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Deciphering Rubber Particle Destabilisation by Hevea *Bark Extract*

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Filtration of Hevea *bark extract through Millipore spin cartridge (molecular weight cut-off at 100 kDa) yielded a brown slurry retentate and a clear liquid in the flow through fraction. The retentate was then separated by preparative reverse phase high performance liquid chromatography (HPLC) and the brown bark pigment was recovered in a broad peak. The whole bark extract, the spin cartridge retentate, and the brown HPLC fraction, readily destabilised the high density rubber particles (Zone 2 and Zone 3) suspensions but only negligible destabilising activity was observed with the clear spin cartridge flow through fraction. Destabilisation of* Zone 1 *rubber particles by whole bark extract, spin cartridge retentate, and the brown HPLC fraction occurred only when C-serum was substituted with* Zone 3 *rubber particles in the reaction mixture. Polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG), which are known to bind phenolic compounds/tannins, effectively precipitated the brown pigment of the bark extract. The clear supernatant recovered from the PVP and PEG treated bark extract also failed to induce rubber particle destabilisation. The presence of substantial levels of phenolics in the bark extract was confirmed by a modified Folin-Ciocalteau assay. Analyses on thin layer chromatography indicated that the brown pigment of the bark extract consists of highly polymeric condensed tannins. Analytical reverse phase HPLC revealed that three different peaks with UV spectra similar to those of tannins, and one peak that resembles phenolics could be separated from the bark extract, and its spin cartridge retentate.*

Key words: rubber particle; destabilisation; bark extract; chromatography; polymeric tannins; phenolics

As latex flows from the tapping cut, flocs of destabilised rubber particles form at the severed ends of laticifers. In the early stages, any flocs formed at the cut ends would be swept away by the high flow rate due to release of turgor. In the later stages, the outflow pressure decreases and flocs accumulate progressively and obstruct flow, initiating a complete cessation of latex exudation. Electron-microscopic studies revealed that obstructions to latex flow could originate at different locations near the tapping cut within the severed laticifers, and a cap of coagulum formed over the cut ends[1–3].

Latex contains significant levels of vacuole like organelles called lutoids, the membrane bound lutoids are liable to damage by osmotic shock[1] and physical shear[4] during the flow, hence this results in release of its

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fluid (B-serum) that could induce destabilisation[5].

Rubber particle destabilisation in latex by B-serum is largely dependent upon the electrostatic interaction on rubber particles, which has been shown by their rapid creaming and flocculation in a dilute aqueous suspension when B-serum is added. Besides electrostatic interaction, other complementary mechanisms involved in rubber particle destabilisation, mainly by B-serum proteins, and by fluid extracted from the soft bark tissue (bark extract) have been reported[6–8]. Unlike B-serum however, bark extract has a net negative charge, therefore destabilisation of rubber particles is unlikely to arise *via* electrostatic interaction alone. The involvement of severed bark tissue in impeding latex flow[8] and subsequently coagulation of high-density rubber particles (from *Zones 2* and *3* of centrifuged latex) by bark extract have been clearly demostrated[9,10]. In addition, both B-serum and bark extract has been shown to synergistically induce rubber particle destabilisation[11]. It was then surmised that cessation of latex flow results from a cumulative effect of turgor loss, internal laticifer plugging mainly by rupture of lutoids (release of B-serum), and formation of cap coagulum at the laticifer ends (involvement of bark fluid)[12].

Previous related studies revealed that the bark tissue/extract contains metabolites that include glucosides[13], quinones[14], tannins[15] and an array of proteins at low to mid range molecular weights as observed on a two dimensional polyacrylamide gel, prominent among them is a peroxidase (E. Sunderasan and R. Philp, unpublished data). Insofar the properties of bark extract that directly contribute to rubber particle destabilisation remain unclear. In this study, experiments were performed to investigate the properties of bark extract that directly contribute to destabilisation of rubber particles.

