JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. **2010,** *58,* 4939–4944 **4939**
DOI:10.1021/jf904336k

Purification of *Azadirachta indica* Seed Cake and Its Impact on Nutritional and Antinutritional Factors

Mohanji Saxena, Kotagiri Ravikanth,* Abhishek Kumar,* Ashish Gupta, Brijpal Singh, and Anirudh Sharma

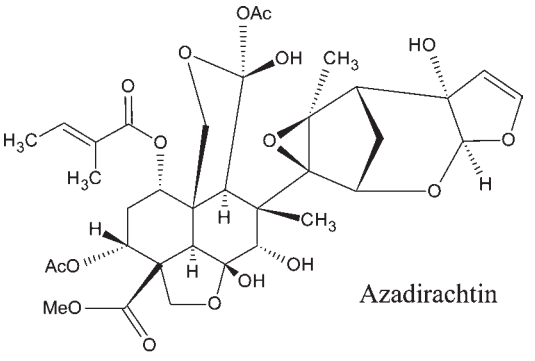
Phytochemistry and Analytical Laboratory, R and D Centre, Ayurvet Limited, Baddi, H.P., India

Azadirachta indica Juss. (family Meliaceae) is a vital plant with multiple agricultural and medicinal utilities. The seed cake after oil extraction can be a good source of nutrition in animal feed. The limitation to its use is the presence of azadirachtin, salannin, and other bitter constituents. To make it palatable for use as a source of animal nutrition it was detoxified using 50 and 80% methanol and was analyzed for contents of azadirachtin, salannin, and nutritional contents such as total carbohydrates, protein, crude fiber, in vitro protein digestibility, and trypsin inhibitor activity (TIA), prior to and after purification. The contents of azadirachtin and salannin were quantified using HPTLC and HPLC. Various validation parameters were also investigated. A highly significant decrease of antinutritional factor (TIA) was recorded after purification of samples, retaining the contents of protein, carbohydrates, crude fiber, and in vitro protein digestibility. The purified seed cake was found to be free of azadirachtin and salannin contents.

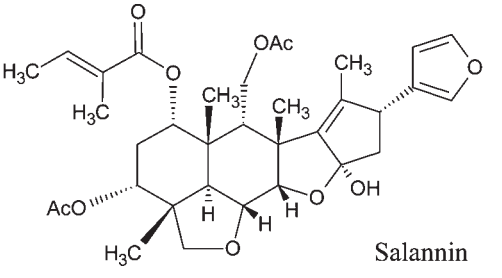
KEYWORDS: Purification; *Azadirachta indica*; nutritional factors; azadirachtin; salannin; seed cake

INTRODUCTION

Azadirachta indica Juss., commonly known as neem (Indian subcontinent), belongs to the family Meliaceae. The medicinal and commercial use of *A. indica* has been well-known. Each part of the neem tree has some medicinal property (*1*). Seeds of *A. indica* are a chief source of neem oil. Neem seed kernels are known to be very rich in oil, reaching 40−48.9% w/w, thus providing an excellent commercial value to the plant (*2*). The oil is a broad-spectrum pesticide, nematicide, fungicide, and miticide, and its cost-effective application makes it relevant as an organic pesticide. The remnant portion (marc), also known as neem spent seed cake, obtained as a byproduct after oil extraction is rich in true protein (80−90% of total protein). Apart from being rich in proteins it also has a relatively balanced amino acid profile (*3*). Due to its rich protein profile, the neem seed spent cake can be used as a nonconventional and alternative source of nutrition in animal feed. The limitation to its use as feed in the livestock and poultry sector is the presence of antinutrition or toxic principles, chiefly trypsin inhibitors, azadirachtin, salannin, and other bitter principles (*4*, *5*). Several attempts have been made to process the seed cake to enhance its nutritive value and palatability ever since the realization of its nutritive potential for livestock (*6*−*10*). Gowda and Sastry (*11*) reported the change in chemical composition of the neem seed cake depending on types of processing such as solvents or expeller extraction of whole or decorticated seed. James et al. (*12*) carried out a study to exhibit the effect of different concentrations of alcohols and water on the crude protein content (in vitro protein digestibility and amino acid profile). Studies carried out so far report the effect of various methods on different nutritive constituents and effect on livestock.



Azadirachtin



Salannin

However, a simple method to determine the concentration of toxic components in the seed cake remains to be worked out. The purpose of the present study was to develop a method for the

*Address correspondence to either author at the R and D Centre, Ayurvet Ltd., Vill: Katha, P.O. Baddi Distt. Solan, H.P., India [e-mail (A.K.) akumar@ayurvet.in or abhishekkumaronline@gmail.com; (K.R.) krk@ayurvet.in, telephone 01795247690, ext. 135].

