



PROJECT NO. : TOX-355F
PRODUCT : NHH 44 Bt-COTTON SEEDS
STUDY : ALLERGENICITY STUDY
REPORT NO. : 000046991
DATE : 18.5.2007

ALLERGENICITY STUDY
WITH
NHH 44 Bt-COTTON SEEDS

Report for:

**UNIVERSITY OF AGRICULTURAL SCIENCES
AGRICULTURAL RESEARCH STATION
DHARWAD-580007
KARNATAKA**

Guidelines:

**‘DBT, Guidelines for Toxicity and Allergenicity Evaluation of
Transgenic Seeds, Plants and Plant parts’**

Prepared by:

**DEPARTMENT OF TOXICOLOGY
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QUALITY ASSURANCE STATEMENT

This is to certify that the work described in the study report entitled ‘Allergenicity study’ with ‘NHH 44 Bt-Cotton seeds’ has been examined with respect to the study protocol and the Standard Operating Procedures in accordance to ‘DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts’ for non clinical laboratory studies.

The report provides true and accurate record of results obtained.

Sr. SCIENTIST
QUALITY ASSURANCE



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STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

We, the undersigned take overall responsibility to conduct the work described in the study entitled ‘Allergenicity study’ with ‘NHH 44Bt-Cotton Seeds’ performed with respect to the study protocol and the Standard Operating Procedures in accordance to ‘DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts’ for non-clinical laboratory studies.

All the raw data, documentation, protocol and copy of final report are retained in the archives at Shriram Institute for Industrial Research, Delhi.

STUDY DIRECTOR

HEAD, DEPT. OF TOXICOLOGY

Approved for issue

**DEPUTY DIRECTOR
(MANAGEMENT)**



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SCIENTIFIC PERSONNEL INVOLVED IN THE STUDY

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SUMMARY

This study was carried out to determine the allergic reactions induced by test substance ‘NHH 44 Bt-Cotton seeds’ with reference to ‘NHH 44 Non Bt-Cotton seeds’ in rabbits. The following battery of tests were conducted to determine the allergic potential of the ‘NHH 44Bt-Cotton seeds’.

1. Passive Cutaneous Anaphylaxis test (PCA)
2. Prausnitz-Kustner test (PK)
3. ELISA test

1. The Passive cutaneous Anaphylaxis test (PCA) involves the observation of well defined blue areas indicating the sites of antigen-induced extravasation of fluid due to interaction with tissue fixed antibody when 0.1 ml of test substance is injected intradermally. After 24 hours, 0.6 ml of test substance was injected intravenously together with 0.4 ml of Evan’s blue.

2. The Prausnitz-Kustner (PK) test involves the measurement of diameter and intensity of the developed skin lesions, when 0.05 ml of the test substance injected intradermally followed by challenge with 0.05 ml of the test substance intradermally after 24 hours.

3. Enzyme linked immunosorbant assay (ELISA) detect the levels of IgE (if present in the test serum) and solid phase anti-IgE, which can be measured spectrophotometrically.

On the basis of the Passive cutaneous anaphylaxis (PCA) test, Prausnitz-Kustner (PK) test and ELISA, the test substance ‘NHH 44Bt-Cotton seeds’ was found to be non-allergenic.



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INTRODUCTION

- This study was carried out to determine the allergic reactions induced by the test substance 'NHH 44Bt-Cotton seeds' in rabbits. The test substance was administered to the test animals by oral route in dietary preparation.
- This study is designed to evaluate the allergenic potential of test substance 'NHH 44 Bt-Cotton seeds'.



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OBJECTIVES

- (a) To determine the allergic potential of the test substance when administered orally (dietary).

- (b) To obtain information on allergic reactions likely to arise after repeated exposure.



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TEST SUBSTANCE

The sponsor is responsible for necessary characterization and evaluation of the test substance. The details of the test substance provided by the sponsor are as follows:

PRODUCT NAME : NHH 44 Bt-COTTON SEEDS
& NHH 44 NON Bt-COTTON SEEDS

SPONSOR : UNIVERSITY OF AGRICULTURAL
SCIENCES, DHARWAD

MATERIAL DESCRIPTION : DARK BROWN COLOURED
SEEDS

PACKED IN : WHITE COLOURED PLASTIC
BAGS

DATE OF COMMENCEMENT : 16. 12. 2006
OF STUDY

DATE OF COMPLETION : 28.03. 2007
OF STUDY



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EXPERIMENTAL DESIGN

Name of species : Rabbits

Strain of the animals : New Zealand white

No. of animals used per group : 10

Age of the animals used : 6 to 8 weeks

Weight range : 1.5- 2.0 Kg

Acclimatization period : 7 Days

Vehicle : Conventional diet



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HUSBANDRY

The room temperature was maintained at $20 \pm 2^{\circ}$ C with 30 - 70 % relative humidity.

The room was ventilated at the rate of approximately 15 air changes per hour.

Lighting was controlled to give 12 hours artificial light (8 a.m. - 8 p.m.) each day.

DIET

Water and standard pelleted feed (Amrut feeds ltd.) was freely available to the animals of both the groups *ad-libitum*.



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EXPERIMENTAL PROCEDURE

A dose of 10% of the total diet of transgenic and non-transgenic foodstuff were incorporated into the feeding pellets of the rabbits of respective groups and fed for sixty days for sensitization with test proteins.

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1. Passive Cutaneous anaphylaxis (PCA)

Principle- PCA is produced with the sera of allergic animal by challenging intradermally sensitized sites with intravenously injected antigen/allergen plus dye. Well-defined blue areas appear, indicating the sites of antigen-induced extravasations of fluid due to interaction with tissue fixed antibody.

