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Larvicidal efficacy and mode of action of compound composition from Adiantum latifolium on Oryctes rhinoceros

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Abstract

Oryctes rhinoceros Linn., a well known pest of coconut palm, causes irrecoverable damage, reduction in yield and death of the plant. Insecticides with novel mode of action, more target selective and biodegradable are needed to replace the impacts of synthetic pesticides. Unexplored phytochemicals may have unknown site of action, which are selective to insects and, are safe for humans and the environment. *Adiantum latifolium* is a shade loving fern resistant to pathogens due to the presence of phytocompounds. The study was undertaken to find out the larvicidal efficacy of a compound composition of triterpenoids isolated from the leaves of *A. latifolium* against *O. rhinoceros* and its mode of action. The compounds showed antibacterial activity against gut bacteria of the larvae and larvicidal activity (LD50, 19.55 mg/kg), weight loss, 20-hydroxyecdysone titre and precocious metamorphosis of the larvae.. It also induced immunotoxicological effects via cellular and numerical changes of haemocytes in the haemolymph. The synergistic interaction of the triterpenoids is useful in the preparation of improved biopesticides.

Key Message

- *Oryctes rhinoceros* is a serious pest of coconut palms.
- Triterpenoid composition from the leaves of *Adiantum latifolium* is proved for its larvicidal activity against *O. rhinoceros.*
- The extract showed antibacterial activity against the gut bacteria isolated from the larvae.
- Ecdysone surge and disruption of metamorphosis, cellular and numerical changes of haemocytes are the other effects.
- The compound composition could be considered as a source for novel biopesticides.

Introduction

The coconut rhinoceros beetle, *Oryctes rhinoceros* Linn., is a well known pest of coconut palm and other plants of the family Arecaceae. Larvae are detritivorous and live deep inside coconut logs or cow dung pits and are well protected from natural enemies (Hinckley, 1967; Zelazny and Alfiler, 1986). Adults are the damaging life stage with flight muscles and host on plants. They damage the meristematic tissues in the crown of the palm and feeds on exuding tissue juices. The spathes on the leaf axil are damaged and the flower bunches are destroyed, resulting in the loss of nuts directly (Vargo, 2000). The wounds caused by the pests also provide entry for harmful bacteria and fungi as well as make the way for secondary infections by *Rhynchophorus ferruginesis* causing irrecoverable damage and death of the plant (Nayar *et al.,* 1976; Bedford, 1980).

Continuous use of synthetic chemical pesticides to control herbivorous insects leads to the development of resistance in insects and becomes ineffective. Therefore, insecticides with novel mode of action, more target selective and biodegradable are needed to replace the impacts of synthetic pesticides (Alkofahi et

al. 1989; Arnason et al. 1993; Isman 2006). As a part of co-evolution, plants use different biosynthetic pathways to produce various photochemicals to resist the attack of pests. Many phytochemical classes of compounds such as terpenoids, alkaloids and phenolics are reported as sources of insecticidal activity.

Ferns are the most primitive of all vascular plants, rarely fed by phytophagous insects in nature (Eastop 1972). *Adiantum latifolium* is a shade loving fern found in low altitudes and plains. The plant is resistant to pathogens due to the presence of phytocompounds (Archer and Cole 1986). The phytochemical analysis of *A. latifolium* leaves has revealed the presence of steroids, triterpenoids, phenolics, saponins, tannins and alkaloids. Ethanol and methanol fractions from *Adiantum capillus-veneris* were already proved to have insect growth regulatory activity against *Plutella xylostella* and *Aphis craccivora* (Moreira *et al*., 2007). Pyrethrin, rotenone, neem, and essential oils are four major types of botanicals used for insect control (Zibaee 2011).

Mode of action of insecticide is important to develop a strategic plan against pests safely and specifically. Variations in the normal ecdysteroid level impair insect development which can be used as an alternate strategy for the control of insect pests. Phytoecdysteroids, chemically the triterpenoids, have also been isolated from ferns and are believed to deter feeding by mimicking insect moulting hormones (Chaubey, 2018; Lafont and Horn, 1989). The phytoecdysteroids 20-hydroxyecdysone, α-ecdysone, polypodin B and poststerone are known to have toxic effect on larvae of colorado beetle *Leptinotarsa decemlineata* (Zolotar et al. 2001).

Haemocytes play an essential role in defending invertebrates against pathogens and parasite that enter the haemocoel. They are mainly responsible for cell and humoral immune responses including phagocytosis, encapsulation, secretion of antimicrobial peptides, cell adhesion molecules, lysozyme, lectins, detoxification and pro-phenoloxidase system (Hoffmann, 2003; Kanost *et al.,* 2004). Many insecticides have negative effects on number and morphology of haemocytes (Zibaee 2011). Six types of haemocytes have been identified in the haemolymph of third instar larvae of *O. rhinoceros* (Adhira *et al.,* 2010).

