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THE IN VIVO ASSESSMENT OF NEUROTOXICITY IN ALBINO WISTAR RATS FED ON LATHYRUS SATIVUS

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ABSTRACT

Lathyrus sativus, rather profoundly known as grass pea, is a legume commonly grown for human consumption and livestock feed in Asia and East Africa. It is of prime importance in drought-stricken and famine affected areas and is thus thought of as an insurance crop as it produces reliable yields when all other crops fail. The seeds of grass pea contain a neurotoxin that causes a neurodegenerative disease when consumed as a primary period on a daily basis. The present study is a strong attempt to evaluate the various traditional processing methods on the composition of grass pea and select the efficient processing method, which makes the food more safe that can prevent lathyrism and protein energy malnutrition. As a result considering the importance of grass pea and the unparallel contribution of improved varieties in the productivity of the crop, the present study was pursued for the *In* vivo assessment of neurotoxicity using acute oral toxicity tests on Albino Wistar rats using the Rota rod apparatus method and the Incline screen test method. Further, the relationship between the various food processing techniques employed and the neurotoxicological assessments was possible.

KEYWORDS: grass pea, food processing, neurotoxicity, oral toxicity tests, Rota rod, Inclined screen test.

INTRODUCTION

Lathyrus sativus - Kesari dal has immense potential as a food, feed, fodder as well as green manure. An epidemiological association exists between the intake of Kesari dal and a motor neural disease named "Lathyrism"-the paralysis of lower limbs in humans. The chief causative agent is the toxic principle identified to be ß –N-Oxalye-L-α, ß-diaminopropionic acid (ODAP), well known as ß-oxalyl amino alanine (BOAA). This toxin is present in all parts of the plant.[1] The production of this valuable crop and the bright prospects of grass pea are handicapped by the stigma of its toxicity. The paralytic effect occurs because of ODAP toxicity in case the consumption of the seeds 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.

It was found that water soaking of the seed could lower β-ODAP content but not sufficiently for continuous safe human consumption. Physical and chemical treatments have also been used in the detoxification to induce some mutants. The mutants were not widely used as their characters were unstable or β-ODAP content was not sufficiently low. Further efforts would be a necessary for further improvements.[2] Effect of different processing techniques (Extrusion, fermentation, germination and autoclaving) on the nutritive value of grass pea had also studied.[3] Different traditional processing methods including roasting, boiling, preparation of sauce and unleavened bread food samples were collected and assayed for β-ODAP levels.[4] The effect of soaking time and soaking solution on the nutritional quality of grass pea seeds were investigated.[5] 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.[6]

As a result considering the importance of grass pea and the unparallel contribution of improved varieties in the productivity of the crop, the present study was pursued for the *in vivo* assessment of neurotoxicity using acute oral toxicity tests on Albino Wistar rats. The two acute oral toxicity tests employed in the study are Rota-rod and Inclined screen test methods.

Muscle relaxant activity using *in vivo* assessment methods: Rota-rod: In Rota-rod method, loss of coordinated motor movement is one of the pharmacological effects of anxiolytic drugs. The effect of the plant extract on coordinated motor movement was assessed

using Rota-rod test.[7] The equipment consists of a horizontal metal rod covered with rubber to 3cm diameter attached to a motor with speed accustomed to 2 rotations/min. The 75cm rod is partitioned with 6 sections using plastic discs, it facilitate the concurrent testing of six animals. Cages were provided below the sections for prevent the movement of experimental animals. The number of animals falling from the roller during this time was counted.[8] The rota-rod test, in which animals must balance on a rotating drum, is widely used to assess motor deficit in neurodegenerative disease models in rodents. Performance is measured by the duration that an animal stays on the rod as a function of drum speed. Two different protocols are widely used, incremental fixed speeds or an accelerating protocol, but there is little information on their equivalence or the relative power, reliability and sensitivity of the two protocols.[9]

