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Preparation and storage of *Pinnatu* destroys the hypocholesterolaemic effect of palmyrah fruit pulp

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Abstract

It has been suggested that the hypocholesterolaemic effect of palmyrah fruit pulp (PFP) is brought about by the soluble dietary fibre which is dominated by pectin. However, this has not been conclusively proven. The present publication describes studies to determine if *pinnatu* (dried PFP, fruit leather) could possess any hypocholesterolaemic activity.

Neither *pinnatu* (prepared by drying PFP in layers as in the conventional method) nor pectin extracted from *pinnatu* (incorporated at a double dose to above experiment) could indicate any hypocholesterolaemic effect on normal Wistar rats. *Pinnatu* pectin on separation on Sepharose gel chromatography showed a high elution volume pattern indicating the hydrolysis of pectin while processing. Further it was found that *pinnatu* contains exopectinase activity. Subsequently, *pinnatu* was prepared using a modified method in order to minimize the hydrolysis of high molecular weight pectin. The Sepharose gel chromatography pattern of the isolated pectin of the modified *pinnatu* showed no degradation of pectin. However, after storing (6-7 months), this *pinnatu* pectin showed a slight shift to the lower elution volume indicating slight hydrolysis. On feeding Wistar rats with this *pinnatu* showed no hypocholesterolaemic effect ($p=0.85$). It is concluded that the preparation and storage of *pinnatu* destroys the hypocholesterolaemic effect of PFP. High molecular weight pectin may not be the major factor for the hypocholesterolaemic effect of PFP. It is also possible that storage of *pinnatu* causes not only chemical but also physical changes in pectin.

Key words: *Pinnatu*, hypocholesterolaemic effect, Pectin

Introduction

The hypocholesterolaemic effect of Palmyrah Fruit Pulp (PFP) has been originally reported in 2002 (Ekanayake and Chandrika, 2002) . Subsequent studies carried out in 2005 (Pathberiya, 2005) have scientifically proven the effect and it has been suggested that this effect is brought about by the SDF especially pectin. The present study was carried out to study the hypocholesterolaemic effect of *pinnatu* and if so whether the effect is due to pectin. *Pinnatu* is a dried fruit leather of PFP.

Materials and Methods

Pinnatu was prepared from PFP from the fruits obtained from Kalpitiya for the first part of the study. *Pinnatu*, prepared in Jaffna (using the traditional method) was obtained from the Palmyrah Development Board, to study the activity of inherent pectinases. Palmyrah fruits from Hambantota district were used to prepare *pinnatu* using a modified method for the final experiments. Out bred, male, weanling Wistar rats were obtained from the Medical Research Institute (MRI), Colombo, Sri Lanka.

Preparation of *pinnatu*

PFP was layered (upto 1 cm thickness) on a piece of cloth and dried at 65°C-67°C using the Mitchel dryer. When the first layer was dried the second PFP layer was layered on top of it and similarly dried. In this manner the whole sample (5.3 Kg) was dried in successive layers (six layers) for 50 hours in order to prepare brownish, sticky, chewing gum like '*pinnatu*'.

Analysis of some components of *pinnatu*

Moisture content was determined by drying in an oven at 105°C (AOAC, 1984). SDF and IDF were determined using the enzymatic method of Asp *et al.* (1983) SDF fraction was isolated omitting the incineration step and was analyzed for pectin by the carbazole reaction (Dekker and Richards, 1972).

Animal model

Experiments were conducted at the animal house, University of Sri Jayewardenepura. Wistar rats (age-7-8 weeks) were divided into two groups (n=6/group). Basal serum total cholesterol levels of the animals were determined (Allain et al., 1974). The control animals were fed with WHO standard rat and mouse breeding feed[7]. Test group was fed with an isocaloric diet containing 11% *pinnatu* in place of 11% maize. Feed intake of the animals was recorded daily and their body weights measured weekly. The serum total cholesterol levels of the animals were determined on the 14th, 28th and 42nd days.