In our previous studies, two triterpenoids adiantobischrysene and 22- hydroxyhopane have been isolated from the leaves of *A. latifolium* having larvicidal activity by impeding metamorphosis (Pradeepkumar et al. 2018, 2019a). A bioactive fraction of structurally related terpenoid composition is also characterized from the leaves of the plant (Pradeepkumar et al. 2019b) (Fig.1). The multiple mechanisms of action of phytochemicals make them very useful in today's agricultural industry (Copping and Menn, 2000). The mixture of secondary metabolites may be deterrent to insects for a longer period compared to the effect of a single compound, because of their different physical and chemical properties along with multiple mode of action. This is evident from the fact that most successful plant species typically synthesize a wide array of moderately toxic defense compounds or a small number of highly toxic substances (Rattan 2010). Mixtures of terpenes from the plant *Porophyllum gracile* is more toxic with longer persistence of defenses against the lepidopteran pest *Ostrinia nubilalis* than monoterpenes alone (Guillet et al. 1998).

The larvicidal efficacy of the compound composition of triterpenoids isolated from the leaves of *A. latifolium* against *O. rhinoceros*, its mode of action including disruption of metamorphosis, changes in enzyme activity, cellular and numerical changes of haemocytes in haemolymph are the subject matter of the present study.

Materials And Methods

Plant material

Theleaves of *A. latifolium* Lam. were used for the study(Pradeepkumar et al. 2018).

Isolation of bioactive compound composition from the leaves of *A. latifolium*

Bioactivity guided isolation of the compound composition and its phytochemical characterization were described in our previous study(Pradeepkumar et al. 2019b). The bioactivity was determined based on the antibacterial activity against gut bacteria isolated from *O. rhinoceros* larvae in our previous study(Pradeepkumar et al. 2018).

*In vitro*study of antibacterial activity

Antibacterial activity was evaluated by agar well diffusion methodusing 24 h grown gut bacteria of strainsgram +ve*Bacillus cereus* andgram-ve*Stenotrophomonas maltophilia* (Pradeepkumar et al. 2018). The plates were incubated at 37°C for 48 h after adding 100 µL samples in the wells. The diameter of clear zone was measured against test culture.

Larvicidal activity of the compound composition

The larvae of *O. rhinoceros* maintained on sterilized cow dung(Mini and Prabhu 1986)were used for the experiment. Third instar larvae (body weight 8.5 ± 1 g) were used for testing larvicidal activity. The compound composition thoroughly mixed with sterilized cow dung after dissolving it in 25 mL of distilled water with 0.02 % Tween 80, at various concentrations (1, 2, 3, 4, 5 mg/100 g), was provided to larvae as food. The controls were fed on cow dung mixed with appropriate concentration of Tween 80. The larvae were reared in plastic containers, each group including six larvae. The body weight and mortality of larvae were recorded for three weeks. The medium was changed once in every three days.

Haemolymph sample preparation for enzyme studies

Haemolymph from both the control and treated larvae were collected, by cutting its 3rd prothoracic leg and gently squeezing its body, stored in sterile eppendorf tubes in a deep freezer. Circulating haemocytes were separated from the haemolymph by centrifugation at 14,000 g at 4°C for 15 min.

Bioassay of the level of 20-hydroxyecdysone (20E) in haemolymph

20E level in haemolymph was assayed by the method described byPorcheron *et al*. (1989) using a commercial ELISA kit (Cayman Chemical, USA) as explained in our previous study(Pradeepkumar et al. 2019a). Pure acetylcholinesterase from the electric eel *(Electrophorus electricus)*has been covalently coupled to a 20-hydroxyecdysone-6-carboxymethoxime derivative and the conjugate used as a tracer. Immunological reaction was performed in a 96-well microtiter plate precoated with secondary antibody.

Assay of cathepsin D

The assay of cathepsin D was done following the method ofMycek (1970)using 100 µL haemolymph as the test sample. One unit of cathepsin activity has been arbitrarily defined as the amount of enzyme in the test solution that caused the absorbancy of the supernatant to increase by 1.0 in 1.0 min.

Assay of phenol oxidase

Activity of phenoloxidase was estimated by the method ofLerch (1987). One unit of enzyme activity is defined as the amount of enzyme which catalyses the oxidation of one µmol of L-DOPA/ min at 30°C. This corresponds to the absorption change of 0.6/min at 475 nm. Specific activity is expressed as enzyme units/ mL haemolymph.

Total haemocyte count (THC)

THC was done by using Neubauer haemocytometer.Haemolymph is collected from both the control and treated larvae as explained earlier, diluted tenfold using WBC diluting fluid and stored in deep freezer. 100 µL of diluted haemolymph was immediately placed over haemocytometer and kept undisturbed for 3 min. The haemocytes in the four corner ruled squares were counted under a light microscope at 400X magnification. The number of circulating haemocytes per cubic millimeter was calculated. Replicates of the experiments were performed to confirm the results obtained.

