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Studies on effect of Flabelliferins and Dietary fibre on the hypocholesterolaemic effect of Palmyrah fruit pulp.

L.G. Pathberiya and E.R. Jansz
Department of Biochemistry, University of Sri Jayawardenpura, Gangodawill, Nugegoda, Sri Lanka.

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Abstract

The fruit pulp of all 4 main types of Palmyrah *(Borassus flabellifer L.,)* has been previously shown to have a significant hypocholesterolaemic effect in ICR mice.

Beta-sitosterol, the parent sterol of flabelliferins, is known to reduce cholesterol uptake but is not present in fresh PFP and these studies indicate that it cannot be formed in the small intestine. *In-vivo* and *In-vitro* studies show that colonic bacteria can hydrolyse flabelliferins but this is not a significant source of absorbable β-Sitiosterol. Hydrolysis of flabelliferins with nariginase to shorten the carbohydrate moiety results in no significant differences in serum cholesterol on feeding to mice compared to the non-hydrolysed PFP (P=0.78) showing that small carbohydrate chain flabelliferins cannot mimic β-sitoserol action in lowering cholesterol absorption. The study of dietary fibre of 4 types of PFP show that content is high (12.3-24.3% dryweign) but variable as is the ratio of soluble and insoluble dietary fibre. The bile salt content of faeces of mice fed on 10% PFP feed measured using enzymatic colorimetric assay results in significantly higher bile salts (67%) compared to the control in faeces. (p=0.0009).

Key words: dietary fibre, flabelliferins, hypocholestrolaemia, palmyrah fruit pulp, β-sitoterol

1. Introduction

Plant sterols are well known to be cholesterol-lowering agents[1]. The most common are the sitosterols. These sterols affect the absorption of dietary and biliary cholesterol from the small intestine, thus lowering serum cholesterol[1].

Palmyrah *(Borassus flabellifer L.,)* fruit pulp (PFP) possesses a hypocholesterolmic effect in mice[2]. It has been shown that 4 types of palmyrah reduce total cholesterol significantly by 24% to 34%. However, it has been shown

that no free β-sitosterol is present in PFP. Sitosterols are present in PFP in the form of saponins termed flabelliferins[1]. Among these flabelliferins, F-II, a tetraglcoside (MW 1030)[4] is an inhibitor of intestinal glucose uptake by inhibiting ATPase[5]. It was possible that F-IIwhile decreasing glucose uptake could lower acetyl coenzyme A and thus cause lowering of cholesterol synthesis. Alternatively this intestinal ATPase inhibitor could lower cholesterol uptake[5]. It is also possible that the reduction of cholesterol bears no relationship to F-II content. Therefore, other components of PFP may be involved in the mechanism of the hypocholesterolamic effect.

Therefore the general objective for this study was to get some insight into the mechanism of lowering of serum cholesterol in mice by PFP

The specific objectives were:

(1) To determine if hydrolysis of flabelliferins could affect serum cholesterol level.

(2) To determine if gastro - intestinal enzymes could hydrolyze flabelliferins to yield β-sitosterol

(3) To test the ability of colonic bacteria to hydrolyse flabelliferins to yield β-sitoserol

(4) To study the content of dietary fibre (DF) using the 4 common types of PFP as DF causes hypocholesterolaemic effects by many mechanisms[7],[8].

2. Materials and methods

Palmyrah fruit pulp

A large batch (2 kg) of metabisulphite stabilized, stored bitter Palmyrah fruit pulp (PFP) was obtained from the Palmyrah Development Board, Colombo, Sri Lanka. This was used for debittering studies in mice. Fruits collected from Kalpitiya (n=60) were separated to in the 4 main types[6]. The pulp was extracted as described previously[4]. Samples of pulp (10g) were used for dietary fibre analysis.

Debittering of PFP

A large PFP sample (1kg) was incubated with naringinase[4] (2g) (which hydrolyses large carbohydrate moieties of flabelliferins) extracted from *penicillium decumbens,* (Sigma, St. Louis, USA) at 37°C in acetate buffer pH = 5.2. An identical sample was not treated with naringinase. A control with WHO standard feed was also used.

