Protease Inhibitors and *in vitro* Digestibility of Karanja *(Pongamia glabra)* Oil Seed Residue. A Comparative Study of Various Treatments

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ABSTRACT: Trypsin and chymotrypsin inhibitor activities (TIA and CIA) were assayed before and after processing of Karanja oil seed residue. Upon treatment, the loss of TIA and CIA varied with the type of processing or extraction: the reduction after solvent extraction ranged between 34 and 15%, respectively. These activities were completely removed in 2.4% HCl; 83.35 and 54.86% were removed on autoclaving; 33.86 and 15.30% on fermentation; and removal was 4.36–83.69% and 3.66–77.59% after exposure to different doses of gamma radiation (1, 5, 10, and 50 KGy). An *in vitro* digestibility study was carried out to confirm the inactivation of the inhibitors and showed improvements from 45 to 81%, with the maximum observed in 2.4% HCl solvent-refluxed residue. A linear relationship was observed between the reduction in protease inhibitor activities and the improvement in digestibility.
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KEY WORDS: Antinutrients, autoclaving, detoxification, fermentation, *in-vitro* digestibility, irradiation, Karanja oilseed residue, nutritive value, protease inhibitors, solvent treatment.

Karanja *(Pongamia glabra)* trees are widely distributed throughout humid lowland tropics. They are grown in many parts of India, with an annual seed production of 1.3×10^5 tons. Karanja seeds are classified as nonedible; only one-fourth of the total production is used for the extraction of oil, which is utilized for medicinal purposes. The oil is reported to have curative effects on eye diseases, rheumatism, leucoderma, and itching wounds (1). After hydrogenation, it is also used in leather tanning and in the soap industry. The seed residue is mainly used as fertilizer (2). Karanja seeds contain 42% oil and 21% protein (3). The protein content in the residual cake ranges from 28 to 30%, and the residual fat content in the cake depends on the oil extraction process. Despite being rich sources of protein, Karanja seeds and their residues have limited utility as human food, or even as animal feed, owing to poor palatibility and the presence of antinutritional/toxic factors (4–9). To increase utilization of these sources, detoxification is essential.

Protease inhibitors (PI) are well-known antinutrients that are responsible for reduced digestibility of plant food proteins (10). Methods to reduce antinutrients include solvent extraction (11,12), fermentation (13), and heat treatment (14–16). Irradiation has recently been used in the study of antinutrients, and for the first time, the effect of gamma radiation on PI has been shown in soybeans (17). In our laboratory, gamma radiation was used for the detoxification of safflower seed residue (18). There is a single report on the PI in defatted Karanja cake (19), wherein the effect of solvent (2.0% HCl) extraction on the inhibitory activities has been shown. The present paper gives comparative data on detoxification effects of various treatments on PI in commercial Karanja oilseed residue.

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MATERIALS AND METHODS

Commercial Karanja cake was obtained from fertilizer suppliers in Shirur, India. All chemicals used for the experiment were of AR grade. Pepsin, trypsin, and chymotrypsin were obtained from Sigma Chemical Company (St. Louis, MO). Pancreatin was from Tuga Biochemicals (Pune, India).

Total fat and protein contents were analyzed by Soxhlet and Kjeldahl methods (20); the cake contained 30% protein and 5% fat. PI were quantitatively determined (18).

Solvent extraction. The commonly used solvents for extraction of PI are phosphate and borate buffers (18,21,22). The inhibitors were extracted by continuously grinding the sample manually in a mortar and pestle with buffer (0.1 M, pH 7.6) in the ratio of 1:20 (wt/vol) at room temperature for 1 h. The presence of trypsin and chymotrypsin inhibitor activities (TIA and CIA) was checked in both buffers and solvents.

Heat treatment. The sample was made into a paste by grinding in water (1:2, wt/vol) in a mortar and pestile, transferred into a breaker, covered with aluminum foil, and autoclaved at 115°C and 15 psi for 1 h.

Fermentation. The process of fermentation was similar to that described by Fretzdorff and Brummer (23), except for the variation in solute-to-solvent ratio. The sample was made into a fine powder, mixed with citrate buffer, pH 5.5 (1:4, wt/vol), and incubated at 55°C for 24 h. The dried powder was used for the assay of inhibitory activities.

Irradiation. The sample was exposed to different doses (1, 5, 10, and 50 KGy) of gamma radiation with a cobalt-60 source at a dose rate of 0.17 Mrad/h.

PI were extracted from the autoclaved, fermented and irradiated residues in 2.4% HCl for the assay.

Assay. Procedures used for the extraction and estimation of PI were identical to those described by Joseph and Dikshit (18). The protein content of the supernatant was estimated by the method of Lowry *et al.* (24).