Differential haemocyte count (DHC)

A drop of haemolymph was placed on clean microscopic glass slide and a thin smear was made by drawing the second slide across the first one at 45° angle. The smear was air dried and stained using Giemsa stain for 20 min. The slides were then washed in running water to remove excess stain then air-dried. Cells were observed, differential count was done and microphotographed using Labomed digital camera (400X). One hundred cells per slide were analyzed. The haemocytes were identified by their distinguishing characters as described byMathur (2002) and Wigglesworth (1972). The percentage of different types of haemocytes in both the treated and control samples were calculated.

Detection of apoptosis of haemocytes by flow cytometry

Apoptosing cells were quantified using FITC Annexin V cell apoptosis assay kit (Invitrogen, Thermo Fischer Scientific). The percentage of apoptotic haemocytes were assessed by flow cytometry(Shapiro 2003). Haemocytes were fixed in 70 % ethanol before staining. Washed the cells twice with cold PBS and

then resuspended in binding buffer. 100 µL of the FITC Annexin V followed by propidium iodide were added to the samples. The tubes were mixed thoroughly by vortexing at a medium speed for 3 to 5 sec followed by incubation for 20 min at room temperature in the dark. The cell populations were distinguished using a flow cytometer with 488 nm line of an argon-ion laser for excitation.

Cell cycle analysis

Cell cycle analysis was done by using standard kit (Muse® Cell Cycle Assay Kit, Merck Millipore). Haemocytes were separated from the haemolymph asdescribed earlier. Appropriate volume of PBS was added to each tube and the contents were mixed by gently vortexing. The cells were centrifuged at 3,000 g for 5 min. The supernatant was discarded without disturbing the cell pellet, leaving approximately 50 µL of PBS. Resuspended the cell pellet in the residual PBS by gently vortexing. The resuspended cells were added drop wise into the tube containing 1 mL of ice cold 70 % ethanol. Caped and freezed the tube at –20 ºC.After overnight incubation, the samples were centrifuged at 3,000 g for 5 min at room temperature. The supernatant was removed and 250 µL PBS was added to the pellet. Again centrifuged and discarded the supernatant. 250 µL of cell cycle reagent was added to the cell pellet.This was incubated at dark for 30 min. After this it was analyzed using a flow cytometer. Gating was performed with reference to untreated control cells and samples were analyzed. Quantitative measurement of the percentage of the cells in G0/G1, S, and G2/M phases of cell cycle was done on Muse Cell Analyzer. The experiments were performed in triplicate.

Statistical analysis

The statistical significance of data for control and treated groups was assessed by analysis of variance (ANOVA) using SPSS 13 for Windows. Statistical significance was accepted when $P \leq 0.05$. Post-hoc testing was carried out using Duncan's new multiple range test (DMRT). LD_{50} values were calculated by probit analysis.

Results

Antibacterial assay and isolation of bioactive compounds

Among the eight sub fractions of ethyl acetate fraction, fifth, sixth, seventh and eighth fractions showed antibacterial activity, the fifth fraction with highest activity (Table 1, Fig.2). The fifth fraction was used for larvicidal activity study.

Larvicidal activity

The compound mixture showed significant mortality of *O. rhinoceros*larvae in a dose dependent manner (Table 2). Estimated lethal concentration LD_{50} value for the compound composition was 19.55 (13.24-23.99) mg/kg, $\chi 2 = 7.04$, df =28, S = + 0.357, I = - 0.700. The duration of feeding for causing significant reduction in body weight decreased with increasing concentration of the compounds. At low

concentrations of 1, 2 and 3 mg/100 g6 %, 4 % and 2 % of larvae entered prepupal stage respectively, but could not pupate.

Phenol oxidase and cathepsin D levels in haemolymph of larvae

The proteolytic enzymes phenoloxidase and cathepsin D showed decreased activity in treated larvae compared with the control (Table 3).

Levels of ecdysteroids in haemolymph

Ecdysteroid concentration was measured in the haemolymph from day 4 to day 12 of treatment (Fig. 3). In the control larvae ecdysteroid levels in the haemolymph showed a passive increase from 250 pg/ mL on day 4 to 810 pg/ mL on day 12. In the larvae treated with compound mixture, the hormone level varies from 370 pg/ mL on day 4 to 5340 pg/ mL on day 12 with alarge decline in ecdysteroid concentration on the day 10 followed by an ecdysone surge. This induced prepupation even in 7.28 g weighed larvae preventing further development.

Haemocytes in haemolymph of larvae

Significant reduction in THC occurred in treated larvae compared to the control in a dose dependent manner. The decrease in number of cells ranged from 11.03 % in1 mg treated larvae on day 8 to 63.84 % in larvae treated with 5 mg of compound composition on day 12 compared to the control. (Table 4).

Regarding the differential haemocyte count, six types of haemocytes were identified in the haemolymph of the larvae (Fig.4). These include prohaemocytes, plasmatocytes, granulocytes, adipohaemocytes, oenocytoids and Spherulocytes. Granulocytes are the most common type of haemocytes.

