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EFFECT OF VARIOUS PROCESSING METHODS OF *LATHYRUS SATIVUS* SEEDS COLLECTED FROM DIFFERENT STATES OF INDIA ON THE MUSCLE COORDINATION ACTIVITY IN WISTAR ALBINO RATS

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ABSTRACT

Legumes are the major sources of proteins in diets consumed in India. Out of these, pulses are consumed more by Indians. Grass pea is one of the rich sources of protein obtained from the pulses. The grass pea seeds cultivated in different States of India like Andhra Pradesh, Kerala, Odisha, West Bengal, Chattisgarh and Bihar were taken and the seeds were treated by using different processing methods of wet roasting, boiling and soaking + boiling. The ODAP and Tannin content was estimated in all the processed samples collected from different states of India. Further, *in-vivo* assessment of the muscle co-ordination activity in Albino Wistar rats was carried out using methods like Rota-rod apparatus and inclined screen test models. The ODAP and Tannin content were found to be low in samples collected from Andhra Pradesh and in soaked + boiled processed seeds of *L. sativus*. The seeds of *L. sativus* collected from Andhra Pradesh showed better muscle co-ordination activity than all the other States of India and the soaked + boiled processed seeds showed a better muscle co-ordination activity compared to the other processed *L. sativus* seeds. The improved muscle coordination activity with processed seeds of soaked + boiled might be due to their decreased ODAP and Tannin levels. The better agroclimatic conditions might be responsible for good muscle coordination activity showed by the samples collected from Andhra Pradesh.

Keywords: Grass pea, *Lathyrus sativus*, processing methods, Rota-rod, Inclined screen test, ODAP, Tannins

INTRODUCTION

There are different kinds of legumes cultivated and consumed largely by Indian population as a chief source of protein. Although legumes constitute one of the most abundant and least expensive sources of protein in human/animal diets, their utilization is limited largely due to the presence of anti-nutritional/ anti-physiological compounds (Vijayakumari *et al*., 2007). Grass pea-*Lathyrus sativus* has immense potential as a food, feed, fodder as well as green manure. An epidemiological association exists between the intake of grass pea and a motor neural disease named Lathyrism or neurolathyrism (Hanbury et al., 2000).

The chief causative agent is the neurotoxic non-protein amino acid identified to be β–N-0xalye-L-α, β-diaminopropionic acid (ODAP), also well known as β-oxalyl amino alanine (BOAA). This toxin is present in all parts of the plant (Campbell et al., 1994). In the case of *Lathyrus sativus*, the seeds and vegetative parts contain ODAP or BOAA (Neurochem Int. 2002). The production of this valuable crop and the bright prospects of grass pea are handicapped by the stigma of its toxicity (Urga *et a*l., 2005; Tsegaye *et al*., 2007). In India, Pakistan, Bangladesh and Nepal, the most common use of grass pea is a *dhal* (a soup-like dish). Even though large number of population used to cultivate and consume *L. sativus* because of biophysical factors such as drought, low soil fertility, and water-logging pose severe constraints to production, in addition to many socio-economic factors (poverty, limited health care, etc) (Oliver, 1997).

The paralytic effect occurs because of ODAP toxicity in case the consumption of the seeds of *Lathyrus sativus.* Grass pea is taken as a staple food, that is, 75% of the diet intake-whereas, it is rendered safe when consumed at 5-30% of the total diet intake (Tsegaye *et al*., 2007). Almost all recent research publications emphasize that there is no neurolathyrism when the pulse is consumed as part of a normal diet and this is very true in the Indian context. No legume other than *L. sativus* has in fact ever served as a staple food (Surya S. Singh and S.L.N. Rao, 2013).

Tannins are secondary compounds of various chemical structures widely occurring in plant kingdom (Francis *et al.,* 2001). They are defined as high-molecular-weight polyphenolic compounds that have the ability to bind with protein and preserve animal hides. These include the formation of tannin/protein complexes that make the protein unavailable, inhibition of the digestive enzymes, increased synthesis of digestive enzymes due to inadequate enzyme digestion, and increased loss of endogenous

proteins such as the mucoproteins of the gastrointestinal tract (Cambell, 1997). Many factors such as soil composition, water stress, temperature and humidity can affect levels of phenolics present in plants (Kouki and Manetas, 2002; Monteiro et al., 2006). Tannin content alters during the development of the plant and also as a response to the environmental changes (Hatano et al., 1986; Santos et al., 2002; Salminen et al., 2001).

