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Preservation of Pulses with the Help of *Nerium Indicum* Linn. Foliage Dust and *Ricinus Communis* L. Seed Oil; Study of Antinutritional and Nutritional Parameters of Five Commonly Utilized Pulses.

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A B S T R A C T

Castor bean oil (Ricinus communis L.) has shown excellent results in preservation of green gram and soybean. After 12 months of storage, no infestation has been seen when 2 ml/kg seed oil is applied to green gram and soybean. As much as at 1 g/kg, leaf dust of Nerium indicum Linn. was seen ineffective in protecting the pulses. We have selected Vigna radiata (L) Wilczek (green gram) as it is most and Glycine max L. least susceptible host of Callosobruchus chinensis L. (Coleoptera: Bruchedae). In studying antinutritional factors of 5 commonly utilized pulses red grams were found to have a high amount of cyanogenetic glycosides. Trypsin inhibitor activity is high in soybeans. There is a remarkable difference in the tannin and oxalate contents as well as haemagglutinating activity of different pulses. The larva of C. chinensis is penetrating seed coat and lives inside. That space also utilized by larvae for pupation, therefore seed coat thickness and composition could also be the descending factors for infestation. Nutritional value and germination ability of pulses which are subjected to preservation by leaf dust and seed oil was monitored every 2 months up to 12 months. In the control set reducing sugar level declined significantly and marginal decline in total carbohydrates, proteins and protease activity has been seen than to the treated sets. In contrast to these, the bacterial and fungal count in control was found to be almost double, and percent germination and percent sprouting decreased to half as compared to treated. Flavonoids mixture isolated from Nerium indicum showed almost 90% mortality of C. chinensis in 2 hours. The aqueous leaf extract was used to isolate flavonoids from N. indicum and mortality was achieved at 4-6 mg/ml concentration in filter paper diffusion assay.

Keywords: Flavonoids, *Nerium indicum*, *Callosobruchus chinensis*, pulses, antinutritional.

Introduction

The hazards due to chemical pesticides are so high that Denmark has set the aim of double the organic food production from 2010 to 2020 in its green growth action plane since 1996. France has also decided to reduce "if possible" by 50% the use of pesticides from 2008 to 2018. Due to their best usefulness, biopesticides are also supported by consumers' organizations. There is plenty of promising work going on in the use of natural microbials. In the last years 165 species have been widely used for pest biocontrol and 250 are to be used in 2011.[1] Mixture of natural Rutaceae plant extracts protects crop against phytopathogenic fungi, especially botrytis and powdery mildew. It can be an alternative to chemical fungicides during the harvest period and in organic agriculture. Agricultural crops and stored grains are under constant assault by insect pests, making insecticides essential to reduce losses. The great numbers of chemicals are biosynthesized in plants, can be called natural laboratories. Many plants have developed natural defense mechanisms against weed competition and animal, insect and fungal attacks. These chemicals discourage feeding insects and other herbivores. Plant extracts have long been used to control insects. Dating as far back as 400 B.C. children were deloused using a powder obtained from the dried flowers of the pyrethrum plant (*Tanaceturm cinerariifolium*). The first botanical insecticide dates to the 17th century when it was shown that nicotine from tobacco leaves killed plum beetles.[2] Botanicals show, Insect growth regulators, feeding deterrents, repellents, confusants, plat allelopathy, *etc*. type of mode of activity.

Nerium indicum Mill has two synonyms: *Nerium oleander* L. and *Nerium odorum* Aiton.[3] Oleandrin and oleandrigenin are the toxic compounds of *Nerium* known as cardiac glycosides. Having a narrow therapeutic index can be toxic when ingested. Toxicity studies of animals administered *oleander* extract concluded that the rodent and avian species were observed to be relatively insensitive to *oleander* "cardioactive glycosides".[3]

Other mammals, however, such as dogs, and humans, are relatively sensitive to the effects of cardiac glycosides and the clinical manifestation of "glycoside intoxication".[3, 4, 5, 6] However, despite the common "poisonous" designation of this plant, very few toxic events in humans have been reported. According to the Toxic Exposure Surveillance System (TESS) in 2002 there were 847 human exposures to *oleander* reported to poison centers in the United States.[7] Despite this exposure level, from 1985 through 2005, only three deaths were reported. One cited death was apparently due to the ingestion of *oleander* leaves by a diabetic man.[8]

Experimental

Pulses, Insect Pests and Extraction: Pulses of varieties *viz.* green gram (*Vigna radiata* L Wilczek, K-851, Phule M-9339 and Kopergaon), black gram (*Vigna mungo* L TAU-1, TPV-4), Bengal gram (*Cicer arietinum* L Vijay) and Soybean (*Glycine max* L Merr, Moneta and MACs-13) were brought from Indian Council of Agricultural Research Mahatma Phule Krishi Vidyapeeth and dealers.

