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# Use of Chemical Treatments to Reduce Tannins and Trypsin Inhibitor Contents in Salseed (*Shorea robusta*) Meal

**Mahmood, S.[1], M. Ajmal Khan[2,*], M. Sarwar[3], M. Nisa[3], W. S. Lee[2], S. B. Kim[2], T. Y. Hur[2] H. J. Lee[2] and H. S. Kim[2]**

[1] Department of Poultry Husbandry, University of Agriculture, Faisalabad, Pakistan

**ABSTRACT :** This study investigated the effect of chemical treatments on tannins (condensed and hydrolysable) and on the trypsin inhibitor (TI) activity in salseed meal. Triplicate samples of ground salseed meal (1 kg) were mixed with 820 ml of either distilled water (pH 5.3), 0.67 M acetic acid (pH 2.4), 0.67 M sodium bicarbonate (pH 8.2) or 2% polyvinyl-pyrrolidone (PVP) solution. The material was placed in airtight plastic containers and incubated at 37°C for 0, 3, 6, 12, 24, 48 and 72 h. Samples of untreated salseed meal which had not been subjected to soaking or incubation were run through the analysis to serve as control. Addition of water, acetic acid, sodium bicarbonate and PVP solutions to salseed meal and subsequent anaerobic incubation at 37°C significantly reduced chemically detectable tannins. At each incubation time, alkali solution was more effective than its counterparts. The effect of acidic solution on hydrolysable tannin was least among the treatments. All the treatments reduced TI activity of salseed meal. The reduction in TI activity by these treatments was similar and ranged between 80-84%. Treatment time effected a decrease in the contents of antinutritional substances. However, the effect of the treatment with the reagents, even for zero incubation time, was quite pronounced. It may be concluded from the present results that the treatment of salseed meal with sodium bicarbonate (0.67 M) is more effective in reducing hydrolysable and condensed tannin contents than PVP, water and acid solutions. Treatment with sodium bicarbonate solution is more economical and easier to handle than acid and PVP treatments. Incubation of the treated material for 12 h is reasonably effective, economical and safe from any mould growth. (**Key Words :** Trypsin Inhibitor, Condensed Tannins, Hydrolysable Tannins)

## INTRODUCTION

Tannins are the polyphenolic compounds with various molecular weights and of varying complexity. They are present in a large number of products of vegetable origin used as human foods or animal feeds (Evers et al., 1999; Kamalak et al., 2005; Rana et al., 2006). Their multiple phenolic hydroxyl groups lead to the formation of complexes with proteins, metal ions, and other macromolecules like polysaccharides (Choct et al., 1999; Ozkan and Sahin, 2006; Kondo et al., 2007). Many previous studies have shown that tannins in the diet result in reduced weight gain and poor feed efficiencies in chicken (Ahmed et al., 1991; Santos-Buelga and Scalbertm, 2000), rats (Elkin et al., 1990; Mole et al., 1990) and pigs (Mitaru et al., 1984). Our previous study (Mahmood et al., 1997; Mahmood et al., 2006) has indicated that digestive capacity and growth of chicken were depressed when diets containing salseed meal were given to birds; the major contribution was due to the presence of tannins in the diets. Trypsin inhibitor (TI) is another antinutritional component in many legumes. It is a small protein (21 kDa) which inhibits the digestive enzyme trypsin with very high specificity and thereby impairs digestive functions in the lower gut (Susmel et al., 1995)

Several treatments have been employed to reduce or eliminate antinutritional effects associated with tannins and TI. The methods commonly used are physical removal of testa (Chibber et al., 1978), soaking in water and aqueous alkali or acid solutions (Reichert et al., 1980), ammoniation (Gandhi et al., 1975), addition of tannin binding agents (Marquardt, 1989), and anaerobic storage of the ingredients (Teeter et al., 1986). Mechanical abrasion of seed coat in legume seeds and cereal grains has been shown to reduce tannin content; however, it also results in increased losses

* Corresponding Author: M. Ajmal Khan. Tel: +82-41-580-3397, Fax: +82-41-580-3419, E-mail: ajmals1@yahoo.com

[2] Dairy Cattle Research Division, National Institute of Animal Science, Cheonan, South Korea.

[3] Institute of Animal Nutrition and Feed Technology, University of Agriculture, Faisalabad, Pakistan.