Light microscopic studies of giemsa stained haemocytes of treated larvae showed many cellular changes such as distortion of cell shape, abnormal staining pattern, rupturing of cell membrane, denucleation, release of cytoplasmic granules, shrinkage and vacuolation of nucleus etc.

DHC showed a significant increase (up to 73.03 %) in the proportion of granulocytes in the compounds treated larvae on day 10 compared with untreated larvae. All other cells showed decrease in their proportions. Up to 34.20 % decrease of prohaemocytes, 34.50 % plasmatocytes, 23.60 % adipohaemocytes, 3.36 % oenocytoids and 2.82 % spherulocytes occurred in treated larvae in a dose dependent manner (Table 5).

Flow cytometric analysis ofhaemocytes

Flow cytometric analysis of apoptosis of haemocytes showed four distinct sub-populations. The results were analyzed in flow cytometric data figures with four quadrants (Table 6, Fig. 5). The lower left quadrant represents viable cells, lower right quadrant early apoptotic cells, upper right quadrant late apoptotic cells, and the upper left quadrant the necrotic cells. The study showed that the compound composition induced apoptosis in haemocytes of *O. rhinoceros.* The live cells decreased from 97.75 % in

control to 88.78 % in treated larvae, whereas total apoptotic cells increased from 1.90 % in control to 11.22 % in treated larvae in a dose dependent manner.

Assuming that the compound composition also could cause DNA damage and cell cycle alterations in the haemocytes of *O. rhinoceros*, the flow cytometric analysis of haemocyte's DNA content and cell cycle distribution were performed on control and treated larvae. It revealed the proportion of cells in G0/G1, S and G2/M phases during the cell cycle. Treated larvae showed high G0/G1 peak and low the proportion of S and G2/M phases compared to control. Such cell cycle distribution in the haemocytes is an indicative of a G0/G1 arrest (Table 7, Fig. 6).

Discussion

In the present study a compound mixture isolated from *A. latifolium* leaves was evaluated for its larvicidal activity against *O. rhinoceros*. It revealed weight loss of larvae and increase in mortality in a dose dependent manner.Five triterpenoids have been characterized from the mixture in our early study(Pradeepkumar et al. 2019b). Terpenoids generally play a major role in plant's defense mechanism by harming, deterring, repelling, or reducing the fitness of potential invaders. More than hundred such molecules, including azadirachtin, having insecticidal or repellent activities have been identified in the crude neem oil(Akhila and Rani, 2002). Terpenoids also synergize the effects of other toxins by acting as solvents to facilitate their passage through membranes. In our previous studies, we have isolated two hopanoid triterpenoids adiantobischrysene and 22- hydroxyhopane from the leaves of *A. latifolium* having larvicidal activity by impeding metamorphosis(Pradeepkumar et al. 2018, 2019a). Out of the five terpenoids in the extract, three are structurally related to 22-hydroxyhopane and two to adiantobischrysene. It is thus evident that action of the various terpenoids present in compound composition fraction of *A. latifolium* leaves may have synergistic action in causing larvicidal effects on *O. rhinoceros*.

The results of the study showed multiple mode of action of the compounds against the larvae. The extract showed antibacterial activity againstthe gut bacteria*Bacillus cereus* and *Stenotrophomonas maltophilia.* In *O. rhinoceros* larvae, the middle region of hind gut has an enlargement called proctodael dilation in which fermentation of food takes place and gut bacteria have a profound role in cellulose digestion(Taylor and Crawford, 1982; Rani *et al*., 2002; Sari, 2016).In our previous study, eight strains of bacteria have been isolated and identified from the gut of *O. rhinoceros* larvae(Pradeepkumar et al. 2018). Of these, two strains representing gram +ve *B. cereus* and gram –ve *S. maltophilia*were used in this study. *B. cereus*bacterium has beenreported from the gut of *O. rhinoceros*larvae with cellulolytic and hemicellulolytic activity, *S. maltophilia* in the midgut of *Lutzomyia longipalpis* (Oliveira et al. 2000; Desai and Bhamre 2012; Sari 2016).These findings suggest starvation induced by the extract by impeding digestive process resulted in weight loss of the larvae. Weight loss and other stressful conditions followed by simultaneous release of 20E in exponential quantities induced precocious prepupation even at a very low weight of 7.28 g. Similar observations on *O. rhinoceros* larvaehave been reported on feeding antibiotic treated-cow dung mixture since it eliminates microbes(Rani *et al*., 2002). Likewise, elevated 20E

leading to precocious pupation was also observed in *Drosophila*(Terashima et al. 2005). Earlier study showed that larvae of *O. rhinoceros* must attain a critical weight of 12.17 g for precocious pupation on prolonged starvation(Rani et al. 2002). The starvation, weight loss of the larvae and hormonal mimicking by the terpenoids present in the extract induced early precocious metamorphosis much before attaining critical weight leading to death of the larvae. The triterpenoids in plants can disrupt hormone levels in insects in different pathways. Phytoecdysteroids are chemically triterpenoids and are reported to be present in ferns(Chaubey , 2018). These compounds either mimic 20E activity inducing precocious metamorphosis or prevent the physiological and morphological changes induced by the hormone. Two terpenoids, cucurbitacins B and D, isolated from seeds of *Iberis umbellata* could prevent 20-hydroxyecdysone induced morphological changes in the *Drosophila*(Dinan *et al*., 1997). The triterpenoids adiantobischrysene and 22-hydroxyhopane from *A. latifolium* caused 20E titre in the haemolymph inducing precocious pupation in *O. rhinoceros*(Pradeepkumar et al. 2018, 2019a). Terpenoids structurally similar to these compounds are also present in the compound composition.