Our laboratory at North Maharashtra University has stored grain insect pest rearing facility; the cultures therefore are available in our laboratory itself. Pulse (50 gm, green gram, *Vigna radiata* (L) Wilczek) was used as a nutritional source and 15 pairs of insects *Callosobruchus chinensis*, released in each jar. Average daily temperature and humidity were recorded as 30 (±2)ºC and 60-70% respectively.

Fine powder of *Nerium indicum* and *Ricinus communis* leaves from locally collected and shed-dried leaves prepared. Powder of leaves passed through a fine muslin cloth. *Ricinus communis* oil was extracted from the Soxhlet method using hexane as a solvent. The powder of 50 g caster beans was prepared in a kitchen grinder and was Soxhlet extracted in 300 ml hexane for 3 h at 50^0C temperature. Clear oil obtained in vacuum evaporator by removing hexane.

Bioefficacy of *N. indicum* leaf dust and castor oil: Leaf dust of *N. indicum* was tested to control *C. chinensis* infestation in green gram (*Vigna radiata* L Wilczek) and soybean (*Glycin max* L Merr). *N. indicum* leaf dust (1 g/kg), *R. communis* seed oil (0.8, 1.2, 1.6 and 2 ml/kg), treatments were given to 5 kg grains. Leaf dust was applied as top dressing, and castor oil was applied to whole grains evenly. Freshly emerged, 20 *C. chinensis* and *Tribolium castanum* pests were released in jars. Untreated control was run simultaneously. Different biochemical parameters were monitored bimonthly to check the nutritional quality of pulses.

Testing of Biochemical parameters

Total carbohydrates of green gram and soybeans were estimated as per Dubois *et al.* (1956)[9]. Miller (1959)[10] and Lowry (1951)[11] methods were used to determine reducing sugar and total proteins respectively. A method described by Jayaraman, 1992[12] was taken to estimate protease activity.

A pre-weighed LOD dish with 1 g whole grain sample of both the pulses have been kept at 105^0C for 2 h. A loss in weight was recorded until the sample showed constant weight. Percent LOD was calculated by the following formula

A–B/C X 100

Where Weight of LOD dish with pulses before drying = A

Weight of LOD dish with pulses after drying = B

Weight of the pulse sample taken = C

For testing of seed viability, 100 grains were sowed, with 4 cm spacing from all directions, in earthen pots filled with wet sand. Pots were kept open to the Sun and irrigated by half-liter water 2 times a day. Germination frequency was recorded after 15 days.

Antinutritional factors of pulses:

Cyanide content: Cyanide content of 5 commonly utilized pulses *viz.* Bengal gram, green gram, soybean, red gram and black gram were determined by the method of AOAC (Association of Official Agricultural Chemists, Official method of Analysis, Washington DC 240 (1970))[13].

Estimation of tannins: Tannins from 5 commonly utilized pulses were also determined by the method of Association of Official Agricultural Chemists (AOAC, 1970)[13]. For this, 5 g samples of pulses were boiled in 400 ml water for 30 min. It is then diluted to 500 ml. To the 10 ml of the aliquot of the above extract, 25 ml indigo, carmine solution and 750 ml water were added. It is titrated against potassium permanganate, $KMnO_4$. End point changed in color first light green and then bright yellow. The volume of $KMnO_4$ was noted (X ml).

Fifty ml of gelatin solution was mixed with 100 ml of extract, 100 ml of sodium chloride and 10 g of kaolin, shaken well, To the 25 ml of clear solution added 25 ml of indigo carmine and 750 ml of water as above. The volume of $KMnO_4$ sol^n required was noted (Y ml). The quantity of $KMnO_4$ required to oxidize tannin is X-Y ml.

1 ml of 0.1 N oxalic acid = 4.2mg of tannin.

Total oxalates determination: The total oxalates in the form of oxalic acid were determined by the method given by Talpatra *et. al.*, (1948)[14].

1 ml of 0.05 N $KMnO_4$ = 0.00225 mg of anhydrous oxalic acid.

Estimation of Trypsin inhibitor activity: Kakade *et. al*. (1969)[15] method was used to determine the activity of Trypsin inhibitor. The readings were recorded on a spectrophotometer at 590 nm. The percent inhibition of Trypsin activity was calculated.

Standard Optical Density - Control Optical Density = Dab

Experimental Optical Density – Control Optical Density = Eab

Percent inhibition = Dab- Eab/Dab X 100.

