Original Article

Santalbic acid from quandong kernels and oil fed to rats affects kidney and liver P450

Gwyn P Jones[1] PhD, Thomas G Watson[2] PhD, Andrew J Sinclair[3] PhD, Anne Birkett[1] PhD, Nicole Dunt[1] BSc(Hons), Sudheera SD Nair[1] MHN and Susan Y Tonkin[2] PhD

[1]*School of Health Sciences, Deakin University, Geelong, Victoria, Australia*
[2]*School of Biological and Chemical Sciences, Deakin University, Geelong, Victoria, Australia*
[3]*Department of Food Science, Royal Melbourne Institute of Technology, Melbourne, Victoria, Australia*

Kernels of the plant *Santalum acuminatum* (quandong) are eaten as Australian 'bush foods'. They are rich in oil and contain relatively large amounts of the acetylenic fatty acid, santalbic acid (*trans*-11-octadecen-9-ynoic acid), whose chemical structure is unlike that of normal dietary fatty acids. When rats were fed high fat diets in which oil from quandong kernels supplied 50% of dietary energy, the proportion of santalbic acid absorbed was more than 90%. Feeding quandong oil elevated not only total hepatic cytochrome P450 but also the cytochrome P450 4A subgroup of enzymes as shown by a specific immunoblotting technique. A purified methyl santalbate preparation isolated from quandong oil was fed to rats at 9% of dietary energy for 4 days and this also elevated cytochrome P450 4A in both kidney and liver microsomes in comparison with methyl esters from canola oil. Santalbic acid appears to be metabolized differently from the usual dietary fatty acids and the consumption of oil from quandong kernels may cause perturbations in normal fatty acid biochemistry.

Key words: santalbic acid, quandong, absorption, rats, metabolism, P450.

Introduction

Fruit of the sweet quandong [*Santalum acuminatum* (R BR) ADC] are a traditional food of Australian Aborigines[1] and there is considerable commercial demand for the flesh as a food ingredient.[2] The fruit contain an oily seed, with a nutrient composition similar to that of many nuts (Table 1), although Caucasian taste panels have found the seeds to have an objectionable aromatic flavour attributed to the presence of methyl benzoate.[3] The oil content is typically in the range 45–65% and the fatty acids, oleic acid (*cis*-9-octadecenoic acid) and santalbic acid (*trans*-11-octadecen-9-ynoic acid), predominate in the triacylglycerols.[4] Acetylenic fatty acids are unusual components of human foods and are found particularly in two families of plants, the Olacaeae and the Santalacea. They are thought to be synthesized by way of oleic and stearolic (octadec-9-ynoic acid) acids where the insertion of further double and triple bonds continues conjugatively in the direction of the terminal methyl group leading to the biogenesis of a number of acetylenic fatty acids.[5] Santalbic acid (Fig. 1) is one of the most common fatty acids found in the seed oils of these plants and has been isolated from several species of the genera *Santalum*, *Exocarpus* and *Ximenia*.[6]

Table 1. Nutrient content of quandong kernels and other nuts[13,11]

	Composition as eaten (%)				
	Quandong	Almond	Brazil	Hazelnut	Pecan
Moisture	1.6	4.7	5.3	6.0	3.0
Protein	15.3	18.6	14.4	12.7	9.4
Fat	67.6	54.1	65.9	60.9	73.0
Free sugars	3.1	4.4	1.5	3.2	2.2
Starch	tr	2.6	2.2	1.6	3.9
Ash	1.3	3.0	3.4	2.7	1.6

tr, trace.