Hence, the present study was aimed to evaluate the effect of different processing methods on the in-vivo assessment methods on Wistar Albino rats fed on *Lathyrus sativus* seeds collected from different States of India and also correlate the effect of Tannins on the muscle coordination activity in Wistar Albino rats.

MATERIALS AND METHODS

CHEMICALS

Reagents used for analysis were purchased from Sigma Aldrich Company. All chemicals and reagents used were analytical reagent grade except H2O2, which was laboratory reagent grade.

SAMPLE COLLECTION

Lathyrus sativus (LS) seed samples were collected from Andhra Pradesh (LS- AP), Odisha (LS- OD), Kerala (LS- KE), West Bengal (LS- WB), Chhattisgarh (LS- CH) and Bihar (LS- BI).

SAMPLE PREPARATIONS

The seeds were cleaned manually to remove foreign matters, immature and damaged seeds. Different traditional processing methods are (Teklehaimanot et al., 1993):

RAW

The cleaned seeds (1Kg) were washed with tap water, rinsed with distilled water and immediately dried in drying oven at 55 °C for 12 h, under air circulation, and then grind by grinder to pass through a 0.425 mm sieve, packed in air tight bottle and stored at room temperature (in the shelf) until analysis.

WET ROASTING

Whole cleaned seeds (1Kg) were washed with tap water, rinsed with distilled water, soaked with distilled water (1:2 w/v seed to water) for 3 hr., decant the soaking water and washed with another distilled water, placed in 2L of distilled boiling water at 96 °C and cooked for 60 min. (until soft) and immediately dried in drying oven at 55 °C for under air circulation, and then grind by grinder to pass through a 0.425 mm sieve, packed in air tight bottle and stored at room temperature (in the shelf) until required for analysis.

BOILING

Whole cleaned seeds (1Kg) were washed with tap water, rinsed with distilled water, soaked with distilled water(1:5 w/v seed to water) at 28^0C (using water bath) for 20 h and then roasted at 200 °C for 40 min in baking oven placed in a baking try and turning with a fork, and then grind by grinder to pass through a 0.425 mm sieve, packed in air tight bottle and stored at room temperature (in the shelf) until required for analysis.

SOAKING + BOILING

100 g sample soaked overnight (8-9 hrs) in water under room temperature and then boiled in sufficient water until the pulse seed is easily pressed soft by hand/spoon/ladle.

CHEMICAL ANALYSIS

METHOD FOR ESTIMATION OF ODAP

Concentration of β-ODAP in the seeds was measured spectrophotometrically using the *o*-phtalaldehyde method of Rao as modified by Briggs *et al*. The modification involved the two times extraction of 0.5 g of the flour with 60% ethanol followed by hydrolysis of the flour with 3M KOH in boiling water bath for 30 minutes. After centrifugation for 15 minutes, an aliquot (250 µl) of the hydrolysate was diluted with 750 µl water and reacted with 2 ml *o*-phthalaldehyde (Sigma, St. Louis, MO, USA) reagent. The mixture was incubated at 35 0C for 2 hours before measuring the absorbance at 425 nm. The β-ODAP standard curve was calibrated using DAP.HCl (Sigma).

METHOD FOR ESTIMATION OF TANNINS

Tannin contents of flour were measured by Folin-Denis method (Schanderi, 1970).

The processed sample (0.5 g) was taken in a 250 ml conical flask and 75 ml distilled water was added to it. It was heated and boiled for 30 min and then centrifuged at 2000 rpm for 20 min. The supernatant was collected in 100 ml volumetric flask and volume was made up to the mark. In a 100 ml flask containing 75 ml water, 1 ml sample extract, 5 ml Folin-Denis reagent and 10 ml sodium carbonate solution were added and volume was made up. Contents of the flask were shaken well and then absorbance was measured at 700 nm after staying for 30 min. A blank was prepared with water instead of sample and standard graph was produced by using 0-100 µg tannic acid.

IN VIVO ASSESSMENT OF NEUROTOXICITY

ACUTE ORAL TOXICITY

The acute oral toxicity study was done according to OECD 423 guidelines. The study was conducted on albino mice of either sex weighing between 25-35 g and was divided into 4 groups containing 3 mice each. They were fasted overnight and maintained with water *ad libitum*.

EXPERIMENTAL ANIMALS

Animal were obtained from the Tina laboratories, Hyderabad. Albino Wistar rats (180-200 g) of male were used in the present study. The animals were housed under standard environmental conditions (23±1ºC) with relative humidity of 50±10% and maintain 12:12 dark and light cycle, maintained with free access to water and *ad libitum*

standard laboratory diet (70% carbohydrates, 25% proteins, 5% lipids (Hindustan Lever, Bangalore). After randomization before the experiment, the rats were acclimatized for a period of two weeks. The animal housing and handling were in accordance with CPSCEA guidelines. The College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, was approved by CPCSEA for conducting animal experiments with the registration No. 516/01/A/CPCSEA. The prior permission for the study was obtained from our Institutional Animal Ethics Committee (IAEC).

