Quantification of Karanjin Using High Performance Liquid Chromatography in Raw and Detoxified Karanj (*Pongamia glabra* vent) Seed Cake

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ABSTRACT : Various products of karanj (*Pongamia glabra*) are utilized for industrial, health and animal agriculture applications in the Indian subcontinent. Despite a rich source of protein (CP, 28-34%), karanj cake was found to be slightly bitter in taste and toxic owing to the presence of flavonoid (Karanjin), restricting its safe inclusion in the livestock diets. Feeding trials with raw cake revealed its poor palatability and adverse performance among different categories of livestock including poultry. The present study was, therefore, aimed to detoxify karanj cake by various physico-chemical methods like solvent extraction, water washing, pressure cooking and alkali and acid treatments. The level of residual karanjin in raw and variously processed cake was quantified using high performance liquid chromatography (HPLC). The raw expeller karanj cake was found to contain about 0.19% of karanjin. Though a non-polar solvent, soxhlet extraction of expeller pressed cake with petroleum ether drastically reduced karanjin content (0.01%). Soaking of cake for 24 h in 1% NaOH (w/w) solution was found to reduce karanjin to a major extent with little further benefit by increasing alkali level. Milder alkalies like lime and fertilizer grade urea reduced the karanjin levels marginally. Similar was the case with mineral acids such as HCl and glacial acetic acid. It was, therefore, concluded that solvent extraction of karanj seeds would be the best method of detoxification as well as for more recovery of oil and karanjin. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 3 : 416-420)***

Key Words : Karanj Seed, Karanjin, Detoxification, HPLC, Solvent Extraction, Alkali and Acid Treatments

INTRODUCTION

Inadequate availability of pastures due to shrinking grazing lands in light of intensive cultivation and chronic shortage of protein and energy rich animal feeds due to enhanced needs of ever increasing human population are some of the major constraints for obtaining optimum productivity of livestock and poultry in India and other South East Asian countries. Hence, the animal nutritionists are compelled to explore the possibilities of feeding non-edible and non-competitive agro-industrial by-products to meet the nutritional requirements of animals. These unconventional feeds were though found to be promising, long term feeding that too at higher levels adversely affected the animal performance due to the presence of toxic factors.

Such non-edible agro-industrial by-products, which are hitherto wasted otherwise despite rich nutritional value, can be converted into wholesome animal feeds after evolving suitable processing technology which could be easily adoptable by farmers as well as by the industry. One among them is karanj (*Pongamia glabra* vent) cake which has great potential in animal feeding.

Pangamia glabra vent (Syn. *Pongamia pinnata*), popularly known as karanj belongs to the natural order *Leguminosae* and family *Papilionaceae*, is a medium sized glabrous avenue tree with a spreading crowns upto 18 m, found almost throughout India upto an altitude of 1,200 m and distributed further Eastwards, chiefly in the littoral regions of South-Eastern Asia and Northern Australia. In India the availability of the seed has been estimated to be 1,300,000 t year^{-1} (Ministry of Agriculture, 1992).

The seeds contain 27-39 per cent of fatty oil (The Wealth of India, 1969) which is used for leather dressing, soap making, lubrication, illumination and for medicinal purposes. The non-fatty components of the oil includes the principal furanoflavonoid, karanjin ($C_{18}H_{12}O_4$, mp. 158.5°) and a novel furano-diketone, pongamol ($C_{18}H_{14}O_4$, mp. 128-29°) (Limaye, 1925; Rangaswami and Seshadri, 1940). The other furonoflavones isolated include pongapin, kanjone and pongaglabrone (Aneja et al., 1958; Aneja et al., 1963).

Karanjin is the active principle responsible for the curative effect of karanj oil in skin diseases. Clincal experiments indicate that it is free from the highly irritating and inflammatory effects of coumarin compounds and its application with other vegetable oils such as coconut, sesame or groundnut oil is reported to be better than when incorported in a paraffin base (Seshadri and Sood, 1963).

Karanj cake, containing~28-34% crude protein, is not commonly used as a feed for livestock and poultry due to the harmful effects of antinutritional/toxic factors present in the seed cake (Natanam et al., 1989). The present study was, therefore, under taken to detoxify the karanj cake by adopting various physicochemical methods like viz. solvent extraction, water washing, pressure cooking, alkali and acid treatments and estimate residual karanjin content using high

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performance liquid chromatographic (HPLC) method.