EXPERIMENTAL

Chemicals

All chemicals used in this study are of analytical grade, and were purchased from Sigma-Aldrich (USA) unless otherwise specified.

Preparation of Rubber Particles and C-serum

Latex sample was collected from RRIM 600 trees tapped at the RRIM Research Station. To prepare the rubber dispersion, fresh latex collected in chilled flasks was fractionated by centrifugation at 44 000 g at 4ºC. Rubber cream from *Zone 1*, *Zone 2* and *Zone 3* were recovered after centrifugation at 40 000 g at 4ºC to obtain the basic centrifugation zones described by Moir[16] (*Figure 1*). The *Zone 1* and *Zone 2* rubber particles were washed in 30% sucrose solution and re-centrifuged at 40 000 g at 4ºC for 1 hr; the additional centrifugation essentially removed residual latex serum entrapped in the rubber cream. A suspension of rubber particles in distilled water was prepared from the creamed rubber fraction after the second centrifugation, giving a concentration of approximately 10%–12% w/v rubber. *Zone 3* rubber particles were pipetted directly from the centrifuge tube, and added to a final concentration of *circa* 6% w/v in the destabilisation reaction mixture.

C-serum refers to the resulting clear aqueous phase in between the upper rubber cream and the sediment at the bottom of the centrifuge tube (*Figure 1*). In earlier studies on rubber particle destabilisation by bark extract, C-serum has been considered obligatory for coagulation to take place[9]. In all rubber particle destabilisation reactions in which C-serum was employed, its final concentration after adding the destabiliser (whole bark

extract, or its fractions) is estimated at *circa* 40% v/v.

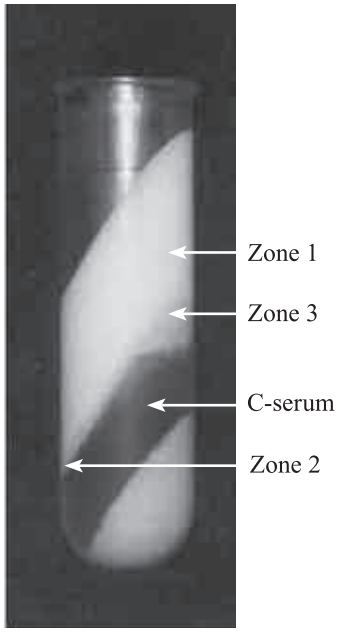

Figure 1. Fractionation of fresh latex by high speed centrifugation. The positions of Zone 1*,* Zone 2*,* Zone 3 *rubber particles, and C-serum are marked.*

Preparation and Fractionation of Bark Extract

Bark extract was prepared essentially as described elsewhere[9]. Briefly, the newly excised bark slivers were collected after tapping, rubber strings and the outer corky layer were removed, and the inner soft bark strips were used immediately for extraction. The soft bark strips were pressed using pinch rollers, and the collected fluid was centrifuged at 15 000 g at 4ºC for 15 mins to obtain a clear dark brown supernatant (whole bark extract). A portion of the whole bark extract was lyophilised, and kept desiccated until further analysis. An aliquot of the whole bark extract was concentrated in Microcon (Millipore, USA) spin-cartridge filter (100 kDa molecular weight cut-off) by centrifugation at 10,000 g for 20 mins that yielded a retentate (fraction F) and its corresponding flow-through fraction.

The brown slurry (fraction F) collected in the cartridge retentate was reconstituted to its original volume with distilled water. An aliquot of the reconstituted fraction F was further diluted 1:10 with water and separated through a SEP-PAK (Waters, USA) C_{18} cartridge. The brown pigment retained on the cartridge was eluted with methanol-water (50:50) and was stored frozen for further separation *via* preparative reverse phase high pressure liquid chromatography (HPLC). The remainder of whole bark extract, fraction F and flow-through fraction were also stored at –20ºC until further use.

