Effects of mustard and *nimbecidine* on the post embryonic development and expression of ovarian protein of - *Alphitobius diaperinus* Panzer, 1779 (Insecta: Coleoptera: Tenebrionidae)

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Abstract- Coleopteran insects constitute major stored grain pests whose control measures are mainly carried out by fumigants. Use of botanical insecticides has expanded impetus in recent insect pest management programme due to minimum health hazards and low toxicity. In present investigation, nimbecidine has been used for the control of *Alphitobius diaperinus*, a minor stored grain pest. So far its effects on the post embryonic development are concerned; substantial decline in survival rate, delayed development, declining levels of fecundity as well as substantial mortality were noticed. Insecticidal efficacy of this phytoproduct is attributed due to its antifeeding and growth inhibition roles. Free amino acid level (showing Alanine, Glycine, Glutamic acid, Histidine, Leucine / Isoleucine, Methionine, Lysine, Proline and Tyrosine) in the post embryonic developmental stages during the mid instar periods of 4th, 5th and 6th instar larvae. male and female of pupae and adults depicted amino acid specific alteration. Except methionine all other amino acids showed substantial decline keeping pace with progression of larval maturity. Variation of amino acids in two sexes showed that except methionine and tyrosine other amino acids presented marked differences; only alanine and glycine showed marked increase in the females while other amino acids presented marked decreased content. Gel electrophoresis study revealed that in the ovary prior to oviposition, a distinct protein band was noted. But in mustard and nimbecidine treated insects this band was very faint which indicated that these phytoproducts exert an adverse effect on the expression of a vitellogenic protein. Nutrition seems to be critical for the release of gonadotropin hormone in initiating vitellogenesis committing to oviposition. As these phytoproducts exert antifeeding activity, it directly affects gonadotropin synthesis which has been reflected in the faint appearance of the protein regulating oviposition. Thus this investigation assesses the role of mustard and nimbecidine in the control of this minor pest by inhibiting the expression of ovarian protein affecting the fecundity of the insect.

Index Terms- *Alphitobius*, Nimbecidine, Mustard, fecundity.

I. Introduction

Coleopteran insects render havoc damage to the stored food grains both for human consumption and sowing. Lesser mealworm *(Alphitobius diaperinus*) is considered as a minor pest causing damage to different stored grains especially in poorly maintained grain processing warehouses. With the advent of chemical insecticides, the most commonly used method of controlling stored grain pests is the application of synthetic contact insecticides and fumigants (Chaube, 2008). The indiscriminate use of broad-spectrum insecticides has created more problems than resolving them. The development of pesticide resistance, accumulation of toxic residues in food, serious health hazards for human applicators and undesirable environmental pollution are acute critical problems that have drawn attention of the scientists to adopt alternative, natural pesticides that are safe and environmentally acceptable (Park et al. 2003; Wang et al., 2011*)* and scientists have recommended use of eco-friendly biopesticides, with special focus on the botanical insecticidal compounds which have long been treated as alternative to synthetic chemical insecticides for pest management, because these are harmless to the environment due to their biodegradation (Cosimi et al. 2009, Nesci et al. 2011, Isman, 2015).

Over past three decades, neem, *Azadirachta indica* has come under close scientific scrutiny as a source of natural pesticide (Schmutterer, 2002). Neem products have been shown to be effective protectants of grains against infestation by grain weevils, grain borers, armyworm and several species of storage moths (Tanzubil, and McCaffery, 1990). Since essential oils of different plants are a complex mixture of components they work together to inhibit resistance to the insecticide. They are strongly recommended to be used in IPM programme. In addition, mustard has also been used as insecticidal agent (Costa et al, 2006; Khater and Khater 2009; Fouad, 2013).

In spite of different attempts to explore all possible measures to control *A. diaperinus* (Watson et al, 2003. Wolf et al 2015) no attempt has been made to show deleterious effects of phytoproducts on this pest.

Most insects have a female specific storage protein which are synthesized in larval fat body and are sequestrated in fat body cells as a protein granule during larval-pupal metamorphosis. Only female fat bodies of pupae degrade storage proteins to use them as source of amino acids and synthesize vitellogenin, a yolk protein precursor to vitellin. In insects, dietary factors stimulate the production of major gonadotropin that regulates the production of vitellogenin (Juliano et al. 2004). Nimbecidine has

a direct anti-feeding role due to its specific odour which directly affects gonadotropin production that eventually reduces the production of distinct ovarian protein that culminates in reduced oviposition (Wegener *et al*, 2013; Amsalem *et al*, 2014).