The compounds induced a decrease in activity of cathepsin D and phenoloxidase. Phenoloxidase enzyme in haemolymph has tyrosinase-like activity and are involved in melanin synthesis, encapsulation against invading microorganisms, tanning of the cuticle, sclerotization and wound healing(Zibaee et al. 2011). So the inhibition of phenoloxidase could be apromising approach for pest control(Gholami et al. 2013). Cathepsins are cellular proteases involved in vital processes like development, metamorphosis, apoptosis and immunity(Saikhedkar *et al.,*2015).

Circulating haemocytes have crucial roles in both cellular mechanisms and producing antimicrobial components in insects. Several insecticides and plant products have negative effects on the number and morphology of haemocytes leading tototal collapse of immune response(Zibaee, 2011; Pandey *et al.,*2012).The present study showed a sharp decrease in THC and DHC with cellular changes such as distortion of cell shape, abnormal staining pattern, rupturing of cell membrane, denucleation, release of cytoplasmic granules, shrinkage of nucleus etc., on treatment with *A. latifolium* leaf compounds. Except granulocytes all other haemocytes show significant decrease in their proportion. Similar observations were reported in haemolymph of third instar larvae of *O. rhinoceros* following infection of *B. thuringiensis*(Adhira *et al.,* 2010). The insecticides chlorozan, marshal, deltamethrin and spinosad were reported to induce a decrease THC in *Schistocerca gregaria* (Halawa et al. 2007). The decrease in total number of haemocytes, changes in their differential count along with cytotoxic changes might have disrupted immunological response of larvae, leading to death.

Apoptotic studies of haemocytes are widely used to assign the effects of various agents on target organisms. The present study also revealed obvious immunotoxicological effects of the secondary metabolites on *O. rhinoceros* larvae. Haemocyte apoptosis is found to be increasing with increasing concentration of compounds. The translocation of membrane phosphatidylserine from inner leaflet to outer cell surface is a hallmark of apoptotic process. Annexin V specifically binds to phosphatidylserine and propidium iodide intercalate with nuclear DNA. In a cell population with FITC annexin V and propidium iodide in the binding buffer, apoptotic cells show green fluorescence, dead cells show red and

green fluorescence, and live cells show little or no fluorescence which can be analyzed by flow cytometry. In insects, appropriate haemocyte numbers are maintained by haemopoietic organs and mitosis of circulating haemocytes(Hinks and Arnold 2008). Cell cycle analysis of haemocytes revealed that in the treated larvae, there is selective loss of G2/M and S cells concomitant with the increase in appearance of cells with decreased DNA content in G0/G1 phase of cell cycle. DNA damage and cell cycle alterations are presumably caused by the compounds present in *A. latifolium* extract.

Continuous use of a single compound may develop resistance in target organisms. It has been suggested that extracts from plants, which contain numerous compounds, are more complex in comparison to synthetic pesticides and therefore, delays the buildup of resistance (Vsllinger 1987). Even after 40 generations, no resistance is noted in aphids that had been treated with neem oil, whereas those treated with purified azadirachtin have developed resistance (Feng and Isman 1995). The present study reveals synergistic action of various compounds, indicating that the extract of the plant should have long lasting effects as a natural product. These compounds are of vital importance considering the biological, medicinal, antimicrobial and insecticidal properties of the plant. The synergistic interaction of many active compounds may be useful in the preparation of improved biopesticides.

Declarations

Author Contributions

The first and second authors designed the work. PKR and EDA did the experiments. The authors discussed the results; the first author wrote the manuscript in consultation with the second author.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study did not require ethics approval.