MATERIALS AND METHODS

Expeller pressed karanj cake was obtained from oil seed cake market of Bangalore, located in South India. The proximate principles of expeller pressed and solvent extracted karanj cake were analysed as per the methods of the Association of Official Analytical Chemists (1995).

Laboratory level detoxification of cake

The following physical and chemical treatments were tried with the expeller pressed karanj seed cake.

Physical methods :

a) Solvent extraction: About 500 g of ground expeller cake was subjected for extraction of residual oil in the cake using petroleum ether (BP. 60-80°C) as solvent by soxhlet apparatus for 10-12 h.

b) Water washing: The supernatant of water soaked and intermittently stirred cake (1:5, w/v) in a plastic trough was siphoned off after 24 h. With the same quantity of water, washing was repeated for another two times at 45 min. interval and then sun dried.

c) Pressure cooking: The cake was pressure cooked with water in the ratio of 1:0.6 (w/v) and 1:1 (w/v) for 60 min. each, from the time of development of pressure which were then sun dried.

Chemical methods :

a) Sodium hydroxide treatment : exactly 250 g of ground karanj cake was soaked in 250 ml of water (1:1 w/v) containing 2.5, 3.75, 5.0 and 6.25 g of NaOH to yield concentration of 1, 1.5, 2.0 and 2.5% NaOH (w/w), respectively. The homogenously mixed samples were left in air tight beakers for 24 h and then sun dried.

b) Calcium hydroxide treatment : Similar to the above method, 2.5, 5.0, 7.5 and 10 g of calcium hydroxide (lime) was added to the cake to yield concentrations of 1, 2, 3 and 4% $Ca(OH)_2$ (w/w), respectively. The rest of the procedure was similar to NaOH treatment.

c) Urea ammoniation : Urea ammoniated cake was prepared by ensiling the cake in water (1:1, w/v) containing fertilizer grade urea at 1, 2, 3 and 4% of cake (w/w) for 5 days and then sun dried.

d) Acid treatments: It was prepared by soaking the cake in water (1:1 w/v) containing hydrochloric acid (HCl) at 0.5 and 1.0% (w/v) for 24 h and then sun dried. Similary, the cake was soaked in water (1:1, w/v) containing glacial acetic acid at 1 and 2% (w/v) for 24 h and sun dried.

Extraction of karanjin from raw and treated cake

Exactly 25 g of ground and thoroughly mixed sample was weighed and transfered into the thimble and extracted for 12 h using 200 ml of freshly distilled methanol as solvent. Methanol extract was then cooled and filtered into a preweighed round bottom flask using Whatman no. 1 filter paper. The excess methanol was distilled off under vacuum. The flask containing the extract was weighed again to obtain the weight of extract by subtracting the empty flask's weight. Sufficient amount of moisture free extract was transfered into vials for futher analysis.

High performance liquid chromatographic analyses of karanjin in raw and processed karanj seed cake

Apparatus : A Thermo Separation Products liquid chromatograph equipped with a variable wave length spectra series UV detector and silica gel prepacked analytical column (RP-18, 7 μm) was used throughout this work.

Solvents and elution : Solvents were filtered using a glass millipore system with a 0.45 μm filter and degassed at room temperature under vacuum with magnetic stirring. Working solutions containing 10 mg of sample per 1 ml of methanol were filtered through a swinny stainless unit with a 0.45 μm filter. The elution solvent system consisited of methanol and water (80:20) run at a flow rate of 1 ml/min at an average pressure of 2000 p.s.i. Samples were dissolved and injected on to the column using a microinjector (20 μl).

Detection : The UV detector was set at 250 nm.

Method to calculate the content of karanjin : Area points were taken into considration to calculate the karanjin levels and the following formulas were used to express the karanjin content in percentages.

a)

$$\text{ppm} = \frac{\text{Area point of test}}{\text{Area point of standard}}$$

b) Karanjin content in methanol extract =

$$\frac{x}{10,000} \times 100\% = \frac{x}{100}\%$$

c) Karanjin content in treatment =

$$\frac{x}{100} \times \frac{y}{100} = \frac{xy}{10,000}$$

x = Karanjin in ppm; y = % methanol extract

The experiment data were subjected to analysis of variance as per the methods of Snedecor and Cochran (1967) by processing as per the programme SPSS 7.5 for windows (1996) in computer.