With a view to this unexplored fields, present investigation was undertaken to report the effects of nimbecidine, on the post embryonic developmental stages of *A. diaperinus*. In addition it also explores possible role of some phytoproducts in the control of pests by evaluating the expression of ovarian protein playing crucial role in oviposition affecting the fecundity of the insect.

II. MATERIALS AND METHODS

Experimental insect: The insects were collected from a neighbouring FCI, ware house from damp, almost dark sites beneath of the polythene sheets of the wheat stacks. In the laboratory, they were maintained in dark containers at 28 +/- 2^{o} C and 70 – 75% RH on wheat. To check fungal infection 2% Nepazin was mixed with the wheat. Temperature and humidity were routinely examined by thermometer and hygrometer. By routine examination from the lower stratum of the culture pots pupae were separated and hatched adults were kept in separate pots to assess the normal developmental profiles. Their incubation period is 4 - 7 days and it takes 40 – 60 days to complete the life cycle. After mating, on average, a female beetle lays about 200 – 400 eggs.

Preparation of phytoproducts:

Nimbecidine: 50 gram semi crushed wheat grains were treated with 0.5 ml, 0.4 ml, 0.2 ml, 0.1 ml of nimbecidine (purchased from T. Stanes and company limited) and in each case the volume was made up to 1 ml. 20 adults were allowed to stay in the treated beaker for several weeks to report detrimental effects of nimbecidine on the post embryonic developmental stages of this beetle.

Mustard oil: Mustard **(***Brassica sp.*) oil (Trade no. W408201 w246611 purchased from Sigma Aldrich company) was used for experiment.

Adult mortality in Nimbecidine treated grains: All the developmental stages were divided into control and four treated groups and their changes were recorded separately. Mortalities were recorded at every 24 hrs until all larvae or pupae either died or developed to the next stage.

The effect of different dosages of nimbecidine treated wheat grains on adult mortality of *A. diaperinus* was assessed. 50 gram of semi crushed wheat grains, were soaked with 0.5 ml, 0.4 ml, 0.2 ml, 0.1 ml of Nimbecidine in individual beakers to ensure the even spread of the material over the surface of the grains. The grains were then kept in a dark beaker and infested with 7 days old 20 adult insects. Each beaker was covered with cotton plug. Mortality was recorded after 1, 2 and 3 weeks.

Biochemical estimations:

Estimation of Protein: For the quantitative estimation of total body protein, weighed larvae of different instars and ovary of adult females were weighed and homogenized in 1N NaOH and the protein was estimated following the process of Lowry et al (1951). For the estimation of total protein levels 2 larvae and one adult insect were used per estimation. Average data of six estimations were considered as final value. Qualitative and quantitative estimation of free amino acids by two dimentional paper chromato- graphy was carried out following the process of Smith and Seakins (1976).

For the qualitative assessment of proteins, separation of bands of respective proteins was carried out by the application of Agarose gel electrophoresis using standard method.

III. RESULTS

Description of post embryonic developmental stages: *Alphitobius diaperinus* shows adult, egg, larva and pupa in its life cycle.

Eggs are about 0.5 mm long, creamy white to tan coloured, slender with slightly rounded ends. Larvae hatched in 6-7 days and development was completed in 40 to 60 days. There are 6 larval instars. The duration of each instar was 3, 5, 5, 6, 8 and 12 days respectively. Mature larvae are7 - 11 mm long. Pupae are creamy white to tan coloured, 6 - 8 mm long. Pupal development was completed in 6 days. The newly hatched pharate adults appeared brown that gradually turned black.

Total protein level during the mid instar periods of 4th, 5th and 6th instar periods appeared more or less ulaltered but in female pupae and adults the levels appeared slightly less than males (Table 3).

Free amino acid level (showing Alanine, Glycine, Glutamic acid, Histidine, Leucine / Isoleucine, Methio- nine, Lysine, Proline and Tyrosine) in the post embryonic developmental stages during the mid instar periods of 4th, 5th and 6th and male and female pupae and adults depicted amino acid specific alteration. Except methionine all other amino acids depicted substantial decline keeping pace with progression of larval maturity. Variation of amino acids in two sexes of showed that except methionine and tyrosine other amino acids presented marked differences; only alanine and glycine showed marked increase in the females while other amino acids presented marked decrease in the amino acid content (Table 3).