One unit of enzyme activity is defined as the amount of enzyme that converts 1 mg of protein to trichloroacetic acid (TCA)-soluble components in a specified time at 37°C and pH 7.6. One unit of inhibitor activity is the amount of inhibitor that depresses the proteolytic activity by one unit.

In vitro *digestibility.* These studies were carried out by following the methods of Boonvisut and Whitaker (25) and Mandal (19) as described by Joseph and Dikshit (18). The TCA-soluble peptides in the filtrates after 24-h digestion were estimated by the method of Lowry *et al.* (24).

The whole study of processing, assay, and *in-vitro* digestion was carried out in triplicate. The percentage reduction in PI was calculated on the initial content in the sample. The F-test for equality of variances was used for the statistical analysis (26).

RESULTS AND DISCUSSION

PI. The results (Table 1) revealed that the untreated sample contained 43.50 and 18.28 U/mg protein of TIA and CIA, respectively. It was also apparent that phosphate buffer was more effective in extracting TIA (72%) than the borate buffer (50%), whereas the latter was found to be more suitable for reduction of CIA, leading to complete removal, compared to only 64% extraction in phosphate buffer. This suggests that PI solubility differs in the two buffers, even under the same pH conditions.

TABLE 1
Effect of Various Treatments on Trypsin and Chymotrypsin Inhibitor Activities (TIA and CIA)[a]

Treatment	TIA (Units/mg protein)	CIA (Units/mg protein)	Protein loss (wt%)
Untreated	43.50 ± 1.55	18.28 ± 2.30	—
Extracts			
Phosphate buffer	31.55 ± 1.05[c]	11.70 ± 1.10[c]	1.44
Borate buffer	21.70 ± 0.88[d]	18.30 ± 0.77[d]	1.20
2.4% HCl	43.55 ± 1.65[c]	18.26 ± 2.42[c]	1.80
2.4% HCl[b]	27.73 ± 1.44[c]	9.46 ± 1.19[d]	3.60
Methanol/HCl[b]	22.08 ± 1.10[d]	8.70 ± 1.21	3.20
60% Methanol	Nil	Nil	0.40
Acetone/hexane/water[b]	18.15 ± 1.10	8.20 ± 1.35	2.10
Residues			
Autoclaved	7.25 ± 2.44[c]	8.26 ± 1.19[d]	Nil
Fermented	28.82 ± 1.20	15.50 ± 1.44	Nil
Irradiated			
1 KGy	41.64 ± 2.65	17.63 ± 2.72	Nil
5 KGy	30.35 ± 1.65	15.77 ± 2.40	Nil
10 KGy	18.25 ± 1.55[d]	13.94 ± 1.75	Nil
50 KGy	7.10 ± 2.87[c]	4.10 ± 1.22[c]	Nil

[a]Values represent mean ± SD of triplicate estimations.
[b]Samples were refluxed for 4 h.
[c]Values are significantly different from the untreated at $P < 0.01$.
[d]Values are significantly different from the untreated at $P < 0.05$.

Complete removal of inhibitory activities was achieved by mechanical shaking in 2.4% HCl. This was confirmed by assaying the inhibitory activities in the residue. In the supernatant obtained after refluxing in 2.4% HCl, PI values were lower, but their absence in the residue confirmed their complete removal, thus indicating degradation of the inhibitors during reflux heating. A similar study on defatted Karanja seeds, refluxed in 2.0% HCl for 5 h, showed reduction of 75.3 and 26.5% in TIA and CIA, respectively (19). The difference in extractability, when compared to our results, appears to be based on the strength of the acid used and the chemical nature of the sample.

Inhibitor activities were absent in methanol extract, but a combination of methanol with HCl could reduce 50 and 48% of the TIA and CIA, respectively. The percentage of TIA and CIA in the solvent mixture acetone/hexane/water was comparatively less, 42 and 44%, respectively. Along with the antinutrients, negligible amounts of protein (0.4–3.6%) were also extracted in all solvents (Table 1).

Protease inhibitors are relatively heat-labile and can be degraded during the process of heat treatment (16). The reduction in TIA and CIA after 1 h autoclaving of Karanja cake was 83 and 55%, respectively. Kapoor and Gupta (27) have reported complete reduction of TIA in soybeans after 30 min of heating, while Nehad (28) reported only 29% reduction on autoclaving Faba beans for 40 min after 12 h of soaking. Variation in the loss of activity in the different samples with time of autoclaving may be due to the structural differences of the inhibitors, moisture content, and particle size of the sample (4).

Activation of the inherent enzymes during the process of fermentation results in degradation of the antinutrients (23,29). The results have shown that, compared to autoclaving (83 and 44%), fermentation was less effective in reducing TIA and CIA (34 and 15%, respectively).