RESULTS

Expeller karanj cake contained: crude protein, 287; ether extract, 121; crude fibre, 40; total ash, 53; organic

matter, 947; total carbohydrate, 539 g kg^{-1}. The corresponding values for solvent extracted cake were 335, 3, 50, 52.3, 946.7 and 609.7 g kg^{-1}, respectively.

The data on yields of methanol extraction and their percentages are given in table 1. While the data on the residual karanjin levels (quantity) in methanol extract and its per centage in raw and processed cakes are given in table 2. The analysis of furanoflavonoid, karanjin by high performance liquid chromotograghy (HPLC) is a sensitive technique which accurate results in minutes compared to classical procedures requiring large amout of material and days, if not weeks, for analysis. For polar substances like karanjin, the reversed-phase (RP) technique is far superior to the normal technique, since there is no danger that some highly polar substance may be retained irreversibly, with the result that the separation characteristics of the column could be gradually changed. This is the first ever report on the standardization of the procedure for karanjin qunantification using HPLC in raw as well as processed cakes. Figure 1A shows the chromatogram of karanjin. Under the present chromatograghic conditions, the retention time of karanjin was about 10.09 min. and other constituents extracted did not interfere since they eluated either before or after the peak of interest.

Raw expeller cake has ~31% methanol extractable material. Treatments with alkali/urea/lime/petroleum ether/pressure cooking did not affect the amount of extractable material, however, water washing has reduced the extractable content to a reasonable extent. Water washing of expeller cake also caused ~23% loss in dry matter content.

Table 1. Methanol extract yield and its percentage[a]

Treatment	Weigh of extract	% of extract in cake
Raw cake	7.78	31.10
Physical treatment		
Solvent extraction	7.50	30.00
Pressure cooking		
Method I	7.55	30.19[a]
Method II	7.50	30.00[b]
Water washing	5.00	20.00
Chemical treatment		
Alkali NaOH		
1.0%	7.28	29.14[a]
1.5%	7.58	30.27[b]
2.0%	7.84	31.35[c]
2.5%	8.17	32.67[d]
Lime treatment [$Ca(OH)_2$]		
1.0%	8.0	32.00[a]
2.0%	8.0	32.05[b]
3.0%	8.18	32.78[c]
4.0%	8.13	32.50[b]
Urea treatment		
1.0%	7.50	30.04[a]
2.0%	7.50	30.00[a]
3.0%	7.52	30.08[a]
4.0%	7.94	31.75[b]
Acid treatement		
a) Glacial acetic acid		
1.0%	7.50	30.00[a]
2.0%	8.00	32.02[b]
b) Hydrochloric acid		
0.5%	7.56	30.26[b]
1.0%	7.50	30.01[a]

[a] Values obtained are the means of triplicate analysis.
Means (among treatments) with different superscript in a column differ significantly ($p \leq 0.01$).

Table 2. Karanjin content in the methanol extract and treated cake[a]

Treatment	Karanjin in methanol extract (ppm)	Karanjin in cake (%)
Raw cake	61.40	0.19
Physical treatment		
Solvent extraction	01.90	0.01
Pressure cooking		
Method I	44.75	0.14[b]
Method II	32.38	0.10[a]
Water washing	80.01	0.16
Chemical treatment		
Alkali (NaOH)		
1.0%	09.57	0.03[a]
1.5%	09.90	0.03[a]
2.0%	09.41	0.03[a]
2.5%	08.54	0.03[a]
Lime treatment		
1.0%	55.20	0.18[d]
2.0%	44.00	0.15[c]
3.0%	35.40	0.12[b]
4.0%	32.60	0.11[a]
Urea treatment		
1.0%	60.67	0.19[d]
2.0%	58.00	0.17[c]
3.0%	50.18	0.15[b]
4.0%	41.43	0.13[a]
Acid treatement		
a) Glacial acetic acid		
1.0%	34.58	0.10[a]
2.0%	32.48	0.10[a]
b) Hydrochloric acid		
0.5%	51.30	0.16[b]
1.0%	40.00	0.12[a]

[a] Values obtained are the means of triplicate analysis.
Means (among treatments) with different superscript in a column differ significantly ($p \leq 0.01$).