Rubber Particle Destabilisation Tests

For the destabilisation test, an aliquot of 500 µL of homogenous suspension of *Zone 1*, *Zone 2* and *Zone 3* rubber particles (10%–12% w/v) were added into vials containing 100 µL bark extract or its fractions and 400 µL C-serum to give a final percentage of 5%–6% w/v rubber particles. The bark extract or its fractions were substituted with distilled water in control reactions. The contents of the vials were vortex-mixed for 5 seconds and immediately examined for formation of flocs or aggregates, indicative of rubber particle destabilisation. Coagulation of rubber particles was deemed to have occurred when the agglomeration and gellation of rubber particles had proceeded to the extent that a cohesive mass could be picked up using a pair of forceps. In this study, evidence of coagulation of rubber particles, if present, appeared approximately 20 min after adding the destabiliser.

Estimation of Total Phenolics in Bark Extract

A method derived from Folin-Ciocalteau, according to Singleton and Rossi[17] was employed to estimate the total phenolics content in lyophilised whole bark extract. Briefly, a known concentration of the extract was prepared using a mixture of methanol, HCl and distilled water (8:1:1 v/v/v). The sample solution was mixed with Folin-Ciocalteau's reagent (10:75 v/v) and left to stand at room temperature for 5 min. Then 750 µL of 6% Na_2CO_3 solution was added to the reaction mixture and left to stand at room temperature for 90 min. Absorbance was measured at 725 nm and the results were expressed in mg gallic acid/g sample (gallic acid equivalent, GAE).

Phytochemical Test for Tannins

A simple phytochemical test for the presence of tannins was performed by adding a few drops 1% $FeCl_3$ solution to the whole bark extract sample. The formation of a blue black colour indicates the presence of hydrolysable tannins while a brownish green colour indicates the presence of condensed tannins.

Chromatographic Analysis of Bark Extract

One dimensional thin-layer chromatography (TLC) silica gel analysis was performed in chloroform/methanol/water (7/3/0.5 v/v/v), toluene/acetone/formic acid (3/6/1 v/v/v), benzene/ethyl formate/formic acid (1/7/2 v/v/v) and ethyl acetate/formic acid/acetic acid/water (100/11/11/26 v/v/v/v). After completion of chromatography the plates were visualized under an ultraviolet (UV) illuminator and further developed by spraying with 2% $FeCl_3$ in ethanol.

Analytical reverse phase HPLC was performed using a Waters Delta 600 gradient pump coupled to a Waters 996 photodiode-array detector. Separation was achieved using a Phenomenex® Luna 5 C_{18} column with dimensions of 250 × 4.6 mm using a 35 min water-acetonitrile-*ortho*-phosphoric acid gradient system (A=85%, B=15% at 0 min, A=75%, B=25% at 12-20 min, A=85%, B=15% at 22-35 min, where A=0.1% ortho-phosphoric acid, B=acetonitrile).

Preparative reverse phase HPLC was performed on the brown pigment (pre fractionated with SEP-PAK C_{18} cartridge) using a Waters Delta 600 coupled to a Waters 2487 UV detector. Separation was achieved using a Waters DELTPAK C_4 column with dimensions of 300 × 7.8 mm in a 30 min run using water-acetonitrile system (85:15) containing 0.06% trifluoroacetic acid. The peak containing the brown pigment was recovered and dried to completion in an oven preset at 70ºC. The dried pigment was then re-dissolved in water to its original volume and used in the rubber particle destabilisation tests.