Effects of phytoproducts: Treatment with nimbecidine and mustard depicted substantial decline in survival rate showing 44% and 42% decline in number respectively (against 26% in control insects). Adverse effects of phytoproducts were pronounced from third instars and increased duration of the larval instars appeared 21.62% in control insects while 65% in nimbecidine treated insects. The weight however did not decline substantially (Table 1). The rate of mortality in nimbecidine treated insects appeared maximum in 0.2 ml dose and minimum in 0.025 ml dose (the effects were noted after 1, 2 and 3 weeks). Each set of experiment was replicated 6 times and average values were considered.

Alteration of protein level in the total body and ovary after treatment with nimbecidine: Protein levels in the whole body, exhibited 80.27% decline (following the treatment with Nimbecidine.

Of the total body protein content, 50.29% was found in the ovary it appeared which exhibited sharp decline following treatment with nimbecidine, depicting 60.6% decline in protein level. Qualitative assessment of the expression of the ovarian proteins following treatment with nimbecidine depicted a contrasting result so far the expression of protein bands are

concerned (Fig. 1). In respect of control insects, the band depicting ovarian protein appeared very faint.

Statistical analysis of data: The statistical calculations like estimation of SE and P values were calculated following the method of Panse and Sukhatme (1978).

IV. DISCUSSION

In spite of the tremendous advent of the chemical insecticides, due to its versatile side effects, different phytoproducts have long been treated as alternative non-hazardous insecticides that have minimum side effects and from the view point of rendering resistance and imparting toxic effects in grains, due to their biodegradable properties, use of phytoproducts has been gaining importance day by day. The insecticidal activity of essential oils and plant extracts against different stored-product pests has been evaluated (Kim et al. 2003; Lee et al. 2003; Cetin and Yanikoglu 2006; Obeng-Ofori, 2007; Ayvaz et al. 2009). Essential oils from different plant species possess ovicidal, larvicidal, and repellent properties against various insect species and are regarded as environmentally compatible pesticides (Cetin et al. 2004).

Nimbecidine is a totally natural neem-oil based product with Azadirachtin as the major active ingredient in addition to other active compounds like Meliantriol, Salanin, Nimbin. Nimbecidine acts as an insect repellant, antifeedant, growth regulator and mating disruptor. As an effective supplement for synthetic pesticides, it has been proved and recognized as ideal phytoproduct in the IPM programme.

In our study, nimbecidine applied at much lower dosages (0.5 ml, 0.4 ml, 0.2 ml, 0.1 ml) reduced progeny production in *A. diaperinus* by 43.1, 42.7, 50.5 and 64.4%, respectively. These results corroborate with the findings of Jilani et al. (1988) reporting a significant reduction in the number of progeny emergence, delayed development, failure of pupation and abnormal pupae and adults of *T. castaneum* treated with calneem oil; Dunkel et al (1990) reporting 70% mortality for adult *S. oryzae* exposed for 2 weeks to neem oil treated wheat; El-Lakwah and El-Kashlam (1999) reporting mortality and progeny reduction of *S. oryzae*, *R. dominica*, *C. maculatus* and *T. castaneum* adults. These antigrowth regulating activities of Azadiractin seems to be attributed for the inhibition of the release of gonadotropic hormone affecting the fecundity of the insects (Banken and Stark 1997; Khater and Khater, 2009). Due to the presence of some additional components other than azadirachtin, our results cannot conclusively state that these are only due to azadirachtin though it seems to be due to azadirachtin.

Free amino acid levels during the mid instar periods directly reflect the balance between food consumption and various synthetic activities. Gradual decline in the level of respective amino acids is due to their involvement in the formation of various organs at the larval-pupal transitional periods. Less amount of amino acids in females might be due to their involvement in the formation of ova.

Substantial decline in the level of amino acids following nimbecidine treatment may be due to the anti-feeding role of nimbecidine. However such effects may indirectly result low synthesis of gonadotropic hormone which is corroborated by the quantitative and qualitative variation of proteins following nimbecidine treatment.

Attainment of threshold level of nutrient stimulates the release of hormones that commit to the developmental transition and reproduction. Most insects require a cumulative feeding threshold to initiate vitellogenesis and commit to oviposition. It is reported that nutritional threshold regulate the level of major gonadotropin - initiating vitellogenesis committing to oviposition (Fronstin and Hatle, 2008).