Inclined screen test method: The method of Allmark and Bachinski (1949) using an inclined screen was originally developed for testing curare-like agents. Later on, it has been used by many authors (e. g. Randall et al 1961) for testing compounds for muscle relaxant activity.[10] The principle of an inclined plane has been used by Ther, Vogel and Werner (1959) for differentiating neuroleptics from other centrally active drugs.[11] Rivlin and Tator (1977) also used an inclined plane to assess skeletal muscle relaxation.[12] The plane consists of two rectangular plywood boards connected at one end by a hinge. One board is the base, the other is the movable inclined plane. Two plywood side panels with degrees marked on their surface are fixed on the base. A rubber mat with ridges 0.2 cm in height is fixed to the inclined plane which is set at 65 degrees. The inclined plane test is to determine the skeletal muscle relaxant activity.[13]

MATERIALS AND METHODS

Sample: Samples of *Lathyrus sativus* seeds- LS

- LS- Andhra Pradesh (LS-AP)
- LS-Odisha (LS-OD)
- LS- Kerala (LS-KE)
- LS- West Bengal (LS-WB)
- LS- Bihar (LS-BI)
- LS- Chhattisgarh (LS-CH)

Chemicals: Reagents used for analysis were purchased from Sigma Aldrich Company. All chemicals and reagents used were analytical reagent grade except H_2O_2, which was laboratory reagent grade.

Sample Preparations: The seeds were cleaned manually to remove foreign matters, immature and damaged seeds. Different traditional processing methods:[4]

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 ^{0}C (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.

***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*. The selected functional foods were administered at a dose level of 2000mg/kg body weight.

Experimental animals: Animals 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. Our University 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).

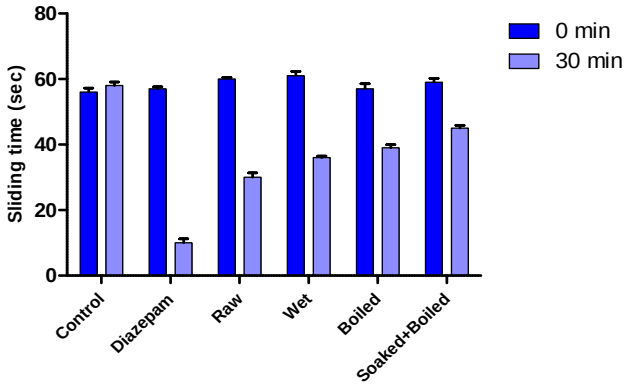
Rota-rod method: Mice were trained to stay in Rota-rod apparatus (3 cm in diameter, 8 rpm) for 120 seconds and at least two times for each animal. Twenty four hours later the animals were injected with vehicle (saline), hydroalcoholic extract of plant (125, 250 and 500 mg/kg) intraperitoneally and placed in apparatus 1 hour later. The latency (in seconds) to drop off the Rota-rod was recorded up to a limit of 100 seconds.

Inclined screen test method: Instead of an inclined wooden board, an inclined screen has been used by Randall et al (1961) and Simiand et al (1989).[10],[14] The Plane consisting of transparent glass was left on an inclined angle at 30º. The mice, try to move out of the plane glass without sliding off, were used for the test. The mice were kept in the superior part of the inclined plane and are given 30sec to hang on or to fall off.[15] 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.[16]

RESULTS AND DISCUSSION

Table 1: Effect of *LS-AP* on muscle relaxant activity (Inclined screen test).