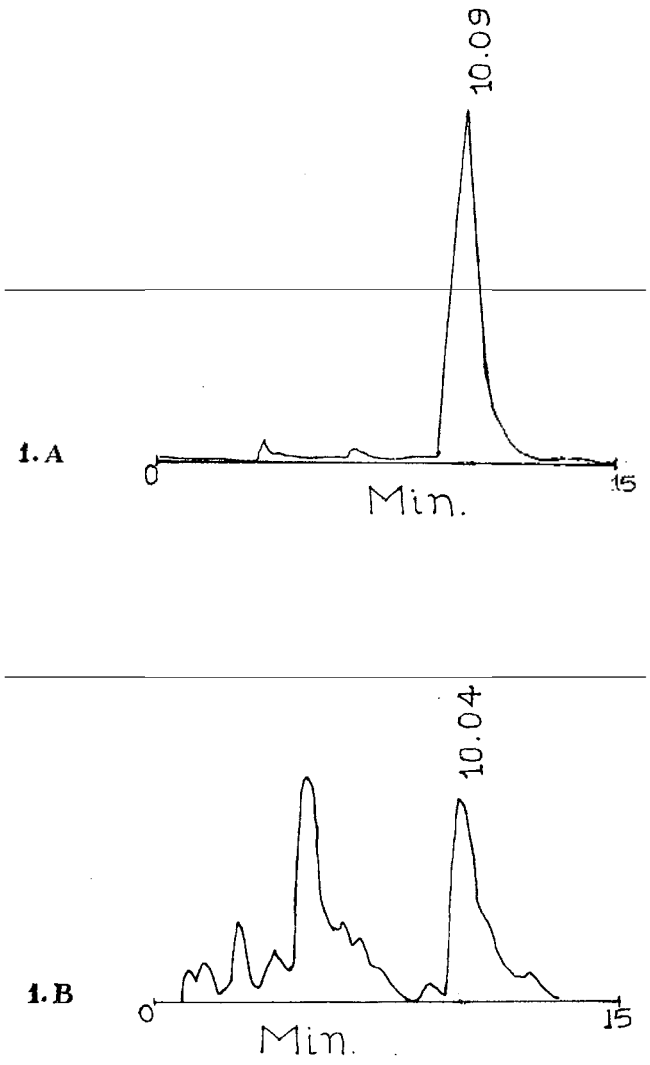

Figure 1. Chromatogram of (A) standard karanjin and (B) karanjin in raw expeller pressed karanj cake

A significant ($p<0.01$) difference was noticed among as well as between treatments with respect to methanol extract yields and karanjin contents in variously processed karnaj cakes (tables 1 and 2). Karanjin content in raw cake was about 61.4 ppm whereas, petroleum ether extraction has removed substantial amount of karanjin, though it is not a polar solvent. However, pressure cooking has reduced only about 50% of karanjin. Water washing on the other hand, had no effect on karanjin reduction. Sodium hydroxide soaking at 1% (w/w) itself reduced karanjin content to a greater extent. However, the milder alkali, calcium hydroxide has reduced karanjin only to a marginal extent. Similar is the result with urea ammoniation. Hydrochloric acid (HCl) and glacial acetic acid treatments have reduced only 1/3rd of karanjin.

DISCUSSION

Both with in and between treatments showed a significant ($p<0.01$) effect on methanol extract yield and karnajin concentration in variously processed cakes. The raw untreated expeller karanj cake contained ~0.19% of karanjin (figure 1B). A drastic reduction in karanjin content was noticed on soxhlet extraction of cake with petroleum ether (b.p. 60-80°C). The cake left after extraction contained only 0.01% of residual karanjin (figure 2A). By far, solvent extraction was found to be the best method of detoxification of karanj seed cake. Incorporation of deoiled karanj cake between 24 and 30% in the concentrate mixtures of growing calves (Gupta et al., 1981; Konwar and Banerjee, 1987) and kids (srivastava et al., 1990) apparently produced no adverse affects in the performance, confirming the reduction of karanjin levels upon deoiling. The alkali treatment of unconventional protein feed supplements (Neem Seed kernel cake, Mahua seed cake, sal seed cake etc.), was successfully tried for their detoxification (Joshi et al., 1990; Katiyar et al., 1991,1993; Bhar and Katiyar, 1996; Gowda et al., 1998). But no such efforts were made with karanj cake.