Determination of Sepharose gel elution profile of pectin in *pinnatu*

Sepharose™ 2B was packed in a column (length-21 cm, diameter-1.3 cm). Void volume was determined using Blue dextran 2000. SDF (1.26 g, equal to 50 mg dry weight) extracted from *pinnatu* was dissolved thoroughly in 2 mL phosphate buffer and this was separated on the Sepharose column. Fractions (2 ml) were collected, freeze-dried and the content of pectin in each fraction was determined by the carbazole reaction (Dekker and Richards, 1972).

Effect of isolated SDF (pectin 92%) of *pinnatu* on the serum total cholesterol levels of Wistar rats

Animals (13-14 weeks of age) were divided into two groups (n=6/group). Basal serum total cholesterol levels of the animals were determined using CHOD-PAP kit. The control group was fed with WHO standard diet. SDF was isolated from *pinnatu* using the method of Asp *et al.* (1983). SDF was precipitated using ethanol. The amount of pectin consumed by a rat per day was calculated depending on the data obtained from the previous experiment. Isolated pectin (after evaporating ethanol) was incorporated to the WHO standard diet (making the dose doubled to that of the previous experiment) substituting the grass powder and was fed to the test group for a period of 31 days and the serum total cholesterol levels of the animals were measured at the end of the experiment.

Detection of pectinase activity

Exo and endo-pectinase activities were detected using a cold water extract of *pinnatu.* Interference by sugar of the extract was overcome by separating it using a Sephadex G-25 column. The column was eluted with acetate buffer and fractions (2 ml) were collected. Each fraction was divided into two equal portions and treated differently. First series of fractions were kept in the freezer immediately after their collection. Second series of fractions were incubated at pH 4.7 and RT for 24 h.

Direct carbazole reaction was done on each sample.

The previous experiment was repeated in the same manner with the following modification (introduction of exogenous substrate). After dividing the fractions of Sephadex G-25 column in to two equal portions, standard pectin (2 mg) was dissolved in each portion. First series of portions were kept in freezer immediately after processing (blank) and the other series was subjected 24 h incubation as described earlier, following the hydrolysis, pectin in each portion of the two series was determined by the direct carbazole reaction (Dekker and Richards, 1972).

To identify the endo-pectinase activity, after running the Sephadex column, Fractions were pooled and divided into two equal portions. Standard pectin (25 mg) was dissolved in each portion and one of them (zero time) was kept in

freezer immediately. Other portion was incubated at pH 4.7 and RT for 24 h. A Sepharose 2B column was packed and was eluted with acetate buffer (pH 3.5). Zero time and incubated samples (2 mL) were run and introduced in to the column separately. Fractions were freeze dried before determining pectin with carbazole reaction (Dekker and Richards, 1972).

Preparation of *pinnatu* using a modified method

Palmyrah fruits were collected from Hambantota. The pH of the fruit pulp was adjusted from 4.6 to 2.5 and heated at 70-80°C for 15 min in order to inactivate the pectinases. pH was readjusted. Fruit pulp was then dried in the normal way. Extraction of the pulp, pH adjustments and boiling were done as quickly as possible using small amounts of fruit pulp at a time, in order to minimize the pectinase activity during processing. This *pinnatu* was stored at -20°C for 6-7 months before testing for hypocholesterolaemic effect using Wistar rats as described earlier.
Pectin was isolated from fresh PFP, fresh *pinnatu* and stored *pinnatu* and separated on a Sepharose gel column in order to compare the molecular weight/ size profile.

Statistical evaluation

Results are presented as mean ± standard deviation (SD). Different data were analyzed statistically by Student's t-test. Differences were considered significant at $p<0.05$. Mean, mode and median were calculated for each gel chromatography elution profiles.

Results

Determination of some components of palmyrah *pinnatu*

Table 1 shows the salient components of palmyrah *pinnatu.* SDF (6.6% dry weight) consists mainly (92%) of pectin.

The effect of *pinnatu* on the serum total cholesterol levels of Wistar rats

The cholesterol lowering effect of *pinnatu* (table 2) was not significant ($p=0.10$, $p=0.50$ and $p=0.79$ for day 14, 28 and 42 respectively). This is different to what was reported previously using PFP (Pathberiya, 2005). The difference may be due to the hydrolysis of pectin during processing. This postulate was supported by the gel chromatography elution profile (Fig 1) of *pinnatu* pectin. Feed intake of the animals was similar; in test group it was 17.0g/day/rat whereas in the control group it was16.7 g/day/rat. Weight gain of the animals was also similar. In both groups it was 108 g/6 weeks/rat.