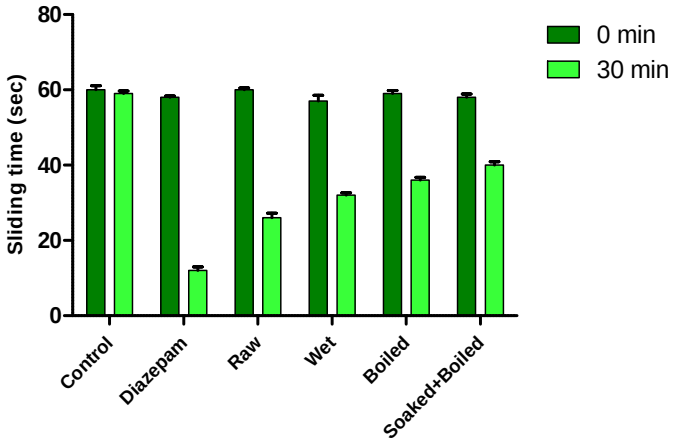
Treatment group	0 min	30 min
Control	56±1.23	58±1.11
Diazepam (2mg/kg)	57±0.65	10±1.26
Raw	60±0.49^{ns}	30±1.38**
Wet roasted	61±1.35 ^{ns}	36±0.49 **
Boiled	57±1.55 ^{ns}	39±0.94 ***
Soaked+Boiled	59±1.26 ^{ns}	45±0.88 ***



Graph 1: Effect of *LS-AP* on muscle relaxant activity (Inclined screen test).

Table 2: Effect of *LS-KE* on muscle relaxant activity (Inclined screen test).

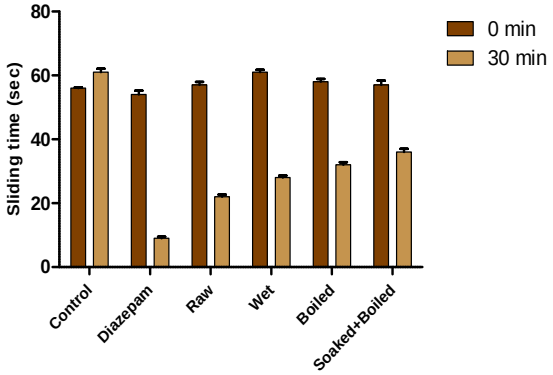
Treatment group	0 min	30 min
Control	60±1.11	59±0.68
Diazepam (2mg/kg)	58±0.36	12±0.94
Raw	60±0.49^{ns}	26±1.20**
Wet roasted	57±1.60 ^{ns}	32±0.66**
Boiled	59±0.84 ^{ns}	36±0.76***
Soaked+Boiled	58±0.92 ^{ns}	40±0.92***



Graph 2: Effect of *LS-KE* on muscle relaxant activity (Inclined screen test).

Table 3: Effect of *LS-OD* on muscle relaxant activity (Inclined screen test).

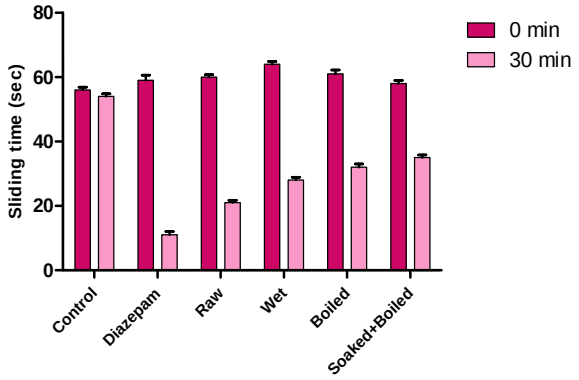
Treatment group	0 min	30 min
Control	56±0.23	61±1.11
Diazepam (2mg/kg)	54±1.23	09±0.49
Raw	57±0.95^{ns}	36±0.73**
Wet roasted	61±0.76^{ns}	46±0.66**
Boiled	58±0.92^{ns}	52±0.86***
Soaked+Boiled	57±1.36^{ns}	54±1.00***



Graph 3: Effect of *LS-OD* on muscle relaxant activity (Inclined screen test).

Table 4: Effect of *LS-WB* on muscle relaxant activity (Inclined screen test).