Determination of flabelliferin profile

The moisture contents of bitter and debitter samples were determined by using the Dean and Stark method (AOAC)[7]. Flabelliferins were extracted into methanol and decarotenised by using petroleum ether (60°-80°C). Flabelliferins were separated from sugar by acetone extraction followed by evaporaion and dry cellulose column[4] chromatography. Weights of mixed flabelliferins measured after the flabelliferins profiles were determined by TLC separaion[4] After which TLC plate were visualized with anisaldehyde[4] the plates were scanned using a computerized dinsitomter[8]. The flabelliferins profiles were calculated using peak areas.

Animal Model

Male Institute of Cancer Research (ICR) mice (weight = 40.7± 2.7g, age: 6-8 weeks) inbred at the Medical Research Institute were used for the study. Mice were separated into groups (n=8) such that their average weights weights were very similar and caged separately.. PFP (10%) incorporated rat & mouse breeding feeds made by bitter (test-1) and debitter samples (test -2) (100g of PFP substituted for 100g of maize per kg feed) were fed to the two tests and the WHO recommended standard diet[10], which was used also as the control. After four weeks the mice were sacrificed after being anaesthetized with diethyl ether and blood was drawn (1-2 ml) by cardiac puncture. Details of animal model have been described previously[13].

Estimation of cholesterol

Serum was collected in the morning and serum separated at room temperature (28-30°C) by centrifuging 1000 g for 10 min. The total cholestrol[14] and HDL cholestrol[15] were determined using enzymatic assay (CHOD-PAP and reactific precipitant, Biolaabos. A., Gardenne, France) immediately.

Collection of faeces

Faeces from the ICR mice, fed on 10% PEP incorporated rat and mouse breeding feed and WHO recommended standard feed10 were collected after two weeks feeding. Faeces (3-5g) were collected as a bulk sample from every mouse for seven days.

Separation of flabelliferin in faeces

Feacal sample (1.5g) was dried at 60°C for 1h, crushed and packed into a Pastuer pipette. The aliquots (5ml) of the solvents, hexane, dichloromenthane, ethyl acetate, methanol and water were passed through the faecal material and the eluents of each solvent collected separately. The eluents were evaporated and re-dissolved in

500μL of solvent before spotting (5μL) on TLC. After TLC separation, spots were visualized by anisaldehyde spray and densitometric scanning[9] was carried out.

Isolation of faecal bacteria

Fresh faeces (0.5g) was collected and dispersed in sterile water (2.5ml). This was used for streaking on agar plates (1.5% agar; 1% glucose; 0.3% peptones; 0.2% beef extract). The culture containing all the bacterial colonies were introduced into sterile culture medium (0.5% glucose; 0.3% peptone; 0.2% beef extract) under sterile conditions.

Hydrolysis of crude flabelliferins

A bacterial suspension (15μL) from the centrifuged culture of mixed bacteria was transferred to mixed flabelliferins in phosphate buffer 1.5ml, pH=7.0 under sterile conditions. Aliquots (15μL) were spotted on TLC at zero time, 24h, 28h, 72h. On the 3rd day the incubation mixture divided into two equal volumes. One portion was extracted to dicloromethane and the other portion was incubated with 2.0mg of lysozyme (E.C 3.2.1.1.7, Sigma, USA) per 1.5ml reaction volume at 37°C for 1h. Following the lysozyme treatment the resulting mixture was extracted with dichloromethane. Constant aliquots (15μL) were spotted before and after lysoszyme treatment the time course of the incubation and also before and after extractions with dichloromethane. TLC spots were visualized by using anisaldehyde[4] followed by densitomertric scanning[11].

Determination of Dietary fibre

Soluble and insoluble dietary fibre of the 4 types of PFP separated by following the the standard procedure of Asp *et al*[16]. The content of soluble and insoluble dietary fibre was calculated on dry basis for each type. Determination were carried out in duplicate.