The metabolic effects of these unusual fatty acids on animals or humans who consume the seeds is not known. However, there have been reports that crepenynic acid (octadec-cis-9-en-12-ynoic acid), a fatty acid with a similar structure to santalbic acid, causes muscular degeneration in sheep.[7,8] Following a series of *in vitro* studies with rat peritoneal leucocytes, it was proposed that acetylenic acids interfere with the metabolism of lipids and fatty acids and that they do this by inhibiting cyclooxygenase and lipoxygenase enzymes.[9] Further, it was found that santalbic acid was a potent inhibitor of the bioconversion of arachidonic acid into prostaglandin E2 in sheep vesicular gland microsomes.[10]

We have conducted preliminary studies with rats and found santalbic acid from dietary quandong oil to be readily incorporated into many tissue lipids, including the following: adipose, skeletal muscle, kidney, liver, blood plasma and heart, although not brain.[11] We also found that animals fed quandong oil had elevated levels of hepatic cytochrome P450 and cytochrome P450 reductase compared with control animals fed canola oil. This indicated the possibility that santalbic acid may not be metabolized like a normal dietary fatty acid but as a xenobiotic compound. In this report we present

Correspondence address: Assoc. Prof. Gwyn P Jones, School of Health Sciences, Deakin University, Geelong, Victoria 3217, Australia.
Tel: 61 3 5227 1369; Fax: 61 3 5227 1270
Email: gwyn@deakin.edu.au
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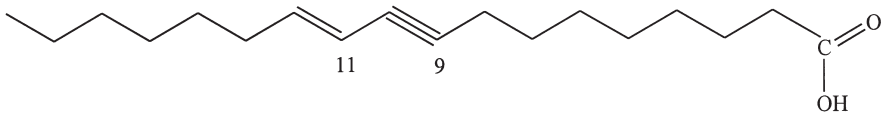

Figure 1. Sructure of santalbic acid.

further data from animal feeding experiments designed to examine the extent to which quandong oil is absorbed from food and whether the observed effects on cytochromes P450 can be attributed to santalbic acid. The experiments were approved by the Deakin University Animal Ethics Committee (A35/92 and A6/95).

Materials and methods

Absorption studies with quandong oil and quandong kernels

Eight-week old male Sprague-Dawley rats were housed individually in metabolic cages. In the first experiment five rats were fed *ad libitum* a semisynthetic diet containing olive oil at 50% dietary energy for 7 days, followed by a similar diet containing 50% energy from quandong oil for 10 days and finally the original olive oil-containing diet for a further 7 days (Table 2 expt 1). The semisynthetic diets were prepared containing casein, corn starch, unprocessed wheat bran, minerals and vitamins providing 26% energy from carbohydrate and 21% energy from protein, 3% energy from oil in processed bran with the balance (50% of energy) being provided by quandong or olive oil. The diets were constructed to meet the nutritional requirements of the animals.[12]

In a second experiment, five rats were fed *ad libitum* diets based on standard rat chow in which the majority of the oil was supplied from either coarsely chopped hazelnuts or coarsely chopped quandong kernels. Chopped nuts were incorporated into powdered chow, moistened and reformed into balls and frozen at –20°C until needed. The feeding regime was to supply the animals with the hazelnut diet for 7 days followed by the quandong kernel diet for 7 days and finally the original hazelnut diet for a further 6 days. The oil content of these two diets could not be standardized at 50% of dietary energy (Table 2 expt 2). This was because analysis revealed the hazelnuts to contain only 35% w/w oil (the Australian food tables give a value of 60.9%) and the batch of quandong kernels to contain 58% w/w oil.[13] The large proportion of hazelnut kernels required to achieve 50% of dietary energy would have compromised the levels of protein and carbohydrate to nutritionally unacceptably low levels compared with those recommended by the American Institute of Nutrition.[12] The hazelnut diet had 45% of energy from fat (37% from hazelnuts), 33% energy from carbohydrate and 22% energy from protein. The quandong kernel diet had 56% energy from fat (50% from quandong kernels), 26% from carbohydrate and 18% energy from protein.

The lipid and fatty acid content of the diets were designed to be as similar as possible in all respects except for the content of santalbic acid. Besides santalbic and stearolic acids, the oils are comprised largely of six fatty acids and three of these predominate, namely, hexadecanoic, *cis*-9-octadecenoic, and *cis, cis,* 9,12-octadecadienoic acid (Table 2). Body weight, food intake, faecal mass and faecal lipids were measured daily in each rat.