Published on Web 03/10/2010

Table 1. Proximate Analysis for Antinutritive as well as Nutritive Factors in *Azadiractha indica* Seed Cake before and after Purification[a]

parameter evaluated	method	AI-1 before	AI-1 after	AI-2 before	AI-2 after	AI-3 before	AI-3 after	AI-4 before	AI-4 after
Antinutritional Factors									
trypsin inhibitor activity (TIA/g)	A	1880.2 ± 15.4	137.66 ± 1.45	1775.8 ± 12.4	143 ± 2.33	1675.5 ± 15.4	144.9 ± 3.23	1770.5 ± 15.4	138.33 ± 0.88
	B		108.5 ± 1.12		117.3 ± 1.92		123.9 ± 2.56		112.7 ± 2.07
Nutritional Factors									
total protein content (% w/w)	A	31.40 ± 2.19	44.0 ± 1.15	29.40 ± 3.43	47.76 ± 0.39	30.72 ± 2.87	46.7 ± 1.15	31.80 ± 3.56	45.5 ± 1.32
	B		56.84 ± 4.78		55.20 ± 5.24		57.52 ± 4.92		56.40 ± 5.34
carbohydrates (% w/w)	A	21.90 ± 3.28	17.66 ± 0.88	20.50 ± 2.56	17 ± 1.15	16.07 ± 1.89	16 ± 1.73	22.08 ± 3.65	17.33 ± 1.66
	B		19.64 ± 1.87		19.80 ± 2.22		18.79 ± 2.08		20.45 ± 2.67
crude fiber (% w/w)	A	8.32 ± 0.12	6.6 ± 0.38	7.56 ± 1.09	6.4 ± 0.52	7.87 ± 0.25	5 ± 0.58	8.45 ± 1.54	6.33 ± 0.44
	B		7.56 ± 0.78		7.03 ± 1.56		6.50 ± 0.82		5.09 ± 1.94
in vitro protein digestibility (% w/w)	A	60.20 ± 3.79	64.0 ± 2.30	61.54 ± 5.67	57.66 ± 1.45	59.80 ± 3.87	56 ± 1.73	58.76 ± 3.98	59.23 ± 0.96
	B		76.75 ± 4.23		72.32 ± 4.35		71.89 ± 4.06		74.62 ± 5.20

[a] Values are expressed as mean ± SE; $n = 3$.

Table 2. Percentage Azadirachtin and Salannin Contents in *Azadiractha indica* Samples Collected from Different Geographical Locations in India by HPTLC[a]

		azadirachtin (% w/w)			salannin (% w/w)		
			after purification			after purification	
sample code	collection place and region	before purification	method A	method B	before purification	method A	method B
AI-1	Gujarat (western India)	0.075 ± 0.0017	0.007 ± 0.0003	nd	0.383 ± 0.011	0.019 ± 0.0008	nd
AI-2	Karnataka (southern India)	0.089 ± 0.001	0.008 ± 0.0002	nd	0.326 ± 0.02	0.020 ± 0.0004	nd
AI-3	Madhya Pradesh (central India)	0.078 ± 0.002	0.008 ± 0.0001	nd	0.464 ± 0.014	0.022 ± 0.0008	nd
AI-4	Orissa (southeastern India)	0.092 ± 0.001	0.0089 ± 0.0002	nd	0.438 ± 0.011	0.022 ± 0.0005	nd

[a] Values are expressed as mean ± standard error (SEM); $n = 6$. nd, not determined.

purification of neem spent seed cake in light of existing studies and to analyze the various limiting factors such as azadirachtin, salannin, and trypsin inhibitor activity.

MATERIALS AND METHODS

Plant Material. Dried *A. indica* seeds were collected from different geographical locations in India. Voucher specimens (RD/AYU/04-09/15G; RD/AYU/04-09/15K; RD/AYU/04-09/15MP; RD/AYU/04-09/15ND) have been deposited in the Phytochemistry Department, R and D Centre, Ayurvet Ltd., Baddi.

Chemicals and Reagents. Precoated thin layer chromatographic plates were obtained from E. Merck (Darmstadt, Germany), HPLC grade methanol was from Rankem (Delhi, India), azadirachtin reference standard was from Sigma (St. Louis, MO), and salannin reference standard was from ChromaDex LGC Promochem (Bangalore, India). Hexane and methanol (LR grade) were supplied by Rankem, and trace analysis grade acetonitrile was supplied by Merck. All other chemicals were of analytical grade.

Preparation of Plant Material. Around 2 kg of *A. indica* seed kernels collected from different geographical locations was ground to obtain coarse powder (sieve no. 20). This was then defatted in a Soxhlet extractor using hexane. The exhausted (defatted) seed cake powder (1.245 kg) was used for further processing.

Purification of Seed Cake. *Method A.* The defatted seed cake obtained above was soaked in 2 L of "aqueous" methanol (50% methanol) for 6 h and extracted for 24 h using aqueous methanol (50%) in a Soxhlet extractor. The spent marc so obtained was dried in a hot air oven at 40−50 °C until constant weight. The defatted and detoxified seed cake was then analyzed.