Procedure- Naive animals were shaved on the back and flanks. Unblemished skin sites were selected and cleaned with 70% alcohol. 0.1 ml of test substance is injected intradermally using tuberculin syringe. After 24 hours, 0.6 ml of test substance was injected intravenously together with 0.4 ml of Evan’s blue (25 % in physiological saline).

Observations- After 30-45 min. of intravenous injection, animals were killed and the lesions on the skin were evaluated for their intensity and diameter (Table-1).



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TABLE-1 Observations (PCA)

Group	Animal No.	Area of dye Extravasations	
		Measurement (mm)	Intensity
Control Group (NHH 44 Non Bt-Cotton seeds)	Rabbit 1		-
	Rabbit 2	5	+
	Rabbit 3	-	-
	Rabbit 4	-	-
	Rabbit 5	4	+
	Rabbit 6	5.1	+
	Rabbit 7	4.8	+
	Rabbit 8	-	-
	Rabbit 9	-	-
	Rabbit 10	3.2	+
Treated (NHH 44 Bt-Cotton seeds)	Rabbit 1	4	+
	Rabbit 2	-	-
	Rabbit 3	-	-
	Rabbit 4	-	-
	Rabbit 5	4.5	+
	Rabbit 6	-	-
	Rabbit 7	-	-
	Rabbit 8	-	-
	Rabbit 9	3.6	+
	Rabbit 10	5.2	+

* The test sample produces area of extravasations of about 15 – 20 mm with at least +++ intensity indicative of positive reaction.



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2. Prausnitz-Kustner (PK) test

PRINCIPLE-When normal skin is injected with reaginic serum, the reaginic antibodies become attached to the skin mast cells and the injected area of the skin acquires the specific skin reactivity towards challenged antigen/allergen,

PROCEDURE- Naive animals were shaved on the back and flanks. Unblemished skin sites were selected and cleaned with 70% alcohol. 0.05 ml of test substance was injected intradermally using tuberculin syringe. Control site were injected with 0.05 ml of physiological saline. After 24 hours, 0.05 ml of test substance was injected intradermally. 30-45 min. later animals were killed. The skin was opened so that the lesions could be evaluated. Diameter was measured and the intensity of the lesions were assessed.

OBSERVATIONS- After challenge wheal and flare formation (>3 mm) on the skin of animals was outlined and recorded (Table-2).



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TABLE-2 Observations (PK Test)

Group	Animal No.	Wheal and Flare response (mm)
Control	Rabbit 1	
	Rabbit 2	2.3
	Rabbit 3	-
	Rabbit 4	-
	Rabbit 5	1.6
	Rabbit 6	2.2
	Rabbit 7	2.3
	Rabbit 8	-
	Rabbit 9	-
	Rabbit 10	1.6
Treated	Rabbit 1	2.3
	Rabbit 2	-
	Rabbit 3	-
	Rabbit 4	-
	Rabbit 5	2.1
	Rabbit 6	-
	Rabbit 7	-
	Rabbit 8	2.2
	Rabbit 9	2.6
	Rabbit 10	1.6

* A wheal and flare formation with >3 mm in the skin indicates positive reaction.



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3. Enzyme-linked Immunosorbent assay (ELISA)

PRINCIPLE-The IgE present in the serum is made to react with solid phase anti-IgE. The label, which in the present assay is an enzyme is taken up by the washed solid phase and is proportional to the IgE content of the sample under test and is then measured spectrophotometrically.

PROCEDURE- Test proteins were adsorbed on microtitre plates. Test substance were pipetted into the wells of the microtiter plate together with ready to use anti-human IgE peroxidase conjugate. After a 30 minutes incubation at room temperature, the plate was rinsed with diluted wash solution. Then the substrate solution was pipetted and incubated for 15 minutes. The colour development was terminated by the addition of a stop solution provided in the kit.

OBSERVATIONS- Optical density of colour of plates were measured spectrophotometrically at the wavelength of 450 nm, using ELISA plate reader. The concentration of the antibodies is directly proportional to the intensity of the colour (Table-3a & 3b).



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TABLE-3a Observations (ELISA)

Standard	I.U./ml	OD
1	0	0.037
2	5	0.269
3	25	0.421
4	100	0.811
5	250	0.831
6	1000	2.914



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TABLE-3b Observations (ELISA)

Group	Animal No.	OD of Test/ control – OD of Blank
Control	Rabbit 1	0.004
	Rabbit 2	0.004
	Rabbit 3	0.005
	Rabbit 4	0.006
	Rabbit 5	0.005
	Rabbit 6	0.003
	Rabbit 7	0.006
	Rabbit 8	0.007
	Rabbit 9	0.005
	Rabbit 10	0.004
Treated	Rabbit 1	0.007
	Rabbit 2	0.006
	Rabbit 3	0.004
	Rabbit 4	0.005
	Rabbit 5	0.005
	Rabbit 6	0.004
	Rabbit 7	0.006
	Rabbit 8	0.005
	Rabbit 9	0.007
	Rabbit 10	0.003

*Test is considered positive when the values are two fold or higher than the control



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RESULTS

Under the conditions of the study, the test substance ‘NHH 44 Bt-Cotton seeds’ was found to be negative for Passive cutaneous anaphylaxis test (PCA), Prausnitz- Kustner (PK) Test and Enzyme Linked Immunosorbent assay (ELISA). Therefore, the test substance ‘NHH 44 Bt-Cotton seeds’ was found to be non-allergenic and is comparable to the ‘NHH 44 Non Bt-Cotton seeds’.