The degradation of PI on exposure to gamma radiation was directly proportional to the radiation dose. CIA and TIA retained in the cake on exposure to 50 KGy dose was 22 and 16%, respectively. An irradiation study on soybeans reported 26% reduction of TIA, with CIA being marginally affected at dose exposure of 100 KGy (17). The study on safflower oil cake has shown complete loss of TIA at 42 Gy dose level, while 15.26% of the CIA remained, even after exposure to 10 KGy (18). This difference in the extent of degradation of PI in different samples on exposure to gamma radiation could be due to preliminary processing, which affects the chemical composition, and to the nature of the material. It is generally observed that, in complex materials, the radiation effect is less extensive than might be expected in pure components. From the overall results it is quite apparent that the percentage reduction in TIA was more than CIA, suggesting the latter to be more stable.

In vitro *digestibility.* The percentage of residual oil in the seed residue has been shown (Table 2) to influence protein digestibility. This is supported by the inverse relationship of digestibility and residual oil in the cake. Two different samples of Karanja cake, containing 5 and 15% oil, showed digestibility of 43 and 39%, respectively. Probably, the presence of oil-soluble antinutrients may be responsible for this variation.

Digestibility studies have confirmed the inactivation of PI. The results showed improvement in the digestibility ranging from 45 to 81%, with the greatest being in the refluxed residues. The improved digestibility on refluxing the sample in 2.4% HCl with or without methanol is probably due to the reduction in other antinutrients, such as phytate, lectins, and phenolics, which are known to influence digestibility (30–32).

The residue of methanol-treated samples, which otherwise failed to reduce PI, showed 37% improvement in digestibility. This is probably due to the removal of the fat-soluble phenolic compounds, thus supporting their apparent role in influencing protein digestibility (32). The digestibility of acetone/hexane/water-treated residue was also improved to 71%. Organic solvents not only reduce antinutrients but also affect protein conformation (32), whereas acid treatment hydrolyzes the protein. Hence, the effectiveness of solvent treatment in improving *in vitro* digestibility appears to be a collective one.

The improvement in digestibility of the autoclaved and fermented residues was 70 and 60%, respectively. This has been related to the degradation of the PI during heat treatment and reduction of phytate along with PI by the inherent enzymes that are activated during fermentation (29). The irradiated samples showed improvement in digestibility proportional to the dose of exposure. The digestibility obtained for the 50-KGy irradiated sample was 62%, compared to 58% for 10 KGy, which is the highest permissible dose (33).

The choice of a technique cannot be based just on the percentage reduction of antinutrients. When selecting a detoxification technique, changes in organoleptic properties and improved utilization have to be taken into account.

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TABLE 2
Effects of Various Treatments on *in vitro* Protein Digestibility[a]

Sample	Trypsin (g/100 g protein)	Pepsin followed by trypsin (g/100 g protein)	Pepsin followed by pancreatin (g/100 g)
Untreated[b]	39.00 ± 0.85	42.50 ± 1.78	42.77 ± 1.85
Untreated[c]	34.00 ± 0.88	37.50 ± 0.95	38.87 ± 1.65
Solvent-treated			
Phosphate buffer	48.85 ± 1.25	56.38 ± 1.56	n.d.
Borate buffer	41.80 ± 1.90	48.00 ± 1.88	n.d.
2.4% HCl	58.98 ± 1.85[f]	63.20 ± 1.45[f]	63.80 ± 2.45[f]
2.4% HCl[c]	68.38 ± 1.45[e]	80.80 ± 0.44[e]	80.77 ± 0.89[e]
Methanol/HCl[d]	60.03 ± 1.35[f]	n.d.	80.80 ± 1.88[e]
60% Methanol	45.65 ± 1.40	58.00 ± 1.56	58.70 ± 1.35
Acetone/hexane/water[d]	61.80 ± 2.40[f]	65.82 ± 1.08[f]	70.84 ± 1.75[e]
Autoclaved	51.00 ± 1.35	69.03 ± 1.28[e]	69.87 ± 1.30[e]
Fermented	49.55 ± 1.86	59.72 ± 2.10	60.33 ± 0.85
Irradiated			
1KGy	41.33 ± 1.28	45.00 ± 0.77	45.26 ± 2.72
5 KGy	48.50 ± 1.35	51.25 ± 1.59	51.90 ± 1.50
10 KGy	50.60 ± 2.65	57.00 ± 2.50	58.30 ± 2.38
50 KGy	58.33 ± 1.44[f]	60.18 ± 1.75	62.88 ± 1.50[f]

[a]Values represent mean ± SD of triplicate estimations.
[b]Sample contained 5% residual oil.
[c]Sample contained 15% residual oil.
[d]Samples were refluxed for 4 h.
[e]Values are significantly different from the untreated at $P < 0.01$.
[f]Values are significantly different from the untreated at $P < 0.05$. Abbreviation: n.d., not detected.

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