Most insects have a female specific storage protein - synthesized in larval fat body cells and are seques- trated in fat body as a protein granule during larval-pupal metamorphosis. Pupae of female fat bodies synthesize vitellogenin from this storage protein which is taken up by the follicle cells of ovaries and store it as a yolk protein granule. Vitellogenins are precursors of the major egg storage protein, vitellin (Tufail and Takeda, 2008). In insects, nutrition is the sole regulating factor that often stimulates the production of the major gonadotropins that controls reproduction. The maximum titer of gondotropin always occurs before oviposition (Hatle et al., 2003a) which suggests that threshold feeding by the females is needed to allow gonadotropin production inducing production of vitellogenin mRNA (Juliano et al., 2004; Fei et al. 2005).

We predict a statistically significant interaction of diet and timing of gonadotropin production on the onset of vitellogenesis and timing of oviposition. Substantial decline in the level of ovarian protein corroborates this data. Disruption of feeding process may be attributed to the decline in the concentration total body protein in other organs.

Gel electrophoresis study in respect of the nimbecidine treatment reveals that in the ovary, prior to oviposition, a distinct band occurs depicting the occurrence of a protein whose actual characterization has not yet been possible but it seems to be vitellogenin. In nimbecidine treated insects this band is very faint which indicates that nimbecidine has a distinct effect on the inhibition of vitellogenin like protein. Nimbecidine has a direct effect on the rate of feeding due to its specific odour and less assimilated nutritional factors seems to reduce the production of gonadotropin which eventually affects the production of distinct ovarian protein that culminates in oviposition (Wegener et al, 2013; Amsalem et al, 2014).

Appearance of very indistinct protein band depicting the location of the protein that is mainly responsible for oviposition is mainly attributed to the anti-feeding role of these phytoproducts and indirect synthesis of JH that controls ova production. This finding corroborates many previous findings in which workers directly or indirectly pointed out that phytochemicals can play a crucial role in the control of pests by altering the levels of gonadotropin - particularly JH. We however cannot conclusively state that declining level of distinct ovarian protein is vitellogenin but may be the mixture proteins that collectively affects reproduction (characterization of this band needs be identified in our further study). Of the various concentrations of phytoproducts the lowest concentrations were approved since from the view point of feasibility of such applications, higher concentrations cannot be recommended because treatment of such phytoproducts rendered obnoxious smell that could not encourage feeding of such grains.

V. CONCLUSION

With a view to the afore said discussion it can be inferred that neem derived products would gain commercial importance not only in the protection of stored commodities, but a practical alternative for effective and sustainable crop protection that could substantially minimize potential for environmental contamination and human health risks. Whether new insecticides based on these plant metabolites will be eventually developed in the future for pest control remains to be proved in practice since field application reports of most of the phytoproducts are scarce.

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Table – 1. Gradual decline in the survival rate (number), duration (days), size (mm) and weight (mg) of different post embryonic stages in response to nimbecidine treatment against untreated insects:

Parameters	**Egg**	**1st Instar**	**2nd Instar**	**3rd Instar**	**4th Instar**	**5th Instar**	**6th Instar**	**Pupa**	**Adult**
Survival rate									
Control	50+/- 9	48+/- 8	47+/- 9	46+/- 7	44+/- 9	42+/- 8	40+/- 8	38 +/-10	37+/-12
Nimbecidine	50 +/- 6	42 +/- 5	45 +/- 4	41+/- 6	38 +/- 5	36 +/- 4	34 +/- 4	32 +/- 4	28 +/- 4
Mustard	50 +/- 3	45 +/- 4	43 +/- 5	44 +/- 6	43 +/- 4	38 +/- 5	36 +/- 4	31 +/- 4	29 +/- 5
Duration									
Control	6-7	3-4	5-6	5-06	6-07	8-09	12-14	6-07	
Nimbecidine	6-7	3-4	5-6	8-11	8-13	8-15	14-19	7-12	
Mustard	6-7	3-4	5-6	5-08	6-11	8-12	12-17	6-9	
Size (mm)									
Control	1.5	2.2+/-0.1	3.2+/-0.2	4.3+/-0.4	5.4+/-0.5	7.7+/-0.2	8.4+/-0.3	5.8+/-0.6	6.1+/-0.5
Nimbecidine	1.5	2.2+/-0.2	3.2+/-0.2	4.0+/-0.2	4.8+/-0.2	6,9+/-0.3	7.6+/-0.1	5.1+/-0.3	5.8+/-0.2
Mustard	1.5	2.2+/-0.2	3.2+/-0.2	4.2+/-0.4	5.0+/-0.2	7.2 +/-0.2	8.1+/-0.3	5.4+/-0.1	6.0+/-0.2
Body Color	Milky white	Creamy white	Light brown	Brown	Deep brown	Dark Brown	Dark Brown	Off white	Black
Weight (mg)									
Control	0.0004	0.0005	0.0010	0.0018	0.0036	0.0071	0.0092	0.014	0.015
Nimbecidine	0.0004	0.0005	0.0010	0.0015	0.0032	0.0066	0.0081	0.012	0.011