References

1. Adhira M Nayar S and DAE (2010) Effect of Bacillus thuringiensis israelensis infection on the biochemical and hematological profile of the hemolymph of Oryctes rhinoceros[L.](Coleoptera: Scarabaeidae) larva. Entomon 35:241–245
2. Akhila A, OF KR-IJ, 2002 undefined (2002) Biosynthesis of some biologically active limonoids in the leaves of Azadirachta indica (the Indian neem tree). Indian J Heterocycl Chem 11:299–302
3. Alkofahi A, Rupprecht J, … JA (1989) Search for new pesticides from higher plants. ACS Symp Ser Chem Soc
4. Archer KJ, Cole ALJ (1986) Cuticle, Cell Wall Ultrastructure and Disease Resistance in Maidenhair Fern. New Phytol 103:341–348. https://doi.org/10.1111/j.1469-8137.1986.tb00620.x
5. Arnason JT, MacKinnon S, Durst A, et al (1993) Insecticides in Tropical Plants with Non-Neurotoxic Modes of Action. Phytochem Potential Trop Plants 107–131. https://doi.org/10.1007/978-1-4899-1783-6_5
6. Bedford GO (1980) Biology, Ecology, and Control of Palm Rhinoceros Beetles. Annu Rev Entomol 25:309–339. https://doi.org/10.1146/annurev.en.25.010180.001521
7. Chaubey M K (2018) Role of Phytoecdysteroids in Insect Pest Management: A Review. J Agron 17:1–10
8. Copping LG, Menn JJ (2000) Biopesticides: A review of their action, applications and efficacy. In: Pest Management Science. pp 56,651–676
9. Desai A, Bhamre P (2012) Diversity of gut bacterial fauna of Oryctes monocerus linnaeus (coleoptera: scarabaeidae). Bionano Front 5:142–144
10. Eastop VF (1972) Deductions from the present day host plants of aphids and related insects. Symp R Entomol Soc No 6 Blackwell Sci Oxford. https://doi.org/10.1590/1413-73722229312
11. Feng R, Isman MB (1995) Selection for resistance to azadirachtin in the green peach aphid, Myzus persicae. Experientia 51:831–833. https://doi.org/10.1007/BF01922438
12. Gholami T, Ghadamyari M, Oliaee AO, Ajamhasani M (2013) Effects of inhibitors on haemolymph phenoloxidase from rosaceous branch borer, ospheranteria coerulescens (Coleoptera: Cerambycidae). J Plant Prot Res 53:324–332. https://doi.org/10.2478/jppr-2013-0049
13. Guillet G, Bélanger A, Arnason JT (1998) Volatile monoterpenes in porophyllum gracile and p. ruderale (asteraceae): identification, localization and insecticidal synergism with α -terthienyl. Phytochemistry 49:423–429. https://doi.org/10.1016/S0031-9422(98)00189-7
14. Halawa S, Gaaboub I, Gad AA, El-Aswad AF (2007) Effect of some insecticides on the haemolymph of desert locust Schistocerca gregaria Forskal. J Egypt Soc Toxicol 36:61–66
15. Hinckley AD (1967) Associates of the coconut rhinoceros beetle in Western Samoa. Pacific Insects 9:505–511

16. Hinks CF, Arnold JW (2008) Haemopoiesis in Lepidoptera. II. The role of the haemopoietic organs. Can J Zool 55:1740–1755. https://doi.org/10.1139/z77-225
17. Hoffmann JA (2003) The immune response of Drosophila. Nature 426(6962):33–38
18. Isman MB (2006) Botanical Insecticides, Deterrents, and Repellents in Modern Agriculture and an Increasingly Regulated World. Annu Rev Entomol 51:45–66. https://doi.org/10.1146/annurev.ento.51.110104.151146
19. Kanost MR, Jiang H, Yu XQ (2004) Innate immune responses of a lepidopteran insect, Manduca sexta. Immunol. Rev. 198,97–105
20. L.Dinan, P. Whiting, J.P.Girault, R.Lafont, S.T.Dhadialla, D.E.Cress, B.Mugat, C.Antoniewski JAL (1997) Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor. Biochem J 327:643–650. https://doi.org/10.1042/bj3270643
21. Lafont R, Horn D (1989) Phytoecdysteroids: structures and occurrence. 36–64
22. Lerch K (1987) Monophenol monooxygenase from Neurospora crassa. Methods Enzymol 142:165–169
23. Mathur.R (2002) Text Book of Entomology, Ist Editio. Campus Books International,New Delhi
24. Mini A, Prabhu VKK (1986) Significance of critical developmental stage on starvation induced endocrine mediated precocious metamorphosis inOryctes rhinoceros (Coleoptera: Scarabaeidae). Proc Anim Sci 95:379–385. https://doi.org/10.1007/BF03179372
25. Moreira MD, Picanço MC, Barbosa LCDA, et al (2007) Plant compounds insecticide activity against Coleoptera pests of stored products. Pesqui Agropecu Bras 42:909–915
26. Mycek M J (1970) Cathepsins. Methods Enzymol 19:285
27. Nayar K, Ananthakrishnan T, David B (1976) General and applied entomology,Tata Mc Graw Hill Publishing Company Limited,New Delhi
28. Oliveira SM, Moraes BA, Gonçalves CA, et al (2000) Prevalence of microbiota in the digestive tract of wild females of Lutzomyia longipalpis Lutz {&} Neiva, 1912 (Diptera: Psychodidae). Rev Soc Bras Med Trop 33:319–322. https://doi.org/S0037-86822000000300012 [pii]
29. Pandey, Suyog, J. P. Pandey RKT (2012) Effect of some botanicals on hemocytes and molting of Papilio demoleus larvae. J Entomol 9:23–31
30. Porcheron P, Moriniere M, Grassi J, Pradelles P (1989) Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. Insect Biochem 19:117–122. https://doi.org/10.1016/0020-1790(89)90081-4
31. Pradeepkumar R, Dinesh Babu KV, Evans DA (2019a) Isolation, characterization and mode of action of a larvicidal compound, 22-hydroxyhopane from Adiantum latifolium Lam. against Oryctes rhinoceros Linn. Pestic Biochem Physiol 153:161–170. https://doi.org/10.1016/J.PESTBP.2018.11.018
32. Pradeepkumar R, Evans DA, Dinesh Babu KV (2019b) Characterization of Multipotent Compounds from Adiantum latifolium Leaves by Liquid Chromatography-Electrospray-Mass Spectroscopy. Anal