Determination of bile salts in faeces

Faeces were colleted as a bulk from individual mice fed on 10% PFP incorporated feed and the standard diet after 2 weeks of feeding. Samples (0.5g) were dried in an oven for 1h at 60°C crushed and extracted with alkaline methanol (3ml x3). The mixture was centrifuged (3000rpm for 10min) and the supernatant was collected. The methanolic extract was extracted with hexane (2ml x 4). The hexane extract was separated and the pH of the initial extract was adjusted to pH=2. Hexane extraction (2ml x 4) was repeated and the hexane extracts were combined before evaporating to dryness. The residue was dissolved in tris buffer (1.5ml) and the pH was adjusted to 8.9 Aliquots (0.5ml) was added to the mixture (2.0ml) of 3α-hydroxy steroid

dehydrogenase (0.1mg) NAD $^{+}$ (4mg) dissolved in tris buffer (pH=8.9) and maintaining the constant volume (2.5ml). The absorbance was measured at 340nm against a reagent blank, before and just after adding the enzyme and at 15 min intervals over a period of 1h during the incubation at 30OC. Sodium cholate was used to plot the standard curve.

Statistical Analysis

All the results are presented as mean±SD. Statistical analysis was carried out in Microsoft Excel. The significance was tested by Student's t-test. A probability level of $p<0.05$ was chosen as the criterion of statistical significance.

3. Results

Effect of Naringinase hydrolysis

Serum cholesterol concentration in mice fed with bitter and debittered PFP and control feed showed no significant difference. The absence of a hypocholesterolaemic effect was noted and is discussed (Table 1). TLC densitometry showed that the content of F-II had reduced from 0.59mg to 0.31mg and a smaller flabelliferin (Fc) had increased in content (table-2), showing that partial hydrolysis of carbohydrate moiety of F-II had failed to increase serum cholesterol.

Extraction of flablliferins from faeces

Aliquots from the hexane, dichloromethane and ethyl acetate extracts were spotted on TIC. On development a large, orange fluorescent, anisaldehyde positive spot, masked the spots. Similar spot appeared in both control and the test. The methanol extract of the test sample contained more intense spots, only one of which was a flabelliferin.This was the antimicrobial flabelliferins[18] (F-B), all the other flabelliferins were not present showing that they had been hydrolyzed. The presence of F-B indicates that intestinal enzymes cannot hydrolyze the characteristic α–rhammnosyl and β-glucosyl bonds and therefore they cannot hydrolyze flabelliferins. But hydrolysis does take place and probably occurs due to colonic bacteria, which are apparently not potent against the anti-microbial flabelliferin F-B.

In-vitro hydrolysis of crude flabelliferins

On incubating crude desugared flabelliferin not containing high F-B with the mixed culture bacterial inoculate, a glucose spot, which were clearly visible by hydrolysis of flabelliferin. Thus declined with time, possibly due to bacterial utilization. There

was an appearance of trace of rhammose that appeared to be not utilized by bacteria. The F-II peak declined to some extent in 24h during which time the intensities of smaller flabeillferin spots increased. The F-B peak did not decline with time. β-sitiosterol was not observed as a hydrolysis product even after lysing the bacteria with lysozyme.

Dietary fibre analysis

Table 3 shows that the 4 type of PFP had soluble (SDF) and insoluble (IDF) dietary fibre However, variation was observed between types.

Bile salt analysis

Incorporation of 10% PFP in to feed (test) showed a significant (p=0.0009) increase (67%) compared to control. (Table 4)