Mustard	0.0004	0.0005	0.0010	0.0017	0.0033	0.0070	0.0088	0.013	0.012

Table 2. Mean number of *A. diaperinus* larvae, pupae and adults emerged from wheat treated with different dosages of Nimbecidine at different times after oviposition period. Data are mean ± SE (n=5 taking 20 insects).

Doses % (v/v)	1st instar larva	2nd instar larva	3rd instar larva	4th instar larva	5th instar larva	6th instar larva	Pupa	Adult
Control	53±5.2	48.6±5.1	45±4.4	44±3.51	42.6±3.8	39±4.72	23± 2.64	32±4.16
0.2ml	31±3.2	26±3.5	24.6±0.3	23±0.7	22±1.5	20±1.5	15±2.8	11±2.8
0.1ml	34.6±2.4	31±2.8	30.3±1.8	27.6±1.2	24.3±2.3	23±2.4	17.6±1.4	16±1.15
0.05ml	38.3±1.2	37±1.9	34.3±1.4	33±1.5	29±2.8	26±3.5	18.6±1.8	16.3±2.1
0.025ml	39±1.5	36.6±0.88	35.3±0.3	32.3±1.2	30±.6.7	28.3±2.2	26.3±2.0	24.6±2.6

Table 3: Variation of total body amino acid concentration (□g / 100 mg body wt.) and protein (mg / 100 mg body wt.) of 4th, 5th and 6th instar larvae, male and female pupae and male and female adults of *A. diaperinus*. Data are mean ± SE (n=6).

Amino acids	Larval instars			Pupa		Adult	
	Fourth	Fifth	Sixth	Male	Female	Male	Female
Alanine	149 ± 2.8	122 ± 2.2	105 ± 3.1	98 ± 2.4	105 ± 4.1	92 ± 3.1	94 ± 3.4
Glycine	89 ± 2.2	71 ± 1.6	53 ± 1.2	67 ± 3.1	74 ± 3.2	64 ± 2.2	69 ± 3.1
Glut. Acid	251 ± 3.9	205 ± 3.8	182 ± 3.8	152 ± 2.9	142 ± 2.8	142 ± 3.1	135 ± 2.4
Histidine	53 ± 1.6	49 ± 1.2	43 ± 2.3	49 ± 2.7	42 ± 2.1	41 ± 1.9	38 ± 1.3
Leu./ Isoleucine	42 ± 1.2	38 ± 1.5	34 ± 1.6	27 ± 1.1	22 ± 1.3	24 ± 1.3	19 ± 0.8
Methionine	15 ± 0.8	17 ± 0.9	16 ± 0.6	09 ± 0.3	08 ± 0.6	07 ± 0.4	05 ± 0.2
Lysine	132 ± 2.1	128 ± 3.9	125 ± 3.1	91 ± 1.9	85 ± 1.8	52 ± 0.9	44 ± 0.6
Proline	311 ± 4.2	291 ± 4.1	285 ± 3.4	268 ± 3.9	259 ± 2.9	228 ± 2.8	219 ± 3.1
Tyrosine	29 ± 0.7	25 ± 0.9	21 ± 0.5	19 ± 0.5	17 ± 0.6	19 ± 0.6	17 ± 0.7
Protein	12.8 ± 0.8	13.6 ± 0.6	14.5 ± 0.5	15.6 ± 0.7	14.1 ± 0.6	16.8 ± 1.2	14.2 ± 2.2

Table – 4. Substantial alterations in the level of amino acids (□g/100 mg body wt.) in control and Nimbecidine (after 3, 6 and 9 days) and mustard treated (after 5 and 10 days) in adults (female) *A. diaperinus*. Data are mean ± SE (n=6).