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of protein content of the material (Eggum et al., 1983). Soaking seeds/grains in alkali solutions (Reichert et al., 1980) have been reported to overcome the harmful effects of tannins in seeds. However, most of the treatments are laborious, expensive and less effective (Salunkhe et al., 1990). Therefore, it is worthwhile looking for inexpensive, mild solutions for the treatment of high tannin materials that are of practical significance even in less developed countries.

Salseed meal is produced from the nuts (seed) of the Sal (*Shorea robusta*), which is a tall (35 m) tree widely grown for timber along the Himalayan foothill belt in India and South Asia. Botanical family of Sal is *Dipterocarpaceae*. The kernel of its nut contains about 15-18% oil, which is removed and used locally in food or sold to the worldwide confectionery industry. The salseed meal is either used by local feed industry or exported to European markets to be used for animal feeding. The presence of salseed meal tannins in the diets of fowl results in lowered digestibility of protein, reduced activities of digestive enzymes (trypsin, chymotrypsin, α-amylase, dipeptidase and disaccharidases) in the gut lumen and increase in the relative weight of the pancreas (Mahmood et al., 1997; Mahmood et al., 2006; Mahmood et al., 2007).

This study investigated the effect of chemical treatments on tannins (condensed and hydrolysable) and on the TI activity in salseed meal.

## MATERIALS AND METHODS

### Treatment of salseed meal

Salseed meal was treated with water, acetic acid, sodium bicarbonate, and polyvinyl-pyrrolidone (PVP). Triplicate samples of ground salseed meal (I kg) were thoroughly mixed with either of 820 ml distilled-water (pH 5.3), 0.67 M acetic acid (pH 2.4), 0.67 M sodium bicarbonate (pH 8.2) or 2% PVP solution. The material was placed in airtight plastic containers and incubated at 37°C for 0, 3, 6, 12, 24, 48, and 72 h (h) in a thermostatically controlled forced-draft oven. After incubation each container was opened and dried in the same oven at 37°C with occasional stirring of the contents. The zero time incubation samples were subjected to moistening with the treatment solution and immediately placed in the oven to start drying. Samples of untreated salseed meal which had not been subjected to soaking or incubation were run through the analysis to serve as control.

### Determination of antinutritional substances in salseed meal

Hydrolysed tannin, condensed tannin, and trypsin inhibitor (TI) contents of treated or untreated salseed meal were estimated using chemical techniques. The salseed meal was finely ground prior to analysis for its antinutritional substance. Details of each of the chemical method employed are given below.

### Condensed tannins

Modified Vanillin-HCl method (Price et al., 1978) was used for the estimation of condensed tannins. A representative sample of salseed meal was extracted for 1 hour with acidified methanol. The extract was centrifuged at 2,500 rpm for 15 minutes and the resultant supernatant was immediately analyzed. Vanillin dissolved in acidified methanol was used as reagent. Twenty minutes after the addition of the reagent to the sample aliquots, the absorbance of the colour developed was measured spectrophotometrically at 500 nm. A portion of supernatant from each sample was mixed with 4% HCl-methanol solution to serve as blank. Catechin was used as standard to compare the values of the samples.

### Hydrolysable tannins

The dye-labelled protein precipitation method (Asquith and Butler, 1985) was used to estimate the hydrolysable tannin contents of treated or untreated salseed meal. Bovine serum albumin (BSA) covalently bound to the dye Remazol Brilliant Blue was used as dye-labelled protein. Representative samples of the meal were extracted with aqueous methanol (50% v/v) for 1 h. The extracts were centrifuged at 2,500 rpm for 15 minutes and the resultant supernatant was immediately analysed for the hydrolysable tannin.

Aliquots of the supernatants were mixed with dye-labelled BSA and allowed to stand for 15 minutes. During this time a tannin-protein complex was formed, the precipitate formed was separated by centrifugation at 2500 rpm for 20 minutes and the supernatant discarded. This precipitate was dissolved in sodium dodecyl sulphate solution and the absorbance of the coloured solution was read at 590 nm. A tube with extracting solution, for each sample was run through the method to serve as a blank. A series of standard solutions containing 0.2-1.0 mg tannic acid per ml aqueous methanol was run through the method to compare the values of the samples.