Method B. The defatted seed cake obtained above was soaked in 2 L of methanol (80% methanol) for 6 h and extracted for 24 h using aqueous methanol (80%) in a Soxhlet extractor. The spent marc so obtained was dried in a hot air oven at 40−50 °C until constant weight. The defatted and detoxified seed cake was then analyzed.

Determination of Azadirachtin and Salannin by HPTLC and HPLC. *Instrumentation.* HPTLC analysis was performed with a Camag (Muttenz, Switzerland) HPTLC system equipped with a Linomat V applicator under nitrogen flow, a twin-trough chamber, a model 3 scanner, and winCATS integration software (version 1.4.1). The samples were resolved on precoated silica gel GF_{254} TLC plates using hexane/ethyl acetate (25:75 v/v) as mobile phase. HPLC analysis was performed on a Waters (Milford, MA) HPLC system equipped with a control module II, pump 515, 20 μL loop injector, PDA detector 2996, and LiChrospher C_{18} column (250 × 4.6 mm i.d., 5 μm; Merck) using an isocratic run of acetonitrile/water (6:4 v/v) as the mobile phase at a flow rate 1 mL /min. Empower 2 software was used for both data collection and integration. Evaluation was done using peak area and linear regression.

Preparation of Standard and Test Solution. About 2.0 mg of accurately weighed azadirachtin and salannin reference standards was dissolved in a small volume of acetonitrile (HPLC grade) in separate volumetric flasks (10 mL), and the volume was made up to the mark. Using these stock solutions further dilutions were made with the various concentration range (50, 70, 80, 110, 130, 160, 180, and 200 ppm) for the determination of the standard curve, limit of detection (LOD), and limit of quantification (LOQ).

The test solution was prepared by refluxing about 1.0 g of accurately weighed sample (powder) with methanol (15 mL; three times) on a water bath. The extract was then filtered, and the volume was made up to the mark in a 50 mL volumetric flask using methanol. An aliquot (10 μL) of the solution was applied on a TLC plate for HPTLC analysis.

For analysis using HPLC, the crude extract (test solution) obtained after refluxing was dried in vacuo and reconstituted in methanol (HPLC grade).

Validation of the Quantification Method. Validation parameters linearity, accuracy, and precision were determined according to the statistical method of validation ICHQ2R1 (*13*).

Accuracy. Three samples from the same batch were extracted with the said method, in parallel.

Table 3. Percentage Azadirachtin and Salannin Contents in *Azadirachta indica* Samples Collected from Different Geographical Locations in India by HPLC[a]

		azadirachtin (%w/w)			salannin (%w/w)		
			after purification			after purification	
sample code	collection place and region	before purification	method A	method B	before purification	method A	method B
AI-1	Gujarat (western India)	0.08 ± 0.001	0.008 ± 0.000	nd	0.40 ± 0.010	0.026 ± 0.002	nd
AI-2	Karnataka (southern India)	0.09 ± 0.001	0.008 ± 0.0001	nd	0.34 ± 0.022	0.032 ± 0.004	nd
AI-3	Madhya Pradesh (central India)	0.08 ± 0.002	0.008 ± 0.000	nd	0.48 ± 0.01	0.031 ± 0.001	nd
AI-4	Orrisa (southeastern India)	0.095 ± 0.001	0.009 ± 0.00	nd	0.462 ± 0.01	0.032 ± 0.002	nd

[a] Values are expressed as mean ± standard error (SEM); $n = 6$. nd, not determined.