Induction of total cytochrome P450 by quandong oil

Eight-week-old male Sprague-Dawley rats housed in groups were fed *ad libitum* diets in which oil provided 30% of dietary energy. The semisynthetic diets were prepared as above and the oil used was either a mixture of quandong (25% energy) with canola (5% energy) in the test diet, or canola alone in the control diet. Rats were numbered and randomly assigned to cages and to diets. All (n = 54) were fed the canola diet for 9 days, following which 12 animals were killed by cervical dislocation and immediately dissected for the extraction of hepatic microsomes. On the 10th day 30 animals were placed on the quandong oil diet and 12 animals remained on the canola oil diet. On each of days 10–15 three animals on the quandong diet were killed and hepatic microsomes extracted. On day 16 the remaining animals were killed and hepatic microsomes extracted. The fatty acid content of lipid in the canola diet was similar to that of the olive oil diet described in Table 2 (experiment 1) except for higher amounts of α-linolenic acid (*cis, cis, cis,* 9,12,15-octadecatrienoic acid) in the former (olive oil contains 0% and canola oil contains approximately 8%).

Table 2. Lipid compositon of diets used in absorption studies

	Lipid compositon of diets			
	Experiment 1		Experiment 2	
	Quandong oil diet	Olive oil diet	Quandong kernel diet	Hazelnut diet
Diet energy from lipid (%)	53.2	53.2	56.4	45.4
Diet energy from quandong oil (%)	50.3	0	50.0	0
Proportion of fatty acids in dietary lipid (%)				
16 : 0	3.8	15.1	4.5	7.5
16 : 1	0.2	1.6	0.4	0.4
18 : 0	1.6	2.7	1.6	2.6
18 : 1	44.9	66.1	45.9	70.7
18 : 2	2.7	12.9	5.6	16.0
18 : 3	2.8	0.6	3.0	0.4
Stearolic	1.0	0	0.9	0
Santalbic	42.0	0	37.5	0

Induction of total cytochrome P450 and cytochrome P450 4A by methyl santalbate/methyl oleate mixture

Three-week-old male Sprague-Dawley rats were housed in pairs and fed a semisynthetic diet for 7 days *ad libitum* containing 20% energy from lipids, of which 17% of dietary energy was supplied by canola oil (basal diet). These rats were younger than those used in previous experiments because of the limited supply of pure methyl santalbate/methyl oleate mixture.

To feed this at a dietary level similar to that known to induce cytochrome P450 when quandong oil was fed necessitated our use of animals with a smaller body weight. After the 7th day lead in the dietary period, one pair of rats (positive control) was maintained on the basal diet *ad libitum* and gavaged daily for four days with 1200 mg/kg of di(2-ethylhexyl)pthalate (DEHP), a known inducer of cytochrome P450 4A1.[14] The remaining animals were switched to a diet in which canola oil contributed 8% of dietary energy and were gavaged daily with oil or fatty acid methyl esters calculated to provide 9% of dietary energy. Pairs of rats were gavage-fed either canola oil, quandong oil, a mixture of pure fatty acid methyl esters from canola oil or a pure mixture of santalbic acid and oleic acid methyl esters obtained from quandong oil. At the end of the fourth day the animals were killed and microsomes prepared from the kidneys and liver.

Preparation of microsomes and measurement of total cytochrome P450 and cytochrome P450 4A

Microsomes from rat liver and kidneys and the assays for protein and cytochrome P450 were prepared and measured as previously described.[11] Cytochrome P450 4A was measured qualitatively using a commercially available western blotting kit employing an antirat cytochrome P450 4A primary antibody and an antisheep Ig biotinylated species-specific secondary antibody (Amersham, UK).