The rats were fasted for 18 h prior to the experiment with water *ad libitum*. During the experiment water was also withdrawn. The doses of the selected plant extracts were fixed based on the acute toxicity study. Control group was administered with distilled water (Group-I), Disease control group treated with Diazepam (2mg/kg), Group III, Group IV, Group V and Group VI serves as different processing *L. sativus* followed as raw, wet roasted, boiled and soaked + boiled. Again each group was sub divided for different states of India (AP, KE, OD, WB, CH and BI).

RESULTS

IN VIVO ASSESSMENT METHODS

MUSCLE RELAXANT ACTIVITY MODELS

ROTA-ROD

In Rota-rod method, loss of coordinated motor movement is one of the pharmacological effects of anxiolytic drugs. The effect of the drug, Diazepam (2 mg/kg, i.p.) on the coordinated motor movement was assessed using Rota-rod test (Tsuda M et al., 1996).

INCLINED SCREEN TEST MODEL

In Inclined screen test method, each group of rats (n=5) were left for 1hr on a flat, slippery, rectangular glass (42cm × 37cm) inclined at 30º to the horizontal, 30 min after the administration of test compounds, Diazepam (2 mg/kg, i.p.) to observe for a paralyzing effect severe enough to cause the rats to slide off the screen (Randall Lo., 1960).

Table 1: Estimation of β- ODAP (mg/100gm) Levels in *L. sativus* seeds of various States of India

Parameters (mg/100gm)	Raw seeds	Wet roasted	Boiled	Soaked+ boiled
LS-AP	519.02±2.36	428.56±3.15*** (17.53)	315.15±2.99*** (39.30)	256.26±3.86*** (50.67)
LS-KE	543.23±2.33	462.23±4.26*** (14.91)	354.12±3.61*** (34.80)	298.16±2.77*** (45.11)
LS-OD	587.39±3.65	486.98±3.46*** (17.20)	404.22±3.46*** (31.17)	335.11±2.56*** (42.93)
LS-WB	621.51±1.97	515.23±2.46*** (17.06)	458.22±2.58*** (26.24)	360.76±3.68*** (42.02)
LS-CH	651.08±3.18	562.33±2.89*** (13.67)	520.19±2.99*** (20.12)	398.11±5.26*** (38.86)
LS-BI	674.26±3.54	620.55±3.28*** (08.11)	583.46±3.33*** (13.50)	456.74±4.30*** (32.34)

*** P<0.001, significance followed 2 way ANOVA, Bonferonni's post test when compared with Raw seeds.

Table 2: Estimation of Tannins (mg/100g) Levels in *L. sativus* seeds of various States of India

Parameters (mg/100gm)	Raw seeds	Wet roasted	Boiled	Soaked+Boiled
LS-AP	632.47±6.32	512.36±7.70*** (18.98)	466.42±6.66*** (26.26)	436.18±3.94*** (31.01)
LS-KE	690.69±3.64	592.44±5.41*** (14.20)	530.15±5.75*** (23.18)	489.92±4.71*** (29.13)
LS-OD	753.45±4.44	604.26±3.64*** (19.78)	566.39±3.67*** (24.83)	532.45±3.58*** (29.34)
LS-WB	781.33±5.19	630.15±8.12*** (19.33)	589.48±4.71*** (24.58)	569.36±6.66*** (27.14)
LS-CH	820.48±4.12	698.26±4.36*** (14.87)	642.12±5.63*** (21.70)	625.45±5.12*** (23.78)
LS-BI	856.45±6.32	726.85±5.55*** (15.18)	691.56±4.84*** (19.27)	658.06±4.36*** (23.13)

*** P<0.001, significance followed 2 way ANOVA, Bonferonni's post test when compared with Raw seeds

DISCUSSION

The food processing methods including soaking and boiling greatly influence the nutritive values of legumes. Of these, soaking prior to boiling plays an important role as it influences the bioavailability and utilization of nutrients and improves palatability, which incidentally may result in enhancing the digestibility and nutritive value (Ramakrishna *et al.,* 2006). The effect of soaking time and soaking solution on the nutritional quality of grass pea seeds were investigated (Urga and Gebretsadik 1993).