RESULTS

Destabilisation of Rubber Particle Suspensions

Whole bark extract and the reconstituted spin-cartridge retentate fraction (brown slurry, faction F) were added to *Zone 1*, *Zone 2* and *Zone 3* rubber particle suspensions in C-serum. Considerable amount of flocs could be seen left on the sides of the vials containing *Zone 2* and *Zone 3* rubber particles immediately after vortex mixing, however, only slight destabilisation of rubber was observed in the vial containing *Zone 1* rubber particles (*Figure 2*). Subsequently, a cohesive mass of coagulated rubber particles developed when the vials containing *Zone 2* and *Zone 3* rubber particles were left standing at room temperature

Figure 2. Rubber particles treated with 10% whole bark extract. Left to right: rubber particles from Zone 1*,* Zone 2 *and* Zone 3*, in 40% C-serum; and* Zone 1 *and* Zone 2 *rubber particles, in which C-serum was substituted with an equal volume of* Zone 3*. Flocs of rubber particles were visible at the side walls of the vials. A similar pattern of rubber particle destabilisation was observed when the spin cartridge retentate or the brown pigment recovered from preparative reverse phase HPLC were used as destabilisers instead of whole bark extract.*

for 20 mins (*Figure 3*). This showed, firstly, *Zone 2* and *Zone 3* rubber particle suspensions were readily destabilised by the bark extract, and that the rubber particle destabilising activity resided in the brown retentate fraction of the spin cartridge (molecular weight cut-off 100 kDa). Another noteworthy observation here is that *Zone 3* rubber particles were most effectively destabilised and coagulated by bark extract or the spin cartridge retentate fraction. On the other hand, when the experiment was performed with the spin cartridge flow through, negligible or no destabilisation (flocculation) of rubber particles was observed. Thus, it was evident that the rubber particle destabilising activity is confined mainly to the retentate fraction (fraction F) while the clear flow-through fraction and its contents had little effect on the rubber particle suspensions.

Subsequently, the spin-cartridge retentate (fraction F) was separated *via* preparative reverse phase HPLC, the fraction separated into two minor peaks and a prominent broad peak, in which the brown bark pigment was eluted (*Figure 4*). The brown pigment recovered from the preparative reverse phase HPLC induced destabilisation and coagulation of *Zone 2* and *Zone 3* rubber particles as observed in earlier tests, thus the brown pigment appeared to be the active rubber particle destabilising factor of bark extract.

In a related experiment, the C-serum component in the reaction mixtures containing *Zone 1* and *Zone 2* rubber particles were substituted with an equal volume of *Zone 3* rubber particles. In both cases, the level of flocculation of rubber particles was enhanced and progressed into a complete coagulation (*Figure 2*).

Effect of Polyvinylpyrrolidone and Polyethylene Glycol on Bark Extract

Polyvinylpyrrolidone (PVP)[18] and polyethylene glycol (PEG)[19] are known tannin precipitants, therefore, their binding to

Figure 3. Effect of adding bark destabiliser (either whole bark extract, the spin cartridge retentate fraction or the brown pigment separated through preparative reverse phase HPLC) to rubber particles. From left; uncoagulated Zone 1 *rubber particles, coagulated* Zone 2*, and* Zone 3 *rubber particles, 20 mins after adding the destabiliser.*

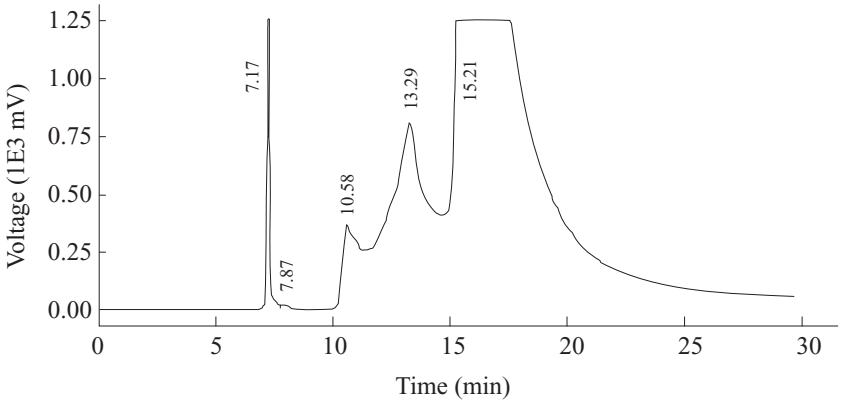

Figure 4. Preparative reverse phase chromatogram of the spin cartridge retentate (F) pre-fractionated via *SEP-PAK* C_{18}*. The brown pigment was eluted and recovered in the broad peak beginning at 15.21 min; optical density was measured at 254 nm.*