4. Discussion

Past studies[3,10] and this study showed that PFP has no free β sitosterol. Therefore the significant lowering of cholestrol reported previously[2,3] for all the main PEP fruit types cannot be due to sitoterol *per se*. According to the literature phytostrols like β sitosterol enter the micelles and prevent the absorption of cholesterol[1]. This is the main mechanism as β sitosterol is poorly absorbed[1]. Previous studies had showed F-11 content had little correlation with lowering of cholestrol[3] despite it being an inhibitor of glucose uptake[4]. One possible hypothesis for the hypocholestrolaemic effect reported previously[2,3,15] that palmyrath also contains small carbohydrate moiety flabelliferins. These may mimic the action of sitosterol in the micelles. However, it was found that naringinase hydrolyzed PFP did not significantly alter (p=0.78) serum total cholesterol and HDL cholesterol in feeding to mice even though naringinase hydrolyses large carbohydrate moiety flabelliferins to give one smaller carbohydrate moiety flabelliferins. Therefore this hypothesis cannot be ruled out. It was further noted that the PFP sample that had been sodium metabusulphite stabilized and stored on a period of time had no hypocholesterolaemic sffect. This may be due to the active hypocholesterolaemic agent being hydrolysed by endo-enzymes of PFP. However flabellifein-II hydrolysis can be ruled out, although pectinase activity remains the more likely possibility. Analysis of faeces for flabellierins showed the presence of only F-B (antimicrobial Flabelliferin) suggesting that colonic bacteria had hydrolysed other flabelliferins. The presence of F-B showed gastro-intestinal enzymes could not hydrolyse the α -rhamnosyl and β-glucosyl residues, which are characteristic of the flabelliferins[13]. β-sitosterol in faeces could not be confirmed, due to masking by other UV active products of the faeces. However this is not important, as β-sitosterol is not absorbed in the colon[1]. Mixed cultures of bacteria isolated from

faeces although hydrolysing a part of carbohydrate chain of flabelliferins did not yield β sitosterol. This is not surprising especially as the *in-viro* hydrolysis technique did not contain anaerobic faecal bacteria. Studies on dietary fiber showed that dietary fiber content was high but varied from one cultivar to other cultivar both in case of shouble dietary fiber and insoluble dietary fiber, both of which are known to lower serum cholesterol by several mechanisms. It is concluded that the high content of dietary fiber may play the major role in lowering of cholesterol with perhaps a minor role by F-II. The latter may have an effect by inhibiting active cholesterol uptake[5] and glucose uptake thus reducing energy change and cholesterol synthesis. Results showed that bile salts are higher in the faeces when mice were fed with 10% PFP and this is a well known mechanism of cholesterol lowering by dierate fiber[8]. Further, another well-known effect of dietary fiber on cholesterol synthesis by absorption of its digestion products (via the enterohepatic circulation), which in turn inhibits the key enzymes in cholesterol snthesis HMG-CoA reductase[7], can also come in to effect.

5. Acknowledgements

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Tabel 1: Effect of incoroporation of 10% bitter and debittered PFP on Total and HDL serum cholesterol.

	Total cholesterol (mg/dl) Mean ± SD	HDL cholesterol (mg/dl) Mean ± SD
Bitter	177.0 ± 24.6 p=0.06*; 9=0.78'	113.6 ± 8.4 p=0.55*; p=0.05'
Debitter	171.3 ± 37.6 p=0.49*	88.6 ± 28.0 p=0.21*
Bitter	187.5 ±11.7	109.0 ± 123.4

*p value with respect to control ± p value with respect to debitter
Each cholesterol detrmination was carried out in duplicate.
Pulp used was metabisulphite stabilized old PFP from Palmyrah Development Board

Table 2 : The contents of Flabelliferins in bitter and debitter PFP

	Bitter (g/100g DW PFP)	Debitter (g/100g DW PFP)
Rhamnose	0.02	0.053
F-I	0.02	
F-II	0.586	0.31
F-B	0.04	
F-C	0.044	0.08
F-D	0.2	0.2
F-E	0.22	0.08
Monoglycoside+sitosterol	0.88	0.39

Table 3 : Dietary fibre contents of 4 types of PFP

	Total (% DW)	Insoluble dietary fibre (% DW)	Soluble dietary fibre (% DW)
Type I	17.8	8.9 8.5	9.1 8.9
Type II	22.5	11.8 12.1	10.9 10.3
Type III	12.3	6.7 6.8	5.3 5.8
Type IV	24.3	16.1 16.4	8.8 8.3

Each estimation was carried out in duplicate on dry weight basis

Table 4 : Effect of 10% PFP incorporated diet on bile salt in faeces.

Content of bile salts ($mg.g^{-1}$)	
Control (mg)	Test
0.048 ± 0.007	0.08 ± 0.015

control : n=5 test : n=6
p= 0.0009