### Trypsin inhibitor activity

Treated or untreated samples of salseed meal were extracted with water for determination of their trypsin inhibitor activity. The extracts were centrifuged at 2,500 rpm for 20 minutes and the supernatant retained. The supernatants of the extracts were diluted with tris-buffer and centrifuged again to remove the precipitated material from the extracts. Supernatant from the diluted samples were used for TI determination according to the method of Liu and Markakis (1989). Porcine trypsin was used as standard

**Table 1.** Effect of water, acid, alkali and polyvinyl-pyrrolidone (PVP) treatments on hydrolysable tannin, condensed tannin and trypsin inhibitor activity of salseed meal (DM basis)

| Variables | Treatments | | | | | SEM |
|---|---|---|---|---|---|---|
| | USSM[1] | Water | Acid | Alkali | PVP | |
| Hydrolysable tannin (%) | 6.83[a] | 3.41[c] | 4.13[b] | 2.28[e] | 2.80[d] | 0.03 |
| Condensed tannin (%) | 1.24[a] | 0.84[b] | 0.73[c] | 0.22[d] | 0.72[c] | 0.01 |
| Trypsin inhibitor (units/g) | 0.96[a] | 0.16b[c] | 0.17[bc] | 0.15[c] | 0.19[b] | 0.01 |

Means in the same row followed by the different superscript letters are significantly different ($p<0.05$).
SEM is among treatment means. [1] Untreated salseed meal.

and the antitryptic activity of the sample was calculated in terms of trypsin units inhibited (TUI)/g of sample.

**Statistical analysis**

The data were subjected to one way and two way analysis of variance. One way analysis of variance was used to test the differences between treatments within an incubation time or to calculate differences due to incubation time within each treatment. Pooled data for each treatment and incubation time were analyzed using two way analysis of variance. Treatment means were compared using Duncan's multiple range test (Steel and Torrie, 1984).

## RESULTS AND DISCUSSION

Addition of water, acetic acid, sodium bicarbonate, and PVP solutions to salseed meal and subsequent anaerobic incubation at 37°C significantly reduced ($p<0.05$) its tannin and TI contents (Table 1). Treatment with alkali solution appeared to be the most effective, followed by PVP, water and acid treatments. The exact mechanism of tannin deactivation is not known. These chemical assays do not distinguish between a genuine biological deactivation and mere transformation to a form which does not respond to the chemistry of the assay. Biological effects must be assessed through animal experiments. It is possible that tannin may undergo oxidation under alkaline conditions to be converted to inert forms (Swain, 1965; Reichert et al., 1980). Several investigations have demonstrated beneficial effects of soaking of high tannin ingredients in alkaline solutions (Chavan et al., 1979; Laurena et al., 1986; Mohammed and Ali, 1988). Salunkhe et al. (1990) concluded that alkali solutions were generally more efficient in deactivating tannins than acidic or ash treatments. In the present study alkali treatment reduced the total tannin contents of salseed meal by 83% when incubated for 72 h. These results are comparable with the findings of Wah et al. (1977) who observed a similar reduction in tannin content of salseed meal when treated with alkaline solution (NaOH) but 37% dry matter was lost when the treated material was subjected to extensive washing with water. Rao and Rao (1986) have also reported 20-71% and 60% losses in dry matter of salseed meal as a result of washing after soaking in sodium hydroxide and sodium carbonate, respectively. These losses in dry matter are undesirable because they lower nutritional quality of feed ingredients due to loss in appreciable amount of protein being extracted with tannin because tannins have greater affinity for protein due to strong hydrogen bonding with carboxyl oxygen of peptide group (Evers et al., 1999; Kumar et al., 2005). Sathe and Salunkhe (1981) reported 21 and 27% losses of protein in winged beans flour soaked in NaOH and KOH solutions due to washing. In this investigation as the treatment solution was merely added to the material and was oven-dried rather than washing with water, therefore, chances of dry-matter loss by leaching were almost negligible. In addition to that soaking of the material requires a large volume of solution which may increase the cost of chemical and subsequent drying. Moreover, NaOH is 56% more expensive than $NaHCO_3$ (Mahmood et al., 1997) and needs more expertise in handling. Therefore, treatment of salseed meal with $NaHCO_3$ may be more useful than NaOH or other expensive alkalis.