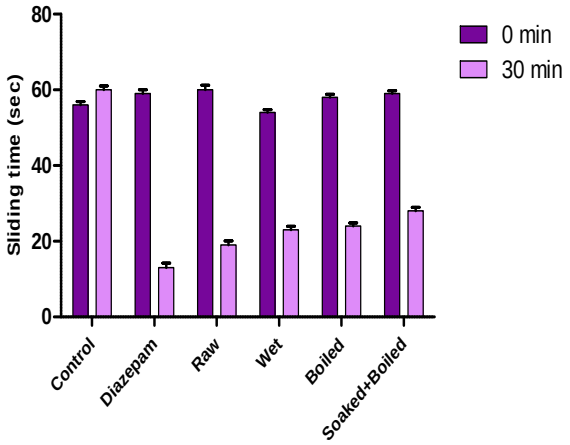
Treatment group	0 min	30 min
Control	56±0.86	58±1.26
Diazepam (2mg/kg)	59±1.03	11±0.88
Raw	60±1.05^{ns}	18±0.76**
Wet roasted	64±0.84^{ns}	20±0.84**
Boiled	61±0.76^{ns}	21±1.20***
Soaked+Boiled	58±0.89^{ns}	25±1.06***



Graph 4: Effect of *LS-WB* on muscle relaxant activity (Inclined screen test).

Table 5: Effect of *LS-CH* on muscle relaxant activity (Inclined screen test).

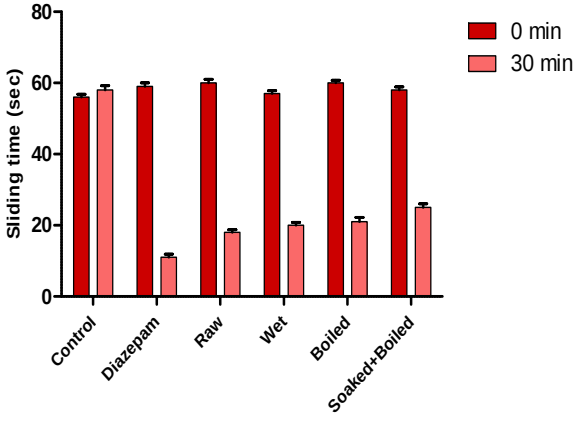
Treatment group	0 min	30 min
Control	56±0.89	60±1.03
Diazepam (2mg/kg)	59±1.06	13±1.23
Raw	60±1.26 ^{ns}	19±1.09**
Wet roasted	54±0.76 ^{ns}	23±0.97**
Boiled	58±0.85 ^{ns}	24±0.86***
Soaked+Boiled	59±0.79 ^{ns}	28±0.95***



Graph 5: Effect of *LS-CH* on muscle relaxant activity (Inclined screen test).

Table 6: Effect of *LS-BI* on muscle relaxant activity (Inclined screen test).

Treatment group	0 min	30 min
Control	56±0.86	58±1.26
Diazepam (2mg/kg)	59±1.03	11±0.88
Raw	60±1.05 ^{ns}	18±0.76**
Wet roasted	57±0.84 ^{ns}	20±0.84**
Boiled	60±0.76 ^{ns}	21±1.20**
Soaked+Boiled	58±0.89 ^{ns}	25±1.06***



Graph 6: Effect of *LS-BI* on muscle relaxant activity (Inclined screen test).

Table 1 denotes the effect of LS-AP on muscle relaxant activity by inclined screen test. The samples of *L. sativus* obtained from farmers of Andhra Pradesh (LS-AP), Kerala (LS-KE), Odisha (LS-OD), West Bengal (LS-WB), Chattisgarh (LS-CH) and Bihar (LS-BI) for the present study. The obtained samples of *L. sativus* were subjected to traditional processing methods basing on people consume them into raw, wet roasted, boiled and soaked + boiled as described in Chapter III (materials and methods). Processing methods for grass pea is very important primarily due to the high content of antinutrients and the difficulty in their digestion.

The control group shows 56±1.23 in 0 minute and 58±1.11 in 30 minutes whereas when treated with Diazepam (2mg/kg), the results are 57±0.65 in 0 minute and 10±1.26 in 30 minutes. The raw seeds showed 60±0.49 in 0 minute and 30±1.38 in 30 minutes and the wet roasted seeds resulted as 61±1.35 ^{ns} in 0 minute and 36±0.49 in 30 minutes. The boiled seeds showed the results as 57±1.55 ^{ns} in 0 minute and 39±0.94 in 30 minutes and the soaked + boiled seeds as 59±1.26 ^{ns} in 0 minute and 59±1.2 in 30 minutes.