Lipid analysis

Total lipid and the fatty acid content of diets and faeces were determined using methods previously described.[11] In addition quandong oil and faecal lipids were fractionated into the major lipid classes by thin layer chromatography on plates of silica-gel, developed with petroleum ether/diethyl ether/glacial acetic acid (73 : 25 : 2 by vol) and visualized with 2,7-dichlorofluorescein (1%) in methanol. The various bands from the plates were collected and subjected to fatty acid analysis by capillary gas chromatography of methyl esters.

Preparation of santalbic acid methyl ester and canola oil methyl esters

Canola and quandong oil were refluxed in methanol with sulphuric acid catalyst. Methyl esters were taken up in hexane and washed with several volumes of water before removal of solvent under vacuum. In an effort to obtain pure santalbic acid methyl ester, quandong methyl esters were chromatographed on a semipreparative scale using a column of silica gel impregnated with silver nitrate.[15] This was only partially successful and a mixture of santalbic acid and oleic acid methyl esters (70 : 30) was collected from the column eluent. However, when examined by capillary gas chromatography these two compounds together accounted for more than 99% of the recorded peaks, thereby indicating the mixture to be essentially free of other fatty acids. The small amount (6 g) of this mixture we obtained was sufficient to gavage two rats for 4 days at 9% of dietary energy.

Statistical analysis

Results are presented as the mean with standard deviation in parentheses. Data have been compared using either Student's *t*-test or analysis of variance and these tests were performed using Minitab Release 8.21 software package (Minitab Inc, State College, PA, USA)

Results

Absorption of santalbic acid

After feeding a quandong oil diet for 10 days the amount of santalbic acid absorbed in 24 h by each of the five rats, calculated from the difference between consumption (ranging from 1.9 to 3.5 g/kg body weight) and excretion (between 0.020 and 0.027 g/kg body weight) showed that on average 99% was absorbed. Similar amounts of total lipid were excreted while the animals were consuming either the quandong diet (5.6 ± 0.9%w/w) or the olive oil (6.5 ± 1.0% w/w) diet and the fatty acid composition of the various faecal lipid fractions were also similar (Table 3). These data show that the absorption of lipid from both diets is almost complete and is consistent with previous reports that excreted lipid is partly of endogenous origin or synthesized by gut bacteria and does not reflect diet.[16,17] Only very small amounts of santalbic acid were excreted by animals fed quandong oil and this was largely found in the triacylglycerol fraction. Twenty-four hours after the animals were returned to the olive oil diet, santalbic acid could not be detected in the faeces. If there was any physiological adaptation to changes in dietary lipid then it was very rapid and unlikely to be of nutritional significance.

The results were somewhat different when rats were fed quandong kernels rather than quandong oil. The proportion of ingested santalbic acid absorbed fell to 91% and the faecal excretion of lipid increased to an average of 11% w/w. Oil in nuts is known to be less digestibile than the isolated oil depending on how thoroughly the nuts are ground by chewing. For example, it has been reported that the digestibility coefficient of oil in hazelnuts was 87.2%, while in peanuts it was 95.5% and in walnuts it was 67.8%.[18] This is probably due to the oil being physically trapped in intact cells of quandong kernel particles as they pass absorptive sites in the small intestine. In our experiment the excretion of santalbic acid continued in decrements for 3 days after the animals were returned to the hazelnut diet, following which it could no longer be detected.

Induction of total cytochrome P450 and cytochrome P450 4A by quandong oil

A one-way analysis of variance showed that the food intake/kg body weight and daily weight gain of animals on both control and test diets were not significantly different throughout the study ($P < 0.05$). The content of hepatic cytochrome P450 after feeding quandong oil for 1–7 days and the corresponding control values are given in Table 4. Significant increments in the content of cytochrome P450 during successive days feeding quandong oil could not be detected, possibly because of the small number of animals tested ($n = 3$), although a trend was evident. However, after

Table 3. Fatty acid composition of major lipid fractions in rat faeces (duplicate assays of pooled samples) after 10 days feeding quandong oil (Qu) or olive oil (Ol) diets