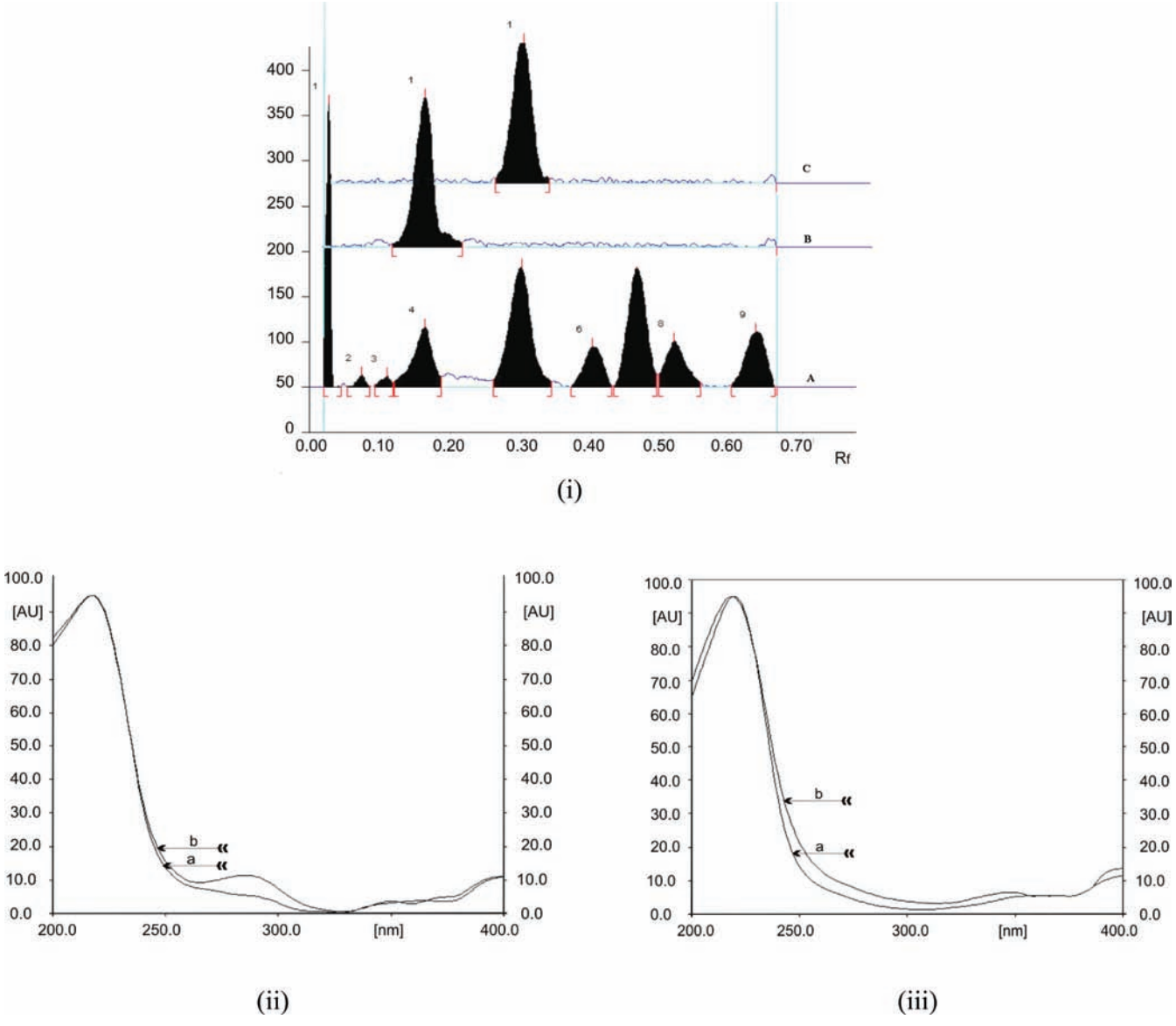


Figure 1. HPTLC chromatogram of methanolic extract of the spent neem seed cake showing the presence of azadirachtin and salannin at 220 nm: (top) chromatogram showing azadirachtin (B), salannin (C), and neem seed extract (A); (bottom left) overlay UV spectra of salannin (a) and seed cake extract; (bottom right) overlay UV spectra of the azadirachtin (a) and seed cake extract.

Precision. Intra day precision: A sample of the marker to be quantified was subjected to analysis on HPLC, six times in the same day for intraday variation.

Interday precision: Interday precision was performed by injecting a freshly prepared concentration of the marker compound on day 1 and day 4.

Recovery. A sample of the crude powder was spiked with known quantities of standards and worked out with the established method. The percentage recovery of standards was computed from the regression equation.

Determination of Antinutritional Factors. Trypsin inhibitory activity was determined using the method of Chitra and Sadasivam (*14*). About 0−1 mL of accurately measured extract was transferred in duplicate sets of test tube (to serve as endogenous (E) and test). Endogenous and test sets were diluted to 2 and 1 mL, respectively, with buffer (Tris-HCl buffer, pH 8.2). One milliliter of trypsin solution (solution of 2 mg of lyophilized trypsin in 25 mL of 0.0001 M hydrochloric acid) was added to each test tube. One milliliter of buffer and 1 mL of trypsin solution was pipetted out to work as standard (S). The test tubes were incubated in a water bath