Amino acids	Control	Nimbecidine treated			Mustard treated	
		After 3 days	After 6 days	After 9 days	After 5 days	After 10 days

Alanine	94 ± 3.4	86 ± 3.9	97 ± 2.9	95 ± 2.7	85 ± 2.5	81 ± 2.4
Glycine	69 ± 2.9	59 ± 2.2	53 ± 2.1	58 ± 2.1	52 ± 3.1	55 ± 2.9
Glut. acid	135 ± 3.2	119 ± 2.8	109 ± 2.9	95 ± 3.1	92 ± 3.5	85 ± 2.8
Histidine	38 ± 1.7	29 ± 1.1	31 ± 1.4	35 ± 1.1	28 ± 2.5	32 ± 2.7
Leu. / Isoleucine	19 ± 0.9	16 ± 0.8	21 ± 0.9	34 ± 0.6	15 ± 1.8	13 ± 1.1
Methionine	05 ± 0.8	04 ± 0.7	04 ± 0.4	05 ± 0.4	06 ± 0.3	05 ± 0.5
Lysine	44 ± 1.9	49 ± 1.8	61 ± 1.9	78 ± 1.7	48 ± 1.7	55 ± 0.7
Tyrosine	20 ± 0.8	19 ± 0.1	21 ± 0.2	19 ± 0.4	17 ± 0.4	19 ± 0.9
Proline	216 ± 2.8	202 ± 3.1	211 ± 3.8	201 ± 3.2	201 ± 3.2	195 ± 3.1
Protein	16.2 ± 2.2	11.1 ± 0.9	11.4 ± 1.8	13.5 ± 1.3	12.9 ± 2.8	14.3 ± 3.1

Table-5. Substantial alterations in the level of amino acids (□g / 100 mg body wt.)) in the ovary of control and Nimbecidine treated (after 3, 6 and 9 days) adults of *A. diaperinus*. Data are mean ± SE (n=6).

Amino acids	**Control**	**Nimbecidine treated**			**Mustard treated**	
		After 3 days	**After 6 days**	**After 9 days**	**After 5 days**	**After 10 days**
Alanine	44 ± 3.4	36 ± 3.9	27 ± 2.9	18 ± 2.7	32 ± 2.8	29 ± 3.1
Glycine	39 ± 2.9	32 ± 2.2	23 ± 2.1	23 ± 2.1	28 ± 2.6	29 ± 3.1
Glut. acid	65 ± 3.2	59 ± 2.8	49 ± 2.9	35 ± 3.1	59 ± 3.2	55 ± 3.2
Histidine	18 ± 1.7	19 ± 1.1	14 ± 1.4	09 ± 1.1	15 ± 2.1	17 ± 2.3
Leu. / Isoleucine	09 ± 0.9	06 ± 0.8	11 ± 0.9	14 ± 0.6	11 ± 1.9	12 ± 2.1
Methionine	05 ± 0.8	04 ± 0.7	04 ± 0.4	03 ± 0.4	06 ± 0.3	05 ± 0.5
Lysine	34 ± 1.9	29 ± 1.8	21 ± 1.9	18 ± 1.7	28 ± 1.3	28 ± 1.2
Tyrosine	10 ± 0.8	09 ± 0.1	11 ± 0.2	09 ± 0.4	11 ± 1.4	12 ± 1.5
Proline	176 ± 2.8	152 ± 3.1	135 ± 3.8	111 ± 3.2	155 ± 2.5	161 ± 2.9
Protein	8.91± 2.2	7.1 ± 0.9	5.4 ± 1.8	3.5 ± 1.3	6.9 ± 1.5	5.5 ± 1.4

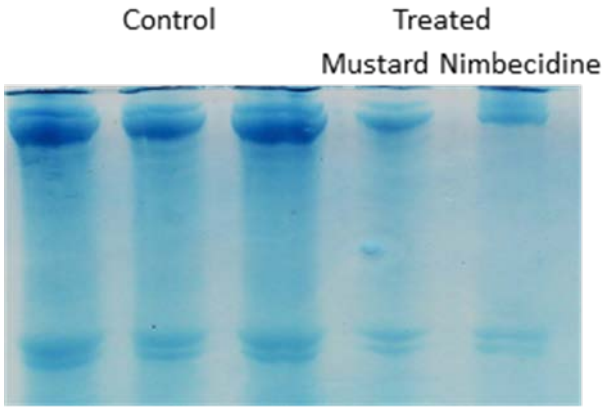

Fig. 1. Differences in the expression of ovarian protein in control and Mustard and Nimbecidine treated *A. diaperinus*.