	Fatty acid in lipid fraction (%)							
	Phospholipid fraction (% of total lipid)		Free fatty acid fraction (% of total lipid)		Triacylglycerol fraction (% of total lipid)		Sterol ester fraction (% of total lipid)	
	Qu (9.7)	Ol (9.6)	Qu (27.8)	Ol (26.8)	Qu (52.2)	Ol (74.0)	Qu (4.3)	Ol (3.4)
Fatty acid								
16:0	27.4	28.7	15.1	26.8	15.5	16.3	12.7	21.2
16:1	nd	nd	nd	0.5	nd	nd	nd	nd
18:0	4.1	3.2	4.9	10.9	2.0	2.1	7.2	10.6
18:1	21.0	19.7	40.1	31.3	25.6	25.8	30.5	27.7
18:2	14.7	18.3	28.0	18.7	41.8	43.9	15.8	19.8
18:3	1.4	1.2	4.9	1.1	3.2	3.0	1.3	2.6
Stearolic	nd	nd	nd	nd	nd	nd	nd	nd
Santalbic	3.1	nd	3.6	nd	4.4	nd	0.86	nd

nd, not detected.

Table 4. Specific content of hepatic microsomal P450 levels in rats fed diets containing quandong oil for 7 days following a 9 day lead in with canola oil compared with controls (canola)

	nmol P450/ mg protein	
Day	Quandong diet	Canola diet
9	nd	1.28 ± 0.52[a] (n = 12)
10	0.98 ± 0.18 (n = 3)	nd
11	1.35 ± 0.05 (n = 3)	nd
12	1.52 ± 0.10 (n = 3)	nd
13	1.73 ± 0.20 (n = 3)	nd
14	1.21 ± 0.53 (n = 3)	nd
15	1.73 ± 0.10 (n = 3)	nd
16	2.30 ± 0.56[b] (n = 12)	1.48 ± 0.45[a] (n = 12)

[a,b]Significantly different (P = 0.002); nd, not determined.

feeding quandong oil for 7 days (day 16) the P450 content was higher (P = 0.002) than in controls fed canola oil. Hepatic cytochrome P450 4A levels were also increased by dietary quandong oil compared with canola oil as shown in a western blot of liver microsomes.

Induction of cytochrome P450 and cytochrome P450 4A by santalbate/oleate methyl ester mixture

The results of the specific content of cytochrome P450 in the microsomes of liver and kidney are given in Table 5. The mean P450 value for the two animals gavaged with santalbate/oleate methyl ester mixture was higher in both liver and kidney microsomes than in the corresponding tissues of the pairs gavaged with canola methyl esters or with quandong oil. If santalbic acid was the responsible agent for elevating P450, then it would be expected that both quandong oil and santalbate/oleate methyl esters would increase the levels of P450 and the reasons why this was not observed are not clear but may be related to the small number of animals tested. The other major fatty acid in the methyl ester mixture, oleic acid, can be discounted as an inducer because in none of our experiments was its consumption, either in the form of triacylglycerols in canola and olive oil or as the methyl esters of canola oil, associated with elevated levels of cytochrome P450 in the absence of quandong oil or santalbic acid. The animals fed DEHP (positive controls) showed levels of P450 comparable to those published by others (0.86 ± 0.22 nmol P450/mg protein).[19]

The results of western blots of cytochrome P450 4A from both liver and kidney microsomes showed that the bands from the animals fed santalbate/oleate and quandong oil were slightly more dense than those from the other treatments except for DEHP. This indicates that santalbic acid induces at least the P450 4A group of enzymes and supports results from the earlier experiment in which animals were fed quandong oil for 7 days.

Discussion

Quandong oil is very effectively absorbed when fed to rats and although slightly less well absorbed when fed in the form of intact kernels, uptake from the diet is still more than 90%. The unusual chemical structure of santalbic acid does not seem to impair its absorption from food. If these data are extrapolated to humans, they indicate that Aborigines and others who periodically eat the kernels could absorb relatively large quantities of this compound on each occasion irrespective of whether the pattern of consumption was frequent or infrequent. For example, a modest snack of 20 kernels would be likely to lead to the absorption of approximately 5–6 g santalbic acid.