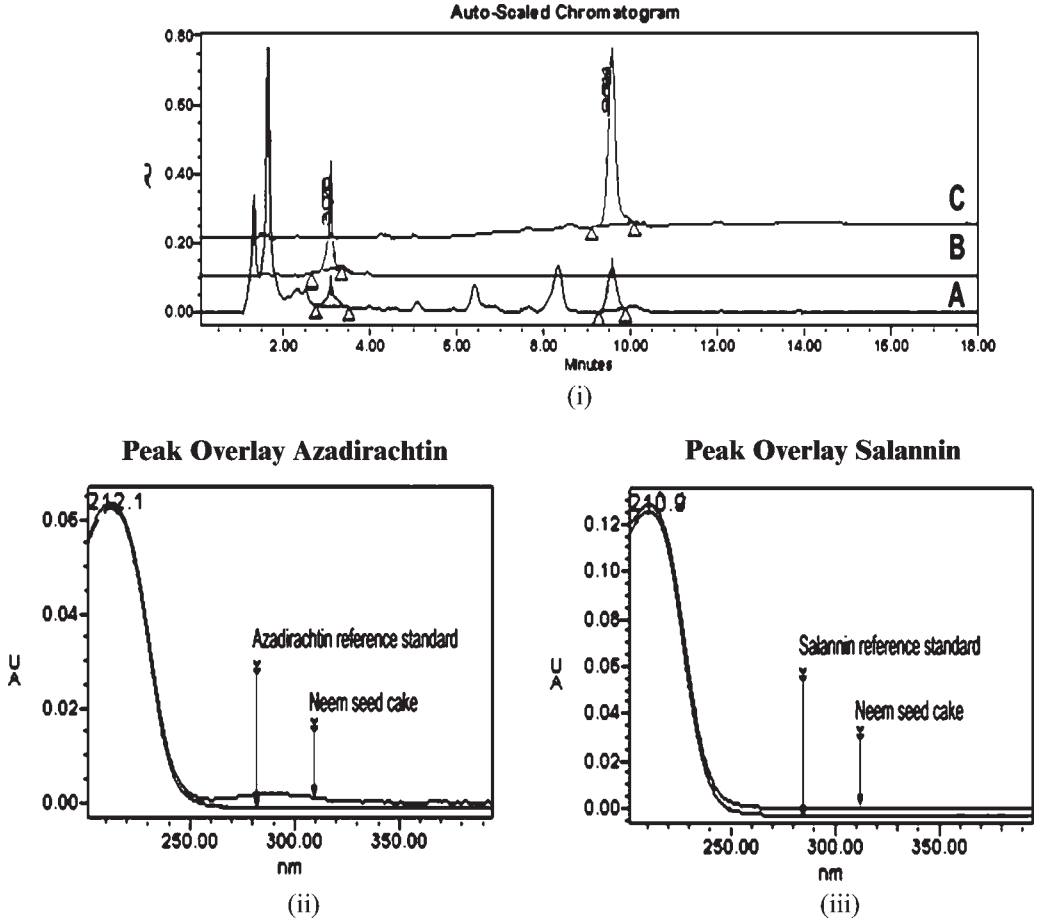


Figure 2. HPLC chromatogram of methanolic extract of the spent neem seed cake showing the presence of azadirachtin and salannin at 217 nm: (top) chromatogram showing azadirachtin (B), salannin (C), and neem seed extract (A); (bottom left) overlay UV spectra of azadirachtin (a) and seed cake extract; (bottom right) overlay UV spectra of salannin (a) and seed cake extract.

at 37 °C. Substrate (1 mg of benzoyl-DL-arginine-paranitroanilide (BAPNA), 2.5 mL) was added to each tube, and the reaction was allowed to proceed for 10−60 min at 37 °C. The reaction was stopped by adding 0.5 mL of 30% GAA. The absorbance was read at 410 nm in a UV spectrophotometer. The protein content was determined by using VELP Scientifica protein analyzer.

Determination of Nutritional Factors. *Carbohydrate Contents.* The contents in the sample were determined according to the anthrone method (*15*). About 100 mg of accurately weighed sample was transferred in a boiling tube. The contents of the tube were hydrolyzed by the addition of 5 mL of 2.5 N hydrochloric acid and heating in a boiling water bath for 3 h. After the tube had cooled to room temperature, the contents were neutralized by adding solid sodium carbonate until the effervescence ceased. The volume was made up to 100 mL. It was then centrifuged and the supernatant collected; 0.5 and 1 mL of aliquots from this solution were used for analysis.

Anthrone reagent (4 mL; 20 mg/10 mL of ice-cold sulfuric acid) was added to different concentrations of the standard (10 mg/100 mL of glucose) and heated on a water bath for 8 min. After rapid cooling, the color (green to dark green) of the solution was read on a UV spectrophotometer at 630 nm. The standard curve was plotted against the concentration and the absorbance.

The amount of carbohydrate present in the sample was calculated as

$$\text{carbohydrate present in 100 mg of sample} = \frac{\text{glucose (mg)} \times 100}{\text{volume of test sample}}$$

In Vitro Protein Digestibility. Accurately weighed powdered sample was mixed with water (10 mL) so as to contain 6.25 mg of protein/mL. The sample was allowed to hydrate for at least 1 h (not longer than 25 h at 5 °C) and equilibrated to pH 8 at 37 °C. In parallel, the three-enzyme solution (TES; 1.6 mg of trypsin, 3.1 mg of chymotrypsin, and 1.3 mg of peptidase/mL of water) was also equilibrated to pH 8 at 37 °C. One milliliter of TES was added to the sample suspension and stirred while being held at 37 °C. After exactly 10 min from the time of addition of TES, 1 mL of bacterial protease solution (7.59 mg of protease/mL of water) was added. The solution was immediately transferred to 55 °C on a water bath. Nine minutes after the addition of the bacterial enzymes, the solution was transferred back to the 37 °C water bath. The pH of the hydrolysate exactly 10 min after the addition of bacterial enzyme was measured (*16*, *17*).

$$\%\ \text{in vitro protein digestibility} = 234.84 - 22.56X$$

$$X = \text{pH after 20 min of incubation}$$

Determination of Total Proteins. Total protein contents were determined with the help of VELP Scientifica protein analyzer (Brinkmann Instruments, Inc., VELP Scientifica UDK 152) (*18*).