polyphenols is due to the high hydrogen bonding affinity[20]. In this experiment, PVP-360 and PEG 4000 were added separately to bark extract to a final concentration of 10% w/v. It was observed that the bark extract turned cloudy immediately after adding PVP or PEG. A brownish precipitate was accumulated at the bottom of the tube while a clear yellowish supernatant was obtained by centrifuging the PVP and PEG treated bark extract. The clear supernatants were then tested for *Zone 1*, *Zone 2*, and *Zone 3* rubber particles destabilising activity. It was observed that at a final concentration of 10% w/v supernatants, only very mild destabilisation of *Zone 3* rubber particles occurred in the suspension after prolonged incubation (results not shown). These findings indicate that PVP and PEG effectively precipitated the brown pigment (presumably polyphenols/tannins) in the bark extract, which were largely responsible for rubber particle destabilisation.

Analyses of Phenolics in Bark Extract

The presence of phenolics in the bark extract was determined by a modified Folin-Ciocalteau method. The colorimetric test revealed the presence of substantial levels of phenolics, *circa* 108.4 ± 3.5 mg GAE/g (average of four tests ± SD) in the lyophilised whole bark extract.

An attempt was then made to characterise the type of bark phenolics using TLC. Despite the use of strong solvent systems for chromatography, the phenolics in the whole bark extract and the spin cartridge retentate (fraction F) remained at the origin, as visualised when the plates were sprayed with ferric chloride reagent (data not shown). This finding indicated that the phenolic compounds in the whole bark extract and fraction F are highly polymeric. Further, a phytochemical test on the bark extract with 1% $FeCl_3$ gave a brownish green colouration, characteristic of condensed tannins. Taken together these results indicated that *Hevea* bark extract contains a substantial amount of highly polymeric condensed tannins. Indication of tannins in the whole bark extract and fraction F were also discerned from the analytical reverse phase HPLC. The reverse phase HPLC chromatograms and the UV spectra of the major peaks for the whole bark extract (sample E) and the spin cartridge retentate (fraction F) indicated the presence of three peaks which have UV spectra similar to those of tannins, and one peak in fraction F that corresponds to phenolics, as shown in *Figure 5*.

DISCUSSION

Various components of *Hevea* bark extract, ranging from acids, tannins, sugars such as cyclitols, sucrose and fructose; have been reported in the past[13–15]. Tannins especially, are a very diverse family of polyphenolic compounds. They can be subdivided into condensed and hydrolysable tannins and both groups are capable of forming complexes with proteins, polysaccharides and other macromolecules[21]. Although the presence of polyphenols/tannins in *Hevea* has been long known, they have never been characterised or directly linked to rubber particle destabilisation. The experimental evidences in this study indicated the presence of highly polymeric condensed tannins and phenolics (brown pigment) in the bark extract. However, further investigation would be required for a detailed characterisation of the bark tannins/phenolic compounds.

The rapid flocculation and aggregation of *Zone 2* and *Zone 3* rubber particles by the spin cartridge retentate and the brown pigment recovered from preparative reverse phase HPLC indicated that the pigment is active as a destabiliser of the bark extract.