Chem Lett 9:682–696. https://doi.org/10.1080/22297928.2019.1674185

33. Pradeepkumar R, John A, Kumar P, et al (2018) Larvicidal efficacy of Adiantobischrysene from Adiantum latifolium against Oryctes rhinoceros through disrupting metamorphosis and impeding microbial mediated digestion. Pest Manag Sci 74:1821–1828. https://doi.org/10.1002/ps.4880
34. Rani R, Jaya S, Chandran D (2002) Effect of antibiotics on larval growth and metamorphosis in Oryctes rhinoceros (Coleoptera: Scarabaeidae). J Plant Crop 30:63–65
35. Rattan RS (2010) Mechanism of action of insecticidal secondary metabolites of plant origin. Crop Prot. 29,913–920
36. Saikhedkar N, Summanwar A, Joshi R, Giri A (2015) Cathepsins of lepidopteran insects: Aspects and prospects. Insect Biochem Mol Biol 64:51–59. https://doi.org/10.1016/j.ibmb.2015.07.005
37. Sari SLA (2016) Cellulolytic and hemicellulolytic bacteria from the gut of Oryctes rhinoceros larvae. Biodiversitas, J Biol Divers 17:78–83. https://doi.org/10.13057/biodiv/d170111
38. Shapiro HM (2003) Practical flow cytometry, 4th edn. John Wiley & Sons, Inc, Hoboken, New Jersey,341–353
39. Taylor E, Crawford C (1982) Microbial gut symbionts and desert detritivores. Sci Rev Arid Zo Res 1:37–52
40. Terashima J, Takaki K, Sakurai S, Bownes M (2005) Nutritional status affects 20-hydroxyecdysone concentration and progression of oogenesis in Drosophila melanogaster. J Endocrinol 187:69–79. https://doi.org/10.1677/joe.1.06220
41. Vargo. A (2000) Coconut Rhinoceros Beetle (Oryctes rhinoceros). Agricultural Pests of the Pacific. Agric Dev Am Pacific
42. Vsllinger M (1987) The possible development of resistence against neem seed kernal extra ct and dfeltamethrin in Plutella xylostella. Schmutterer, H(Ed) Nat Pestic from neem tree (Azadirachta indica) other Trop plantsProceedings third Int neem Conf Nairobi Kenya 10-15July, 1986, Eschborn
43. Wigglesworth VB (1972) The principles of insect physiology. 7th EdN.Chapman and Hall,London
44. Zelazny B, A Alfiler – (1986) Oryctes rhinoceros (Coleoptera: Scarabaeidae) Larva Abundance and Mortality Factors in the Philippines. Environ 15:84–87
45. Zibaee A (2011) Botanical Insecticides and Their Effects on Insect Biochemistry and Immunity. Pestic Mod World - Pests Control Pestic Expo Toxic Assess 55–68. https://doi.org/10.5772/16550
46. Zibaee A, Bandani AR, Malagoli D (2011) Purification and characterization of phenoloxidase from the hemocytes of Eurygaster integriceps (Hemiptera: Scutelleridae). Comp Biochem Physiol - B Biochem Mol Biol 158:117–123. https://doi.org/10.1016/j.cbpb.2010.10.006
47. Zolotar RM, Bykhovets AI, Kovganko N V. (2001) Effect of certain phytoecdysteroids on larvae of colorado beetle Leptinotarsa decemlineata. Chem Nat Compd 37:537–539. https://doi.org/10.1023/A:1014816815996

Tables

Table 1 Antibacterial activity of sub fractions of ethyl acetate fraction of *A. latifolium* leaves against gut bacteria isolated from *O. rhinoceros* larvae

		Inhibition zone (mm)			
	Sub fractions:	5	6	7	8
Gram+ve	*Bacillus cereus*	21.33 ± 0.33	16.33 ± 0.33	13.33 ± 0.33	18.00 ± 0.33
Gram-ve	*Stenotrophomonas maltophilia*	20.67 ± 0.33	14.33 ± 0.33	13.67 ± 0.33	16.33 ± 0.33

Values are mean of three replicates ± SE of zone of inhibition of bacteria.