RESULTS AND DISCUSSION

Spent seed cakes being a good source of proteins have been in use as a source of nutrition in animal feed since time immemorial. Spent neem seed cakes have been reported to possess a relatively balanced amino acid profile (*3*) and rich protein contents. As said earlier, the bitter principles, chiefly azadirachtins, salannin, and other toxic components, limit its use as an animal feed. A relatively better nutritional profile of seed cake was observed after treatment of the spent cake with methanol at a concentration of around 75% (*19*). The researchers demonstrated the effect of various solvents on the crude protein contents and amino acid profile of the seed cake. However, they did not report the presence/contents of toxic components in the seed cake.

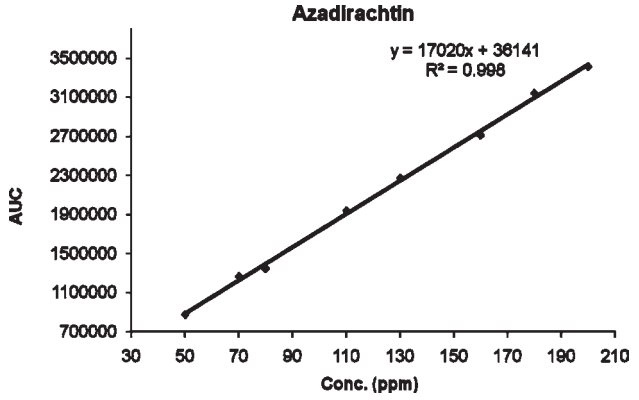


Figure 3. Standard curve plot of azadirachtin obtained using HPLC.

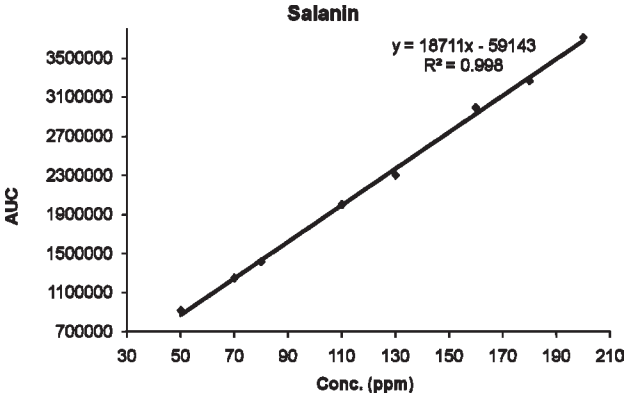


Figure 5. Standard curve plot of salannin obtained using HPLC.

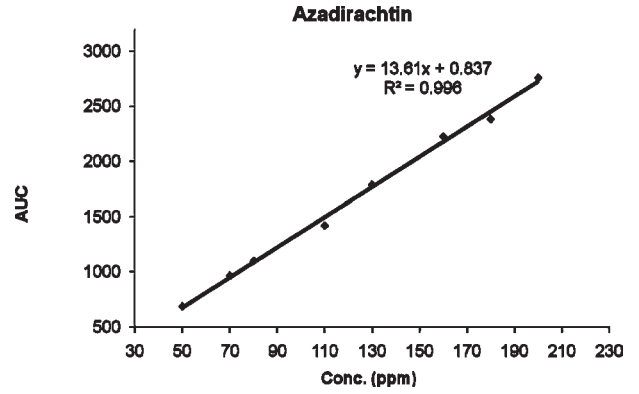


Figure 4. Standard curve plot of azadirachtin obtained using HPTLC.

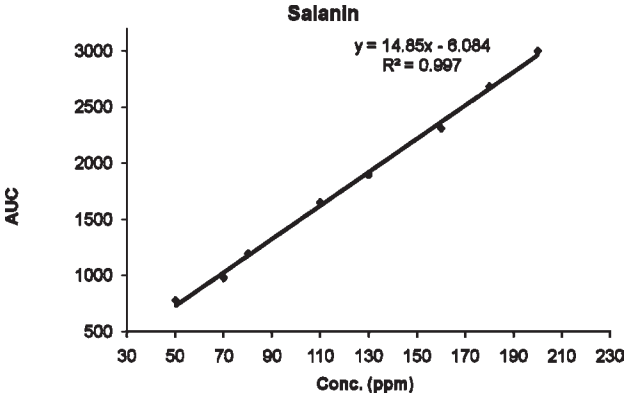


Figure 6. Standard curve plot of salannin obtained using HPTLC.