Estimation of haemagglutinating activity: Serial dilution method of Liener and Hill (1953)[16] with modifications according to the method of Liener (1955)[17] was used to determine the haemagglutinating activity of the seed extract of 5 commonly utilized pulses. Centrifuged 3 ml of blood premixed with anticoagulant at 3000 rpm for 20 min. red blood cells were washed with normal saline (0.85%) and again centrifuged. One gram of samples was suspended in normal saline (10 ml). It was kept in the cold for overnight and was then filtered through Watman no. 41 filter paper. This agglutinin extract. Haemmagglutinin plates (Plexiglass plates with wells, 8X10) were used for carrying out the experiment.

Isolation of flavonoids: Flavonoids the plant secondary metabolite was isolated and purified from the aqueous foliage extracts according to the procedure described by Agarwal (Agarwal 1997)[18]. The extract was concentrated fivefold in volume in a rotaryvacuum evaporator (Rota vapor R- 124, Buchi, Switzerland) at 95(±2)^{0}C and the concentrate was washed with hexane (5X 50 ml) in a separating funnel. A lead acetate solution was added to the aqueous phase to precipitate tannins. The supernatant was diluted with distilled water (1:1 by volume), acidified with HCl (2 M), and heated on a boiling water bath for 30 min. The residue thus obtained was dissolved in methanol and used as partially purified flavonoids.

Bioefficacy testing of *Nerium indicum* partially purified flavonoids:

For bioefficacy, partially purified flavonoids (1 ml) were sprayed on Petri dish pre-loaded with Whatman filter paper no. 1. Ten paires of freshly emerged *C. chinensis* were released into the Petri dishes. Observations were noted at room temperature periodically (every hour) for 12 h. Negative (untreated) and solvent control were run simultaneously (Haliscak and Beeman, 1983)[19]. Each set was run in a triplicate and repeated three times in all three seasons.

Results and Discussion:

Many ancient Rishis and Moonies have said in Ayurvedic literature that no plant is without medicinal properties. Over 80% of the population of developing countries served by traditional medicines, mostly of plant origin. As per a report of the World Health Organization (WHO), at least 25% drugs of modern pharmacopoeia are derived from plants. Many synthetic chemicals are manufactured as per the prototype obtained from plants. All this is because the compounds obtained from plants are less or nontoxic; they are easily biodegradable, have minimum side effects, if any, are easily available and economical. As compared to the number of plants, very little study of their anti-microbial and pesticidal activities is available.

No doubt plants have excellent track record and novel lead in wound healing, but they have not been explored thoroughly for molecules having a wide range of biological activities. There are many advantages to planting products as they are non-polluting, self-contained, solar powered factories synthesizing a wide variety of secondary metabolites in minute quantities. Plant parts or their extracts in different solvents, therefore, find many applications in treating various diseases of human being as well as domestic animals, whereas a significant reduction in bird population (Vulture is now become a endangered species) has been seen due to use of synthetic drugs in their treatments. They have shown promising antimicrobial and insecticidal activities to be exploited in agriculture also (Hostettmann *et al*., 1998)[20]. Increased public awareness about the hazardous effect of synthetic insecticides has necessitated the use of plant derived products as an alternative strategy. Use of various products derived from Neem (*Azadirachta indica*) is one of the best examples in this regard.

Ricinus communis L., (*R. communis*), caster-bean and *Nerium indicum* Linn. (*N. indicum*) are commonly found in India. Castor-bean is a cultivated shrub belonging to family Euphorbeaceae is grown for its seed oil. The oil is known for its purgative properties in the Ayurvedic system of medicine (Joshi, 2000)[21]. Seeds of castor-bean plant are poisonous to people, mammals, due to presence of a toxic protein called ricin which has powerful cyto-toxic and heme agglutinating properties.[22] *N. indicum* is a common ornamental garden plant, belonging to family Apocynaceae.

Lab-scale trial to demonstrate potential of *N. indicum* for post-harvest preservation of pulses: Simple *in vitro* assay with extracts in different solvents has shown good results against pulse beetle. To facilitate application on a large scale, it was decided to explore the use of fine leaf powder of *N. indicum* as well as seed oil of *R. communis* directly on pulse grain during storage. For large scale applications, the technique/procedure should be logistically easy, technically simple, operationally user-friendly, and economically appealing. For this experiment, the pulses chosen were green grams and soybeans.

The leaf dust was manually applied on the grains at the rate of 1 g/kg at 5 kg level in pet jars. The castor oil was applied at the rate of 2 ml/kg.