In the present study it was found that soaking of cake for 24 h with 1% NaOH (w/w) was adequate to remove most of the karanjin (figure 2B). Further increase in the dose of alkali did not improve the detoxification (figure 2C). However, this may be helpful in detannification of karanj cake and subsequent improvement in the efficiency of protein utilization as the karanj cake contains 2.6-3.0% tannin (Chandrasekaran et al., 1989).

Compared to the strong alkali (NaOH), lime [$Ca(OH)_2$] is a milder base and less harmful. However, lime treatment of the cake was only marginally effective in reducing the karanjin content. Similar was the result with urea treatment. Acidulation of the cake with a mineral acid namely HCl and glacial acetic acid at 1.0% (w/v) of the cake brought out ~40% reduction in karanjin content. A simple method like pressure cooking could reduce the karanjin by about 50%.

By solvent extraction most of the karanjin has been removed from the cake as compared to all other treatments. Solvent extraction has an additional advantage of more recovery of oil for further use. As on date there is no specification available on the maximum permissible levels of karanjin or its metabolites in the karanj cake. Further studies could only elucidate the relationship between the karanjin and toxicological effect of the feedstuff.

Nowadays supercritical fluids are used for extraction in place of organic solvents. If the residual cake left after solvent extraction proves to be safe as animal feed supplement there is scope for developing detoxification technology of karanj cake.

Karanjin and karanj oil are valuable industrial products.

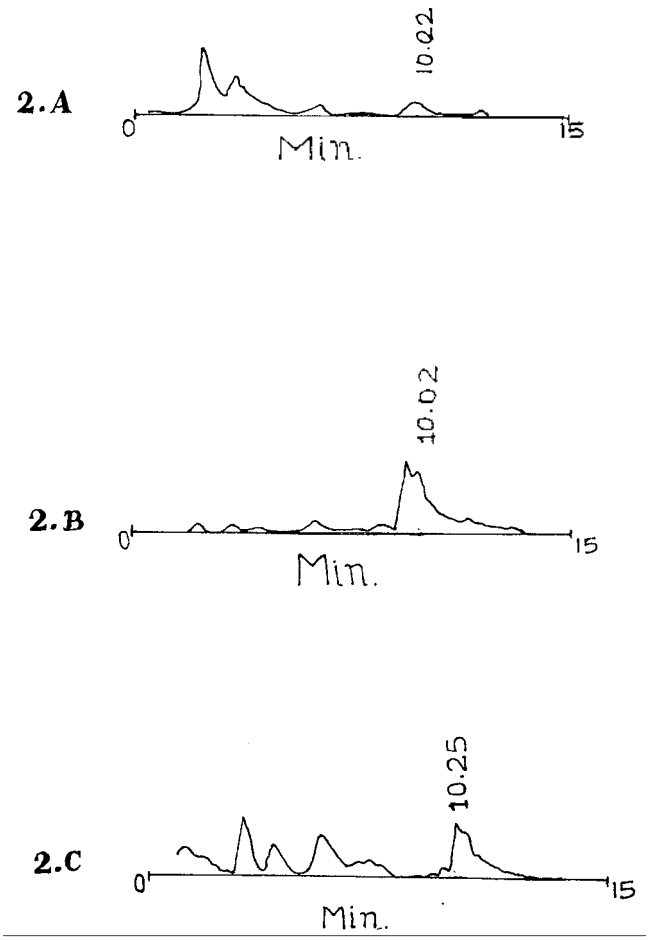

Figure 2. Chromatogram of karanjin in (A) solvent extracted cake (B) alkali (1%. w/w) treated expeller pressed cake and (C) alkali (2.5%. w/w) treated expeller pressed cake

The extraction technology will have dual advantage of producing karanjin free. protein rich animal feed supplement and karanj oil. Experiments are under way in evaluating the wholesomeness of these treated cakes as protein feed supplement.

CONCLUSION

Solvent extraction of karanj seed proves to be the best method of detoxification as it drastically reduced the karanjin content as well as yielded additional commodities like karanjin and karanj oil.

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