Determination of the Sepharose gel chromatography profile of SDF and pectin of *pinnatu*

Pinnatu pectin on separation on Sepharose gel chromatography showed the typical polydisperse pattern with a shift to the high elution volume pattern indicating the hydrolysis of pectin while processing. The mean, mode and median of the distribution were 28.4 mL, 26.0 mL and 24.0 mL respectively. The void volume of the column was 8 mL as determined by Blue dextran 2000.
Previous studies on the same fresh PFP from Kalpitiya has shown mean, mode and median for the molecular weight (M.W) profile to be 20 mL, 18 mL and 18 mL respectively (Rajapaksha, 2005), clearly showing the hydrolysis of pectin in *pinnatu*.

Table 1: Some components of *pinnatu*

Analysis	% Dry Weight
Moisture*	11.4-17.0
Insoluble dietary fibre	23.1
Soluble dietary fibre	6.6
Total dietary fibre	29.8
Soluble dietary fibre-modified method (without incineration)	10.1
Pectin	92.2% SDF

Each result is the average of two determinations, * n = 6

Table 2: Effect of *pinnatu* on serum total cholesterol levels of Wistar rats

Group	Cholesterol (mg/ dL) Mean ± SD			
	D_0	D_{14}	D_{28}	D_{42}
Test (n=6)	76.96 ± 10.45	67.04[1] ± 10.32	54.99[2] ± 10.40	68.48[3]± 6.26
Control (n=6)	71.45 ± 3.58	71.91 ± 13.72	58.57 ± 9.70	69.08 ± 5.86

[1] p= 0.101, [2] p= 0.50 and [3] p=0.79 in comparison to the control

The effect of isolated SDF (pectin 92%) of *pinnatu* on the serum cholesterol levels of Wistar rats

As shown in table 3 *pinnatu* pectin did not produce a significant cholesterol lowering effect (p=0.42) even at a doubled dose. Feed intake of the animals did not differ markedly. In test group it was 11.9 g/day/rat and in control 12.6 g/day/rat, as determined over a period of 31 days. The mean weight gain was also similar; in test group 17.0 g/for 3 weeks and in control group17.3 g/for 3 weeks.

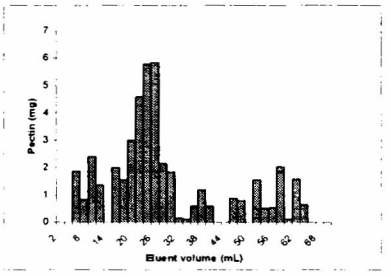

Figure 1: Gel chromatography profile of *pinnatu* pectin

Table 3: Effect of extracted pectin from *pinnatu* on serum total cholesterol levels of Wistar rats

Group	Serum total cholesterol levels (mg/dL) Mean ± SD	
	D_0	D_{31}
Test (n=6)	79.53 ± 4.44	74.74* ± 6.00
Control (n=6)	77.50 ± 2.74	71.27 ± 6.88

*p=0.42 in comparison to the control

Pectinase activity in *pinnatu*

On incubation (24 h) of fractions from Sepharose G-25, fractions 2, 3 and 4 showed higher absorbance values for the direct carbazole reaction showing the presence of exo-pectinase activity in *pinnatu*.
In tests for endo-pectinases activity the control and test showed similar profiles of absorption indicating its absence in palmyrah *pinnatu*.

Comparison of gel filtration chromatography profiles of pectin isolated from fresh PFP, modified process fresh *pinnatu* and stored *pinnatu*

The elution profiles of fresh PFP and fresh *pinnatu* were similar judging from means, modes and medians (fig 2, 3 and 4). However, a slight shift towards the lower M.W. (higher elution volumes) was observed with the pectin of stored *pinnatu* profile.