Treatment time exhibited a decrease in the contents of hydrolysable and condensed tannins of salseed meal (Table 2 and 3). However, the effect of the treatment with the reagents, even for zero incubation time, was quite pronounced; the fall after that time was gradual. At each incubation time, alkali solution was more effective than other treatments. However, at 12 h incubation and onward the effect on hydrolysable tannin was noticed similar between alkali and PVP treatment. The effect of acidic solution on hydrolysable tannin was least among the treatments. Acetic acid and PVP solution had similar effects on condensed tannins at each incubation time. It is notable that deactivation by acid was less than that achieved by alkali treatment. It is possible that some of this difference may be due to different affinities of phenol (-OH) groups in the acid treated material and phenate ($-O^-$) groups in the alkali treated material. It is likely that under such conditions hydrolysable tannin may be hydrolysed resulting into breakdown of the tannins into smaller units which do not have tannin like effect. Alternatively condensed tannin might undergo polymerization in diluted acid medium (Marquardt, 1989), resulting in formation of high polymers which may be insoluble because of their size and lose their ability to precipitate protein (Ahmed, 1991). According to

**Table 2.** Effect of water, acid, alkali and polyvinyl-pyrrolidone (PVP) treatments within each incubation times on hydrolysable tannin contents (% of DM) of salseed meal

| Incubation time (h) | Treatments | | | | SEM |
|---|---|---|---|---|---|
| | Water | Acid | Alkali | PVP | |
| 0 | 5.38[a] | 5.06[b] | 2.98[d] | 4.23[c] | 0.06 |
| 3 | 4.15[b] | 4.63[a] | 2.80[d] | 3.76[c] | 0.10 |
| 6 | 4.11[b] | 4.41[a] | 2.75[d] | 3.11[c] | 0.06 |
| 12 | 3.74[b] | 4.27[a] | 2.66[c] | 2.85[c] | 0.07 |
| 24 | 2.59[b] | 4.07[a] | 2.32[c] | 2.35[c] | 0.06 |
| 48 | 2.07[b] | 3.38[a] | 1.27[c] | 1.92[b] | 0.07 |
| 72 | 1.79[b] | 3.31[a] | 1.16[c] | 1.37[c] | 0.07 |

Means in the same row followed by the different superscript letters are significantly different ($p<0.05$).
All values for hydrolysable tannins in treated samples were significantly different ($p<0.05$) from that of untreated salseed meal 6.83% (SD±0.12).
SEM is among treatment means.

**Table 3.** Effect of water, acid, alkali and polyvinyl-pyrrolidone (PVP) treatments within each incubation time on condensed tannin contents (% of DM) of salseed meal

| Incubation time (h) | Treatments | | | | SEM |
|---|---|---|---|---|---|
| | Water | Acid | Alkali | PVP | |
| 0 | 0.93[a] | 0.83[ab] | 0.30[c] | 0.75[b] | 0.04 |
| 3 | 0.85[a] | 0.79[ab] | 0.25[c] | 0.72[b] | 0.04 |
| 6 | 0.82[a] | 0.73[ab] | 0.21[c] | 0.71[b] | 0.03 |
| 12 | 0.81[a] | 0.73[b] | 0.21[c] | 0.72[b] | 0.03 |
| 24 | 0.81[a] | 0.72[b] | 0.20[c] | 0.71[b] | 0.03 |
| 48 | 0.82[a] | 0.70[b] | 0.20[c] | 0.71[b] | 0.03 |
| 72 | 0.81[a] | 0.63[b] | 0.18[c] | 0.69[b] | 0.02 |

Means in the same row followed by the different superscript letters are significantly different ($p<0.05$).
All values for condensed tannins in treated samples were significantly different ($p<0.05$) from that of untreated salseed meal 1.24% (SD±0.06).
SEM is among treatment means.

**Table 4.** Effect of water, acid, alkali and polyvinyl-pyrrolidone (PVP) treatments within each incubation time on trypsin inhibitor (units/g of DM) contents of salseed meal

| Incubation time (h) | Treatments | | | | SEM |
|---|---|---|---|---|---|
| | Water | Acid | Alkali | PVP | |
| 0 | 0.26[a] | 0.27[a] | 0.27[a] | 0.33[a] | 0.05 |
| 3 | 0.21[a] | 0.26[a] | 0.16[a] | 0.29[a] | 0.05 |
| 6 | 0.16[a] | 0.21[a] | 0.14[a] | 0.19[a] | 0.03 |
| 12 | 0.16[a] | 0.15[a] | 0.16[a] | 0.16[a] | 0.02 |
| 24 | 0.14[a] | 0.15[a] | 0.13[a] | 0.14[a] | 0.02 |
| 48 | 0.11[a] | 0.10[a] | 0.12[a] | 0.12[a] | 0.02 |
| 72 | 0.10[a] | 0.07[a] | 0.05[a] | 0.12[a] | 0.02 |

Means in the same row followed by the different superscript letters are significantly different ($p<0.05$).
All values for trypsin inhibitor contents in treated samples were significantly different ($p<0.05$) from that of untreated salseed meal 0.96 (SD±0.05).
SEM is among treatment means.