Table 2 denotes the effect of LS-KE on muscle relaxant activity by inclined screen test. The control group shows 60±1.11 in 0 minute and 59±0.68 in 30 minutes whereas when treated with Diazepam (2mg/kg), the results are 58±0.36 in 0 minute and 12±0.94 in 30 minutes. The raw seeds showed 12±0.94 in 0 minute and 26±1.20 in 30 minutes and the wet roasted seeds resulted as 57±1.60 in 0 minute and 32±0.66 in 30 minutes. The boiled seeds showed the results as 59±0.84 in 0 minute and 36±0.76 in 30 minutes and the soaked + boiled seeds as 58±0.92 in 0 minute and 40±0.92 in 30 minutes.

Table 3 denotes the effect of LS-OD on muscle relaxant activity by inclined screen test. The control group shows 56±0.23 in 0 minute and 61±1.11 in 30 minutes whereas when treated with Diazepam (2mg/kg), the results are 54±1.23 in 0 minute and 09±0.49 in 30 minutes. The raw seeds showed 57±0.95 in 0 minute and 36±0.73 in 30 minutes and the wet roasted seeds resulted as 61±0.76 in 0 minute and 46±0.66 in 30 minutes. The boiled seeds showed the results as 58±0.92 in 0 minute and 52±0.86 in 30 minutes and the soaked + boiled seeds as 57±1.36 in 0 minute and 54±1.00 in 30 minutes.

Table 4 denotes the effect of LS-WB on muscle relaxant activity by inclined screen test. The control group shows 56±0.86 in 0 minute and 58±1.26 in 30 minutes whereas when treated with Diazepam (2mg/kg), the results are 59±1.03 in 0 minute and 11±0.88 in 30 minutes. The

raw seeds showed 60±1.05 in 0 minute and 18±0.76 in 30 minutes and the wet roasted seeds resulted as 64±0.84 in 0 minute and 20±0.84 in 30 minutes. The boiled seeds showed the results as 61±0.76 in 0 minute and 21±1.20 in 30 minutes and the soaked + boiled seeds as 58±0.89 in 0 minute and 25±1.06 in 30 minutes.

Table 5 denotes the effect of LS-CH on muscle relaxant activity by inclined screen test. The control group shows 56±0.89 in 0 minute and 60±1.03 in 30 minutes whereas when treated with Diazepam (2mg/kg), the results are 59±1.06 in 0 minute and 13±1.23 in 30 minutes. The raw seeds showed 60±1.26 in 0 minute and 19±1.09 in 30 minutes and the wet roasted seeds resulted as 54±0.76 in 0 minute and 23±0.97 in 30 minutes. The boiled seeds showed the results as 58±0.85 in 0 minute and 24±0.86 in 30 minutes and the soaked + boiled seeds as 59±0.79 in 0 minute and 28±0.95 in 30 minutes.

Table 6 denotes the effect of LS-BI on muscle relaxant activity by inclined screen test. The control group shows 56±0.86 in 0 minute and 58±1.26 in 30 minutes whereas when treated with Diazepam (2mg/kg), the results are 59±1.03 in 0 minute and 11±0.88 in 30 minutes. The raw seeds showed 60±1.05 in 0 minute and 18±0.76 in 30 minutes and the wet roasted seeds resulted as 57±0.84 in 0 minute and 20±0.84 in 30 minutes. The boiled seeds showed the results as 60±0.76 in 0 minute and 21±1.20 in 30 minutes and the soaked + boiled seeds as 58±0.89 in 0 minute and 25±1.06 in 30 minutes.