Different traditional processing methods including roasting, boiling samples were collected and assayed for β-ODAP levels by Teklehaimanot et al., 1993; Ramachandran, and Ray. 2008. The effect of cooking, roasting, autoclaving and fermentation on the content of β-ODAP in the whole seeds and flour of grass pea were determined at different levels of temperature, time, pH, degree of soaking and moisture content by Akalu *et al*., 1998.

In the present study, the soaked+boiled samples were found to reduce the ODAP content greatly. The order of ODAP levels is LS-AP<LS-KE< LS-OD< LS-WB<LS-CH<LS-BI. Hence, the present study reveals that the various processing methods reduces the toxicity due to ODAP and, improve in the agroclimatic conditions not only improves the nutritional content but also reduces the levels of ODAP which leads neurolathyrism.

Table 3: Effect of *LS* from various States of India on muscle relaxant activity in Wistar Albino rats (Rota-rod apparatus model)

Treatment	0 Min						30 Min					
	LS-AP	LS-KE	LS-OD	LS-WB	LS-CH	LS-BI	LS-AP	LS-KE	LS-OD	LS-WB	LS-CH	LS-BI
Control	332±2.36 ns	330±3.26 ns	333±2.89 ns	326±3.26 ns	335±3.26 ns	326±2.56 ns	335±2.65*	328±4.03*	330±4.16*	322±2.69*	333±2.89*	328±3.46*
Diazepam	326±4.26	326±4.10	326±2.47	333±4.23	339±2.56	329±2.89	100±3.16	104±2.56	108±3.28	97±4.21	102±2.56	99±3.25
Raw	329±3.51 ns	332±2.49 ns	328±3.29 ns	329±2.87 ns	326±2.48 ns	333±3.25 ns	179±4.62*	160±4.66*	153±2.99*	140±3.64*	133±3.44*	120±2.56*
Wet Roasted	333±4.10 ns	328±3.16 ns	331±2.9 ns 9	330±3.11 ns	326±3.22 ns	326±4.08 ns	230±5.16*	222±3.19*	211±2.81*	206±3.21*	198±3.79*	177±2.79*
Boiled	324±3.98 ns	329±2.87 ns	329±3.46 ns	325±2.76 ns	330±4.13 ns	335±3.11 ns	259±4.23*	244±3.55*	232±3.67*	219±2.79*	200±2.33*	190±3.35*
Soaked +boiled	320±5.12 ns	330±3.84 ns	325±3.28 ns	321±3.55 ns	333±3.77 ns	339±2.84 ns	295±3.87*	281±3.87*	269±3.28*	256±2.90*	232±3.61*	218±3.88*

nsP>0.05, *P<0.001, significance followed 2 way ANOVA, Bonferonni's post test when compared with diazepam.

Table 4: Effect of *LS* from various States of India on muscle relaxant activity in Wistar Albino rats (Inclined screen test model)

Treatment	0 Min						30 Min					
	LS-AP	LS-KE	LS-OD	LS-WB	LS-CH	LS-BI	LS-AP	LS-KE	LS-OD	LS-WB	LS-CH	LS-BI
Control	56±1.23 ns	60±1.11 ns	56±0.23 ns	56±0.86 ns	56±0.89 ns	56±0.86 ns	58±1.11*	59±0.68*	61±1.11*	58±1.26*	60±1.03*	58±1.26*
Diazepam	57±0.65	58±0.36	54±1.23	59±1.03	59±1.06	59±1.03	10±1.26	12±0.94	09±0.49	11±0.88	13±1.23	11±0.88
Raw	60±0.49ns	60±0.49ns	57±0.95ns	60±1.05 ns	60±1.26 ns	60±1.05 ns	30±1.38*	26±1.20*	36±0.73*	18±0.76*	19±1.09*	18±0.76*
Wet Roasted	61±1.35 ns	57±1.60 ns	61±0.76 ns	64±0.84 ns	54±0.76 ns	57±0.84 ns	36±0.49*	32±0.66*	46±0.66*	20±0.84*	23±0.97*	20±0.84*
Boiled	57±1.55 ns	59±0.84 ns	58±0.92 ns	61±0.76 ns	58±0.85 ns	60±0.76 ns	39±0.94*	36±0.76*	52±0.86*	21±1.20*	24±0.86*	21±1.20*
Soaked +boiled	59±1.26 ns	58±0.92 ns	57±1.36 ns	58±0.89 ns	59±0.79 ns	58±0.89 ns	45±0.88*	40±0.92*	54±1.00*	25±1.06*	28±0.95*	25±1.06*

nsP>0.05, *P<0.001, significance followed 2 way ANOVA, Bonferonni's post test when compared with diazepam.