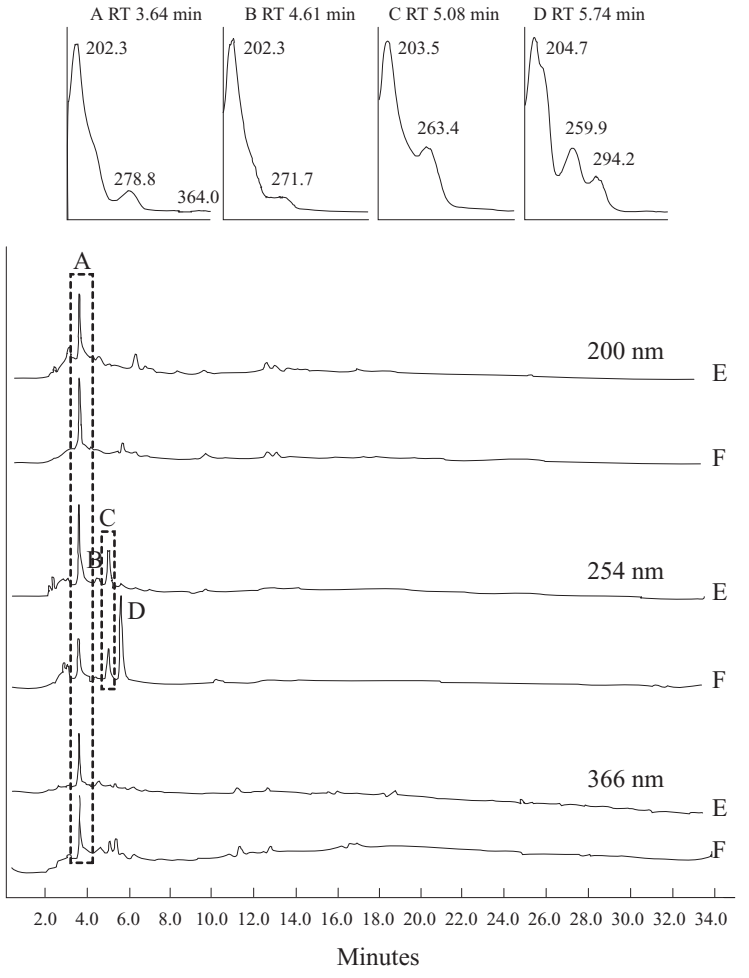

Figure 5. Analytical reverse phase chromatograms and ultraviolet (UV) spectra of the major peaks for the whole bark extract (E) and spin cartridge retentate fraction (F). The UV spectral of peaks A, B, and C are characteristic of those of tannins, while peak D resembles a phenolic compound.

Using the ultra-filtration technique, previous researchers have concluded that the major active components in the bark extract consist of mainly large molecular substances, while it was postulated that proteins were involved, the destabilizing factor was not conclusively elucidated[3]. In this study, the brown pigment in the bark extract was effectively precipitated by PVP and PEG, which indicated that the active factor could be of phenolics/tannin origin. The highly polymeric form of the condensed tannins in the brown pigment was evidenced by the TLC analysis and analytical reverse phase HPLC indicated the presence of three different tannins and a phenolic compound. Thus the results presented in this paper strongly suggest that bark polymeric tannins/phenolics were largely responsible for the destabilisation and coagulation of *Zone 2* and *Zone 3* rubber particles.

Destabilisation and coagulation of *Zone 1* rubber particles was achieved by substituting the C-serum component with *Zone 3* rubber particles in the reaction mixture. Destabilisation and coagulation of *Zone 2* rubber particles were also enhanced when *Zone 3* particles were substituted for C-serum in the reaction mixture. It thus seems plausible that *Zone 3* particles could act as a co-factor in rubber particle destabilisation by the highly polymeric tannins/phenolics of bark extract.

As stated earlier, bark extract contains an array of low to mid-range molecular weight proteins, and prominent among them is peroxidase (E. Sunderasan and R. Philp, unpublished data). Accumulation of bark peroxidase activity due to tapping injury has been reported[22]. It was then postulated that bark peroxidase could convert wound induced latex phenols (substrates) into phenolic polymers, and these phenolic polymers might enhance latex coagulation due to association with proteins. It appears that besides the direct action of bark polymeric tannins/phenolics, as observed in this study, peroxidase released from injured bark cells could also be implicated in rubber particle destabilisation, thus having opposing effects on latex flow.

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