Reichert et al. (1980) the mechanism of tannin deactivation by water or acid treatment may be similar to the reaction which proceeds in grain as it approaches maturity. As the seed ripens, polymers of procyanidins are formed from monomers present in the seed resulting in an increase in the concentrations of soluble and insoluble polymers. They further postulated that the storage of moisture-imbibed grains may simply allow a continuation of the natural polymerization process that was occurring as the seed was drying out. It is likely that such insoluble polymers may not interfere with tannin protein complexes.

Treatment of salseed meal with a solution containing PVP deactivated more than half the tannin. The deactivation of tannin in salseed meal treated with PVP could be attributed to its capacity to bind tannin contents of the material hence preventing the formation of tannin-protein complexes. However, it was slightly less effective than the alkaline solution. Sievwright and Shipe (1986) reported that PVP was partially effective in preventing the formation of tannin-protein complexes. Inclusion of PVP in the diets of chicks containing raw and heated faba bean (Marquardt, 1989) significantly improved the weight gain and feed to gain ratio. The improvement may be related to the binding of tannin contents of the material with PVP. Similar nutritional improvement in high tannin diets by adding PVP have been reported by Armstrong et al. (1973).

The TI activity of all the treated salseed meal samples was lower than untreated salseed meal. The response of TI content of salseed meal to the treatments due to incubation time was quite different from those of hydrolysable or condensed tannins (Table 4). All the treatments had similar effect on trypsin inhibitor contents within each incubation time. The response to incubation time within each treatment showed that tannins and TI continuously decreased with the increase in incubation time. The data revealed that in each case most of the deactivation had already occurred between 6-12 h of incubation. All treatments exhibited a drastic deactivation in hydrolysable and condensed tannin contents of salseed meal even without any incubation (Zero incubation time i.e. wetting, immediately followed by drying). Tannins have been shown to inhibit trypsin (de Lumen and Salamat, 1980). Therefore, the total TI activities of the samples will be the sum of the trypsin inhibitor activities of both the tannin and the proteinaceous trypsin inhibitors. Because all of the treatments also reduce the tannin contents of the samples, we may safely assume that at least part of the reduction in TI activity may be ascribed to the reduction in tannin. The treated material was dried in a forced draft incubator at 37°C; the exposure of the meal to this temperature might have deactivated proteinaceous TI of the material. But it is less likely because TI extracts from winged beans have been stable at 60°C (Kadam and Smithard, 1987).

The physical appearance of acid-treated salseed meal after treatment was similar to that of untreated salseed meal. However, treatment with alkali, water, and PVP resulted in a visible growth of mould at incubation times of 24 or more hours. Growth of the mould increased with the incubation time. Among these treatments, water-treated material had the highest apparent mould growth followed by PVP and alkali. The colour of salseed meal treated with water and PVP after treatment was slightly darker than that of the untreated material. Whilst alkali treated material was notably darker colour. The reduction in the tannin and TI contents of salseed meal was found to be influenced by the anaerobic incubation time. Although tannins and TI continued to decline for 72 h of incubation, the major part of reduction was completed within 12 h incubation. The data of the present study reveal that anaerobic incubation of water, acid, alkali and PVP treated salseed meal for 12 h is reasonably effective incubation time to be used as it needs less storage requirements and is safe from any mould growth.

## CONCLUSIONS

Based on the results of the present study it could be concluded that the treatment of salseed meal with sodium bicarbonate (0.67 M) is more effective in reducing hydrolysable and condensed tannin contents than PVP, water, and acid solutions. Treatment with sodium bicarbonate solution is more economical and easier to handle than acid and PVP treatments. Incubation of the treated material for 12 h is reasonably effective, economical, and safe from any mould growth.

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