On the basis of the previous studies, the present paper provides a process of purification and different chromatographic methods to analyze the various toxic components. It has been reported that these toxic compounds are slightly soluble in water and are freely soluble in organic solvents such as hydrocarbons, alcohols, and ketones (*20*), and a better nutritional profile had been achieved with methanol at 75% (*19*).

In the present study, the seed cake after defatting was treated with methanol at different concentration levels. The seed cake was analyzed for contents of azadirachtin, salannin, and nutritional contents such as total carbohydrates, protein, crude fiber, in vitro protein digestibility, and trypsin inhibitor activity (TIA), prior to and after purification.

This study also determined the effect of solvents on the protein contents as well the ability of the media to detoxify the seed cake.

Nutritional factors such as total protein contents were enriched with the removal of bitter constituents after processing, thereby raising the total contents (% w/w). The results of the nutritional factors (carbohydrates, protein, crude fiber, and in vitro protein digestibility) are given **Table 1**.

Enhanced in vitro protein digestibility values were obtained by processing with 80% methanol (method B). However, there was no significant difference observed among the initial values and those obtained after processing with 50% methanol.

A negligible difference in the values of total carbohydrate contents was observed after treatment with either method. A slight decrease in the values of crude fibers was also noted. However, the decrease was not significant (**Table 1**).

The highly significant decrease in the TIA values was found in the seed cakes processed with different solvent ratios (methods A and B). A significant difference in the values was observed between the two methods used for processing (**Table 1**).

The results from **Tables 2** and **3** show the initial levels of azadirachtin and salannin, procured from different geographical zones of India. When analyzed after purification, the samples showed the absence of azadirachtin and salannin contents on processing with method B (80% methanol). However, traces of both were found in the seed cake processed with 50% methanol. The peaks in the extract were identified by comparison of UV profiles and retention times/retention factors (**Figures 1** and **2**). The figures show the overlay chromatogram of the azadirachtin, salannin, and methanolic extract of the untreated seed cake when analyzed on HPLC and HPTLC, respectively. The contents of azadirachtin (**Figures 3** and **4**) and salannin (**Figures 5** and **6**) were quantified using a standard curve.

The details of the method precision (interday and intraday) and linearity are shown in **Table 4**. The details of accuracy (% recovery) are given in **Tables 5** and **6**. The limits of detection defined as the S/N ratio of 3:1 were found to be 8 and 9 ng for azadirachtin and salannin, respectively, and the limits of quantification defined as the S/N ratio of 10:1 were calculated as 29 and 32 ng for azadirachtin and salannin, respectively, when analyzed on HPLC.

In light of previous studies (*19*) and from the present experiments it was found that the neem seed spent cake can be utilized for the purpose of animal nutrition after processing with methanol (80%). However, as reported earlier (*19*), compared to 80% methanol there was no significant difference observed in the values of various parameters when the seed cake was treated with 75% methanol. Furthermore, the studies need to be confirmed for safety in vivo, before the exploitation of the seed cake on a commercial scale.

Table 4. Regression Equations and Their Correlation Factors for Quantitative Analysis of Different Standards

mode of analysis	compound	range of concentration (ppm)	equation of regression line[a]	correlation coefficient (R^2)	RSD (%) day 1	RSD (%) day 3
HPLC	azadirachtin	50−200	$Y = 17020x + 36141$	0.998	0.467	0.450
	salannin	50−200	$Y = 18711x - 59143$	0.998	0.426	0.868
HPTLC	azadirachtin	50−200	$Y = 13.61x + 0.837$	0.996	0.846	0.924
	salannin	50−200	$Y = 14.85x - 6.084$	0.997	0.782	0.967

[a] Y = peak area response; x = amount of standard.

Table 5. Recovery Study of Marker Compound by Proposed HPTLC Method[a]

marker compound	amount present in cake (mg/g) (A)	amount added (mg/g) (B)	amount quantified (mg/g)	recovery (%w/w)	average recovery (% w/w)
azadirachtin	0.75	0.375	1.125 ± 0.54	100.20 ± 0.25	99.91
	0.75	0.750	1.500 ± 0.78	99.85 ± 0.09	
	0.75	1.125	2.625 ± 0.49	99.69 ± 0.14	
salannin	3.86	1.93	5.790 ± 0.26	98.83 ± 0.15	99.29
	3.86	3.86	7.720 ± 0.45	98.68 ± 0.20	
	3.86	5.79	9.650 ± 0.30	100.35 ± 0.08	

[a] Values are expressed as mean ± standard deviation (SD); n = 3.