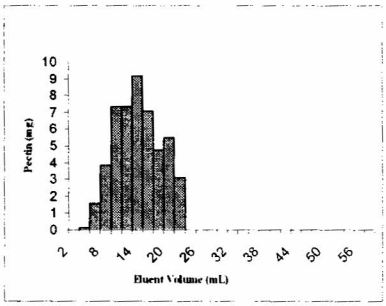

Figure 2: M.W. profile of pectin from fresh PFP (mean = 14.2 mL, mode = 14.0 mL, median = 14.0 mL)

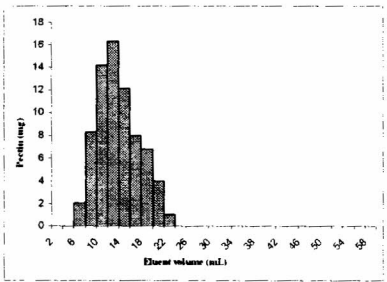

Figure 3: M.W. profile of pectin from fresh *pinnatu* (mean = 14.9 mL, mode = 14.0 mL, median = 14.0 mL)

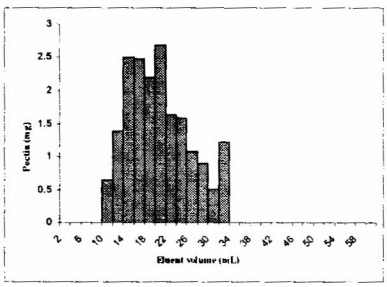

Figure 4: M.W. profile of pectin from stored *pinnatu* (mean = 19.8 mL, mode = 20.0 mL, median = 20.0 mL)

The effect of *pinnatu* prepared by the modified method on the serum total cholesterol levels of Wistar rats

Pinnatu prepared by the modified method did not show significant (p=0.85) cholesterol lowering effect in Wistar rats, as shown in table 4. Feed intake of the animals was not significantly different. In test group it was 14.5 g/ day/rat and in control 14.6 g/day/rat. The mean weight gain was also not significantly different; in test group 87 g/28days/rat and in control group 83 g/28days/rat.

Table 4: Effect of *pinnatu* on serum total cholesterol levels

Group	Serum total cholesterol levels (mg/dL) Mean ± SD	
	Day zero	After five weeks
Test (n=6)	83.03 ± 8.02	59.90* ± 7.12
Control (n=6)	83.33 ± 5.32	60.61 ± 4.36

*p=0.85 in comparison to control

Discussion

The hypocholesterolaemic effect of fresh PFP has been proven previously in 2005. However, it has been recorded that the old PFP (stored in freezer in bulk with sodium metabisulphite preservative) could not produce the effect (Pathberiya, 2005). It was also proved that the hypocholesterolaemic effect was due to neither flabelliferins nor β-sitosterol (Pathberiya, 2005). Pectin was a strong candidate due to the fact that there was a significant (p=0.0009) increase in bile salt excretion (Pathberiya, 2005). Rajapaksha (2005) duplicated the study using Sepharose gel chromatography study with fresh and metabisulphite treated PFP from the same fruits. The results showed that the pectin eluted at a higher elution volume in the treated PFP when compared to fresh PFP indicating hydrolysis. This was further evidence that pectin could be the causative constituent for the hypocholesterolaemic effect in PFP.

However, the present study did not indicate any significant hypocholesterolaemic effect of either *pinnatu* or SDF (at double dose) extracted from *pinnatu.* It was hypothesized that this was due to hydrolysis of high M.W. pectin during *pinnatu* processing. The isolated pectin on Sepharose gel chromatography indicated the hydrolysis of pectin confirming the hypothesis. Further testing *pinnatu* for any remaining pectinase activity, showed exo-pectinase activity, but no evidence for endo-pectinase activity.

Subsequently *pinnatu* was prepared after deactivating the inherent pectinases. It was evident that the pectinase activity had been successfully inhibited. Unfortunately for logistic reasons *pinnatu* had to be kept in a freezer at –20°C for 6-7 months. On testing this stored *pinnatu* pectin it was found that there was a slight shift to lower elution value indicating some hydrolysis. This *pinnatu* showed no hypocholesterolaemic (p=0.85) effect. A number of reasons for this could be postulated; (a) a small change in MW of pectin during storage, (b) a physical change occurring in pectin during *pinnatu* preparation or storage, resulting a difference in solubility or (c) some other hypocholesterolaemic factor present in fresh PFP was being destroyed in the course of *pinnatu* preparation or storage.

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