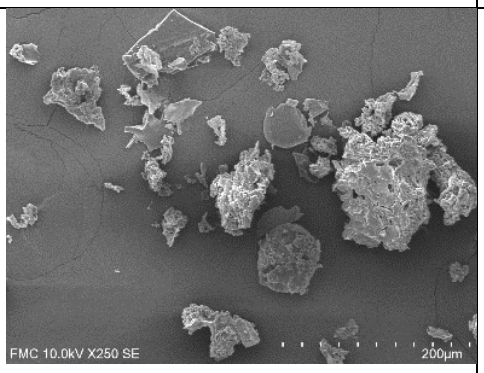
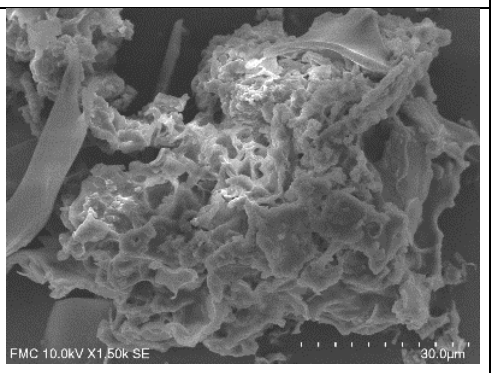
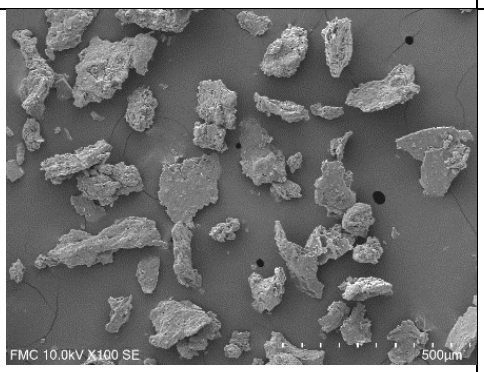
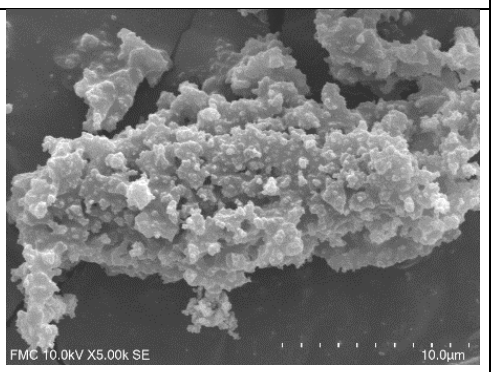
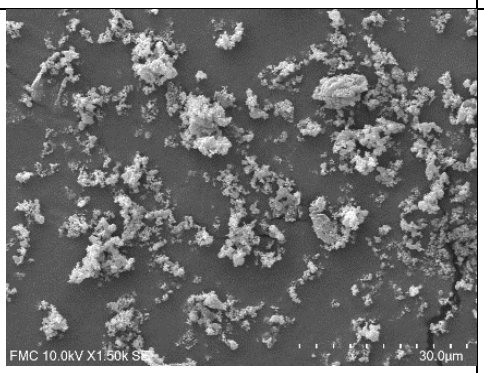
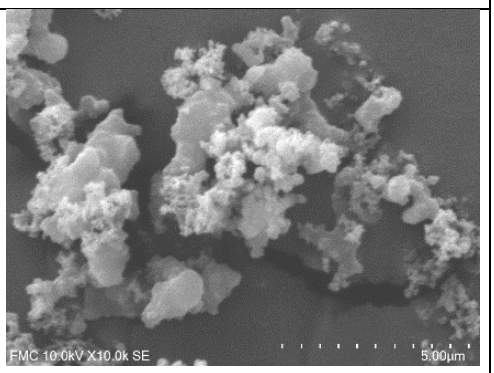
481

482 **(Table 2)**

483

484 Table 2- Comparative of SEM images of encapsulated material, PVP, seeds-extract, and
485 seeds-extract processed by SAS

Original PVP		
Original Seeds Extract		
Seeds Extract SAS Processed		
Seeds Extract in PVP (1:1)		

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