When grass pea is processed, the protein inhibitor and other anti nutritional factors, which inhibit the protein digestibility and chelate the mono, di and trivalent metal ions and form insoluble complexes will be degraded to a smaller molecular form and release the protein and the essential elements. The food processing methods including soaking, germination, decortications, fermentation and cooking greatly influence the nutritive values of legumes. Of these, cooking and germination 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.[17] Therefore, data on the effect of traditional processes on the nutrient composition, mineral contents and ant nutritional factors could be evaluated. It was found that water soaking of the seed could lower β-ODAP content but not sufficiently for continuous safe human consumption. Physical and chemical treatments have also been used in the detoxification to induce some mutants. The mutants were not widely used as their characters were unstable or β-ODAP content was not sufficiently low. Further efforts would be a necessary for further improvements.[2]

Boiling in water or repeated steeping in hot water and discarding the extracts can detoxify the seeds. Roasting of seeds, at 140^{o}C for 15 to 20 minutes, result in 80 to 90% destruction of the neurotoxins. Some people soak the seeds overnight and decant the water before cooking. This eliminates about 90% of the toxin. Toxic amino acids are readily soluble in water and can be leached. Fermentation is useful to reduce ODAP content. Moist heat (boiling, steaming) denatures protein inhibitors, which other wise add to the toxic effect of raw grass pea through depletion of protective sulfur amino acid.[18] Compound β1 is a water- soluble amino acid present in the ODAP that can be leached from seed by soaking in water.[19],[4] Steeping grass pea in a large volume of cold water for 3 min leached out approximately 30% of 1, with greater losers when hot water was employed.[4] Similarly, steeping dehusked seed in hot water for several hours and boiling the seed in water removed 70– 80% of the neurotoxin. Moslehuddin and colleagues, 1987 also found that washing seed partially removed it.[20] Padmaja prasad and associates, 1997 reported that boiling grain and discarding the water reduced 1 level up to 90%.[21] Boiling has widely been used in the preparation of *L. sativus* seed as dahl, in bread-making and in vegetable preparations.[22]

In the present study, the methods of Rot-rod apparatus model and Inclined screen test models were selected to assess the neurotoxicity of *L. sativus* seeds from different States of India. Among all the States of India, the Rot-rod apparatus model and Inclined test models used on the seeds of *L. sativus* showed better muscle co-ordination activity in Andhra Pradesh. In Andhra Pradesh, the soaked + boiled processed seeds showed a showed better muscle co-ordination activity compared to raw, wet roasted and boiled processed *L. sativus* seeds whereas in Bihar, all the processed *L. sativus* seeds showed muscle relaxant activity in both Rota-rod apparatus model and Inclined test model.

The processed samples of *L sativus* collected from different States of India were evaluated for their muscle relaxant activity in Albino Wistar rats by rota rod and inclined screen test. 25mg/kg ODAP was used as a standard. The treatment with the processed samples of *L. sativus* like raw wet roasted, boiled and soaked+boiled reduced the % fall in time from rota rod in the order of ODAP> raw> wet roasted> boiled> soaked+boiled. The persent fall in ODAP levels in soaked+boiled were found to be low which might be due to the presence of low ODAP content. The samples collected from LS AP were found to have less muscle relaxant activity compare to the samples collected from other states of India. ODAP by virtue of its toxicity on neurons might be producing the muscle relaxation (REF). Hence the present

study reveals that the various processing methods will reduce the toxicity due to ODAP. The improving the agroclimatic conditions will not only improve the nutritional content but also reduce the levels of ODAP which leads neurolathyrism.

CONCLUSION

Hence, the efficient use of food processing techniques by manipulating the processing time can improve the optimal use of the legume- grass pea for safe human consumption. Therefore, this investigation constitutes an effort to prevent neurolathyrism simultaneously by the detoxification of ODAP, employing various effective food processing techniques on the grass pea seeds deemed safe for human consumption.

The neurotoxicological methods of Rota-rod and Inclined screen test have been proven to be a simple assay for muscle relaxant activity. Although the muscle relaxant tests satisfy the criteria of sensitivity and relative potency compared with clinically effective doses, the effects of anxiolytics are not clearly differentiated from neuroleptics and even from neurotoxic compounds.

To minimize nutrient loss during adverse processing of grass pea, optimization of processing conditions are recommended for investigation.

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