Table 6. Recovery Study of Marker Compound by Proposed HPLC Method[a]

marker compound	amount present in cake (mg/g) (A)	amount added (mg/g) (B)	amount quantified (mg/g)	recovery (%w/w)	average recovery (%w/w)
azadirachtin	0.75	0.375	1.125 ± 0.54	100.02 ± 0.25	99.91
	0.75	0.750	1.500 ± 0.78	100.01 ± 0.09	
	0.75	1.125	2.625 ± 0.49	99.70 ± 0.14	
salannin	3.86	1.93	5.790 ± 0.26	98.80 ± 0.15	99.29
	3.86	3.86	7.720 ± 0.45	99.02 ± 0.20	
	3.86	5.79	9.650 ± 0.30	100.05 ± 0.08	

[a] Values are expressed as mean ± standard deviation (SD); n = 3.

LITERATURE CITED

(1) Biswas, K.; Chattopadhyay, I.; Ranajit, K.; Banerjee, R. K.; Bandyopadhyaya, U. Biological activities and medicinal properties of neem (*Azadirachta indica*). *Curr. Sci.* **2002**, *82* (11), 1336–1345.

(2) *The Wealth of India*; Publication and Information Directorate CSIR: New Delhi, India, 2004; Vol. 1, A-Ci, 107 pp.

(3) Tewari, D. M. *Monograph of Neem*; International Book Distributor: Dehradun, India, 1992; pp 105−135.

(4) Devakumar, C.; Mukerjee, S. K. Chemistry of neem bitter principles. *IARI Res. Bull.* **1983**, *40*, 1−17 pp.

(5) Devakumar, C.; Dev, S. Chemistry. In *Neem Research and Development*; Randhawa, N. S., Parmar B. S., Eds.; Society of Pesticide Science: New Delhi, India, 1993; pp 63−96.

(6) Agrawal, D. K.; Garg, A. K.; Nath, K. The use of water washed neem (*Azadirachta indica*) kernel cake in the feeding of buffalo calves. *J. Agric. Sci.* (*Cambridge*) **1987**, *108*, 497–499.

(7) Chand, S. *Nutritional evaluation of neem seed meal in chicks*. Ph.D Thesis, Rohilkhand University, Bareilly, India, 1987; pp 66−106.

(8) Katiyar, R. C.; Sastry, V. V. B.; Agrawal, D. K. Urea and alkali treated neem seed kernel cake as livestock feed. In *Annual Report Animal Nutrition Division*; IVRI: Izatnagar, India, 1991.

(9) Musalia, L. M.; Anandan, S.; Sastry, V. R. B.; Agrawal, D. K. Urea-treated neem (*Azadirachta indica*) seed kernel cake as a protein supplement for lambs. *Small Ruminant Res.* **2000**, *35*, 107–116.

(10) Rao, V. K.; Kowale, B. N.; Verma, A. K. Effect of feeding water washed neem *Azadirachta indica* seed kernel cake on the quality lipid profile and fatty acid composition of goat meat. *Small Ruminant Res.* **2003**, *47*, 213−219.

(11) Gowda, S. K.; Sastry, V. R. B. Neem (*Azadirachta indica*) seed cake in animal feeding scope and limitations − review. *Asian-Aust. J. Anim. Sci.* **2000**, *13*, 5–5.

(12) James, D. B.; Ameh, D. A.; Agbaji, A. S. Effect of solvent extraction on crude protein, in vitro protein digestibility and amino acid profile of neem seed cake. *Asian J. Anim. Vet. Adv.* **2007**, *2* (2), 81−85.

(13) http://www.ich.org/LOB/media/MEDIA417.pdf.

(14) Chitra, R.; Sadasivam, S. A study of the trypsin inhibitor of blackgram (*Vigna mungo*). *Food Chem.* **1986**, *21*, 315–320.

(15) Hedge, J. E.; Hofreiter, B. T. In *Carbohydrate Chemistry 17*; Whistler, R. L., BeMiller, J. N., Eds.; Academic Press: New York, 1962.

(16) Hsu, H. W.; Sutton, N. E.; Banjo, M. O.; Satterlee, L. D.; Kendrick, J. G. *Food Technol.* **1978**, *32*, 69.

(17) Satterlee, L. D.; Marshall, H. F.; Tennyson, J. M. *J. Am. Oil Chem. Soc.* **1979**, *56*, 103.

(18) http://www.metrohmusa.com/utilities/download_pdf.asp?DR = downloads%2Fpdf%2Fapplications%2FASandFN = 0802-W102B.pdf.

(19) James, D. B.; Ameh, D. A.; Agbaji, A. S. Effect of solvent extraction on crude protein, invitro digestibility and amino acid profile of neem seed cake. *Asian J. Anim. Vet. Adv.* **2007**, *2* (2), 81–85.

(20) National Research Council. *Neem: A Tree for Solving Global Problems*; National Academic Press: Washington, DC, 1992.

Received for review December 8, 2009. Revised manuscript received February 15, 2010. Accepted February 22, 2010. We acknowledge the Department of Biotechnology, New Delhi, for funding the research project BT/PR9097/AAQ/01/328/2007.