Tannins are polyphenolic compounds having astringent and bitter taste that can be felt after eating unripened fruit. They are known to bind protein and alkaloids and made the meal difficult to digest. Consumption of tannin in large doses may cause bowel irritation, damage the liver, stomach and kidney irritation and gastrointestinal pain, chelate minerals and makes them unavailable to the body. Its prolong consumption may lead to iron deficiency that cause anemia, however tannins reduce the bioavailability of only plant sources iron i.e. non-haem iron by making complexes (McGee and Harold, 2004; Karamac, 2009). Tannins have been claimed to affect adversely protein digestibility from plant-based diets. In the present study, the concentration of tannins was generally high in all the samples analyzed and similar to values reported for grass pea germplasm (Urga K., 1995). Tannin levels in our study were found to generally vary with the intensity of pigmentation, with the darker seed coats generally giving higher levels of tannins as reported by Deshpande and Campbell., 1992.

In the present study, the raw and processed samples of *Lathyrus sativus* collected from different States of India were evaluated for their muscle coordination activity in Albino Wistar rats by Rota-rod and Inclined screen test models. 2mg/kg Diazepam was used as a standard. The treatment with the processed samples of *L. sativus* like raw, wet roasted, boiled and soaked+boiled reduced the percent fall in time from Rota rod as well as Inclined screen in the order of raw> wet roasted> boiled> soaked+boiled. Among the different processing methods, the soaked+boiled ones were found to have good muscle coordination activity compared to the raw, wet roasted and boiled processed *L. sativus* seeds.

Among the seeds of *L. sativus* collected from different States of India, the seeds of *L. sativus* collected from the State of Andhra Pradesh showed better muscle coordination activity whereas all the processed *L. sativus* seeds collected from Bihar showed low muscle coordination activity in both Rota-rod apparatus model and Inclined test model. ODAP by virtue of its toxicity on neurons might be producing the muscle relaxation (Hanbury et al., 2000).

It has been reported that if the legume is boiled for two hours and the water is then decanted, almost 85% of the toxic amino acid- β-ODAP is eliminated. Therefore, this investigation constitutes an effort to prevent the loss of other nutrients, simultaneously to the elimination of toxicity. This indicates that the processing of *L. sativus* seeds found to reduce the content of β-ODAP which might be responsible for its good muscle coordination activity. The improved muscle coordination activity of processed samples compared to raw seeds might be due to the decreased ODAP content.

Processing methods for grass pea is very important primarily due to the high content of anti-nutrients and the difficulty in their digestion. The higher content of tannins is known to inhibit the activity of the digestive enzymes and, thus, interfere with the digestion and absorption of dietary proteins, carbohydrates, minerals and other nutrients, such as vitamin B12. They may also cause damage to the mucosa of the digestive tract and hence, they are undesirable for human consumption from a nutritional point of view (Vijayakumari *et al*., 2007).

The increase in the tannins in any part of the phytochemicals leads to the increase in the muscle coordination activity of Wistar albino rats by promoting the antioxidant defence mechanism against the oxidative stress. Many researchers reported that the plants with rich source of tannins possess better antioxidant activity (Shahidi, 2000; Lopes et al., 1999; Ferguson, 2001; Wu et al., 2004 and Andrade et al., 2005). But in the present study, the *Lathyrus sativus* seeds samples contain sufficient amount of tannins but the increased levels of ODAP, the amount of tannins cannot compensate the toxic effects of ODAP. At the same time, during the processing methods, the amount of tannins present in the grass pea seeds get leached into the soaked media. Hence, in the present study, decrease in the tannin content of the processed ones is noticed and the soaked + boiled *Lathyrus sativus* was not able to control the muscle coordination activity. The order of the activity is The treatment with the processed samples of *L. sativus* like raw, wet roasted, boiled and soaked+boiled reduced the percent fall in time from Rota rod as well as Inclined screen in the order of raw> wet roasted> boiled> soaked+boiled.

CONCLUSION

Hence, for proper utilization of grass pea, especially in developing countries, by following the simple and economic household processing and cooking methods will not only enhance the nutritional quality of the grass pea, also lowers the content of anti-nutrients and increases the bioavailability of essential nutrients and minerals. The samples collected from LS-AP soaked + boiled seeds were found to have good muscle coordination activity when compared to the samples collected from other States of India. Overall, it can be concluded that the processing methods like wet roasted, boiled and soaked+boiled greatly improves the nutritional value of grass pea but also reduces the toxicity of β-ODAP and also the tannin content in *Lathyrus sativus* which might be responsible for its muscle coordination activity.

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