The fact that cytochrome P450 levels are elevated in rats fed quandong oil at 25% of dietary energy indicates that quandong oil contains a xenobiotic compound or compounds

Table 5. P450 content of liver and kidney microsomes in rats (n = 2) fed standard laboratory chow after 4 days daily gavage with quandong oil, canola oil, canola oil fatty acid methyl esters, santalbic acid/oleic acid methyl esters or diethylhexylpthalate

Animal group	Diet (gavage)	Liver nmol P450/ mg protein	Kidney nmol P450/ mg protein
Control	Canola oil	0.37 ± 0.08	0.25 ± 0.18
Test	Quandong oil	0.47 ± 0.03	0.37 ± 0.23
Positive control	Di (2-ethylhexyl) pthalate	1.10 ± 0.08	0.90 ± 0.40
Control	Canola oil fatty acid methylester mixture	0.49 ± 0.11	0.21 ± 0.04
Test	Santalbate/oleate methyl ester mixture	0.86 ± 0.20	0.56 ± 0.24

that induce a phase 1-type reaction. The major quantitative difference between the test and control diets is the content of santalbic acid in the triacylglycerols, other fatty acids being present in relatively similar amounts. Western blotting analysis of hepatic microsomal proteins confirmed our expectation that cytochrome P450 4A levels were increased. This group of enzymes has been reported to hydroxylate the terminal (ω) carbon and to a lesser extent the penultimate terminal (ω-1) carbon of lauric acid, arachidonic acid and eicosanoids.[20] Following studies with the isoenzyme cytochrome P450 4A1, it has been postulated that arachidonic acid adopts a hairpin configuration that can be almost superimposed upon lauric acid and that this serves as a suitable shape for the isoenzyme.[21] If this is true then santalbic acid may induce a different cytochrome P450 4A isoenzyme to that induced by arachidonic acid because the molecule is relatively straight due to the peculiar nature of the unsaturation, that is, a *trans* double bond and conjugated triple bond centrally located in the hydrocarbon chain.

Confirmatory evidence for the role of santalbic acid as an inducer of P450 is given in Table 4 and from the darker bands obtained with western blot analysis. The relatively low levels of total P450 in animals gavaged with quandong oil could be explained by the short duration of exposure, difference in age (3 weeks compared with 8 weeks), the small number of animals or by the possibility that the methyl ester of santalbic acid is a more potent inducer than the triacylglycerol ester. Also, in animals gavaged with quandong oil the cytochrome P450 4A band in a western blot was slightly more intense than the band from canola oil indicating that santalbic acid as both the methyl and glyceryl ester is capable of inducing this isoenzyme.

From this and previously reported results[9,10] on the toxic effects of octadecenynoic acids in animals it would appear that there are potentially two complementary mechanisms which may contribute to alterations in eicosanoid production in animals. The first of these would relate to the proposed formation of an intermediate allene or vinyl hydroperoxide[22] which would bind and deactivate the lipoxygenase. Second, as shown in this study, the elevation of P450 4A by santalbic acid could result in an increase in the hydroxylation of other fatty acids,[23,24] thereby inhibiting eicosanoid production by inducing a deficit of substrate fatty acids such as arachidonic acid.

Santalbic acid from quandong kernels fed to rats is extensively absorbed from the diet and widely distributed in the animal's tissues. It induces total cytochrome P450 and cytochrome P450 4A in the liver and kidneys to a greater extent than do fatty acids usually found in foods. Many dietary factors are known to influence cytochrome P450 activity both in animals and humans;[25] however, in our experiments we provided strong evidence that santalbic acid is an inducer of this enzyme system. The implications of these changes if mirrored in human consumers are unclear. However, it is possible that exposure to santalbic acid may lead to perturbations in the metabolism of arachidonic acid and other eicosanoids. Thus, until these effects are better understood the human practice of eating quandong kernels should be viewed with some circumspection.

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