The efficacy of treatments was measured in terms of number of insects observed (both live and dead) and grains damaged over a period of 12 months at an interval of 2 months. The results are presented in table 1 and 2 leaf dusts of *N. indicum* were found to be ineffective in protecting the grains during storage through the level of infestation was relatively less compared to untreated controls in green gram. In soybeans, no infestation was observed until the first 4 months. Castor oil was found to be better than both the leaf dusts in soybeans as well as green grams. Seed oil applied at the rate of 2 ml/kg showed no infestation even after 12 months of storage. The relatively less infestation in soybean can be attributed to the fact that it contains an inbuilt mechanism of defense in the form of different types of inhibitors (Mendki *et al*., 2001b)[23].

Studies of antinutritional parameters of five commonly utilized pulses: In this as well as all earlier studies (Mendki, 2002)[24]; various pulses have shown differential susceptibility to *C. chinensis* infestation under identical storage conditions. The resistance/susceptibility of pulses will be attributed to a no. of factors like shape, size, color, texture *etc.* The biochemical composition, both in terms of nutritional as well as anti-nutritional factors, may also play an important role. We have analyzed the anti-metabolite profile of five commonly utilized pulses for this purpose (Table 3). Of the five anti-metabolite factors, analyzed, red gram was found to have a high amount of cyanogenetic glycosides. Similarly, soybeans showed the presence of a high amount of Trypsin inhibitor activity. There was not much difference in the tannin and oxalate contents as well as haemagglutinating activity of different pulses.

In general, *C. chinensis* larvae survive well in seeds having low levels of anti-metabolite factors. The larvae must penetrate the seed coat and therefore seed coat thickness and composition could also be the descending factors. Tannin along with other phenolic compounds was found to be present in seed coats. It is also known to combine proteins in the gut and present digestion and subsequent assimilation in some animals (Marquardt *et al*., 1978)[25].

At this juncture, we do not know what role these facts playing host discrimination and consequent behavior of *C. chinensis* females. What all could be said at this point is that an optimal balance of nutritional and anti-nutritional factors decides *C. chinensis* infestation.

Table 1: Efficacy of *N. indicum* leaf dust and *R. communis* seed oil against *C. chinensis* in green gram, during storage

Time	Treatment	Insect count		No. of grains damaged
		Live	Dead	
2 months	*N. indicum* leaf dust (1 g/kg)	12	2	2
	R. communis seed oil (0.8 ml/kg)	0	3	1.4
	Untreated control	16	9	9
4 months	*N. indicum* leaf dust	9	5	4
	R. communis seed oil	12	10	1.9
	Untreated control	21	12	17
6 months	*N. indicum* leaf dust	10	6	8
	R. communis seed oil	16	12	12
	Untreated control	27	16	23
8 months	*N. indicum* leaf dust	12	6	11
	R. communis seed oil	19	13	27
	Untreated control	32	19	37
10 months	*N. indicum* leaf dust	12	6	11
	R. communis seed oil	19	13	27
	Untreated control	32	19	37
12 months	*N. indicum* leaf dust	12	6	11
	R. communis seed oil	19	13	27
	Untreated control	32	19	37

- Twenty *C. chinensis* inoculated on 0 hour, in 5 kg of fresh, un-infested soybean in pet jars.
- Observations were recorded in 100 gm composite sample.

Table 2: Efficacy of *N. indicum* leaf dust and *R. communis* seed oil against *C. chinensis* in soybean, during storage

Time	Treatment	Insect count		No. of grains damaged
		Live	Dead	
2 months	*N. indicum* leaf dust (1 g/kg)	0	0	2
	R. communis seed oil (0.8 ml/kg)	0	0	1.3
	Untreated control	0	0	7
4 months	*N. indicum* leaf dust	0	0	7
	R. communis seed oil	0	0	1.3
	Untreated control	0	4	23
6 months	*N. indicum* leaf dust	2	1	11

Time	**Treatment**	**Insect count**		**No. of grains damaged**
		Live	**Dead**	
	R. communis seed oil	0	0	1.2
	Untreated control	7	8	30
8 months	*N. indicum* leaf dust	6	5	15
	R. communis seed oil	0	0	1.2
	Untreated control	8	13	46
10 months	*N. indicum* leaf dust	6	5	15
	R. communis seed oil	0	0	1.2
	Untreated control	8	13	46
12 months	*N. indicum* leaf dust	6	5	15
	R. communis seed oil	0	0	1.2
	Untreated control	8	13	46

- Twenty *C. chinensis* inoculated on 0 hour, in 5 kg of fresh, un-infested soybean in pet jars.
- Observations were recorded in 100 gm composite sample.