Concentration: 0.5 mg/ mL. No zone of inhibition was observed in controls.

Table 2 Larvicidal activity of compound composition isolated from *A. latifolium* leaves against third instar larvae of *O. rhinoceros*

Dosage (mg/ 100 g) cow dung)	Mortality (%)*	Body weight compared to critical weight**	% transformation into pre pupae
Control	-	above	-
1	31.67 ± 1.67[a]	above	6
2	58.33 ± 1.67[b]	above	4
3	66.67 ± 2.10[c]	below	2
4	70.00 ± 2.58[c]	below	-
5	88.33 ± 1.67[d]	below	-

*After three weeks, values are mean ± SE of six replicates, values marked with different letters indicate significant differences between groups (P ≤0.05). ** Critical body weight for *O. rhinoceros* larvae for

precocious metamorphosis is **12.17 g** *(Rani et al.,* 2002).

Table 3 Effect of compound mixture from leaves of *A. latifolium* on phenol oxidase and cathepsin D levels in haemolymph of third instar *O. rhinoceros* larvae

Enzyme activity	Dosage (mg /100 g cow dung)	Duration of treatment (days)		
		8	10	12
Cathepsin[a]	control	2.95 ± 0.19	2.90 ± 0.29	2.91 ± 0.35
	1	2.82 ± 0.20	1.71 ± 0.34*	0.98 ± 0.09*
	3	2.85 ± 0.20	1.56 ± 0.06**	0.85 ± 0.05**
	5	2.62 ± 0.18	1.06 ± 0.04**	0.62 ± 0.19**
Phenoloxidase[a]	control	14.00 ± 0.72	15.70 ± 0.81	15.85 ± 0.82
	1	12.53 ± 0.81	12.35 ± 1.35*	11.35 ± 0.55**
	3	12.13 ± 0.99	10.99 ± 0.11**	9.38 ± 0.49**
	5	11.44 ± 0.75	10.24 ± 0.80**	9.96 ± 0.17**

Values represent mean of three replicates ± SE, * indicates a p value 0.05 level,

** indicates a p value 0.01 level.

a- unit activity/ min /mL haemolymph

Table 4 Effect of compound mixture from leaves of *A. latifolium* on THC in the third instar larvae of *O. rhinoceros*

Total haemocyte count (number/ mm^3 of haemolymph)			
Dosage (mg/100 g cow dung)	Duration of treatment (Days)		
	8	10	12
control	10976 ± 81.44	11175 ± 136.36	11049 ± 122.77
1	9765 ± 166.07	8755 ± 186.02	6066 ± 41.63
3	9176 ± 180.44	6913 ± 150.70	5039 ± 125.56
5	8693 ± 153.33	6032 ± 133.87	3995 ± 169.95

Values represent mean of three replicates ± SE, all values significant at 0.01evel when compared with control.

Table 5 Effect of compound mixture from leaves of *A. latifolium* on DHC in the third instar larvae of *O. rhinoceros*

Differential haemocyte count (%)						
Dosage (mg/100 g cow dung)	PR	PL	GR	AD	OE	SP
control	23.30 ± 1.51	14.20 ± 0.43	30.30 ± 0.83	20.46 ± 0.52	6.36 ± 0.32	5.32 ± 0.28
1	19.30 ± 0.23*	12.43 ± 0.40*	39.26 ± 0.49*	18.36 ± 0.57*	5.26 ± 0.20*	4.33 ± 0.31
3	17.33 ± 0.26**	11.26 ± 0.49**	45.20 ± 2.08**	17.26 ± 0.20**	4.46 ± 0.08**	3.33 ± 0.26*
5	15.33 ± 0.44**	9.30 ± 0.40**	52.43 ± 2.25**	15.63 ± 0.60**	3.00 ± 0.15**	2.50 ± 0.35**

Values represent mean of three replicates ± SE, * significance at 0.05 level, ** significance at 0.01 level.

PR- prohaemocyte, PL- plasmatocyte, GR - granulocyte, AD -adipohaemocyte, OE- oenocytoid, SP- spherulocyte

Table 6 Effect of compound mixture from leaves of *A. latifolium* on cell apoptosis of haemocytes in the third instar larvae of *O. rhinoceros*

Cells	% Gated			
	Dosage (mg/100 g cow dung)			
	Control	1	3	5
Live	97.75	94.44	92.24	88.7
Early apoptotic	0.25	4.78	7.26	10.82
Late apoptotic/dead	1.65	0.74	0.48	0.40
Debris	0.35	0.04	0.02	0.00
Total apoptotic	1.90	5.52	7.74	11.22

Table 7 Effect of compound mixture from leaves of *A. latifolium* on cell cycle alterations of haemocytes in the third instar larvae of *O. rhinoceros*

Cell cycle stages		G0/G1	S	G2/M
Control	% Gated	53.3	20.2	8.8
	% CV	23.5	10.4	9.9
Treated	% Gated	84.8	6.8	3.7
	% CV	14.4	11.3	9.8

Figures

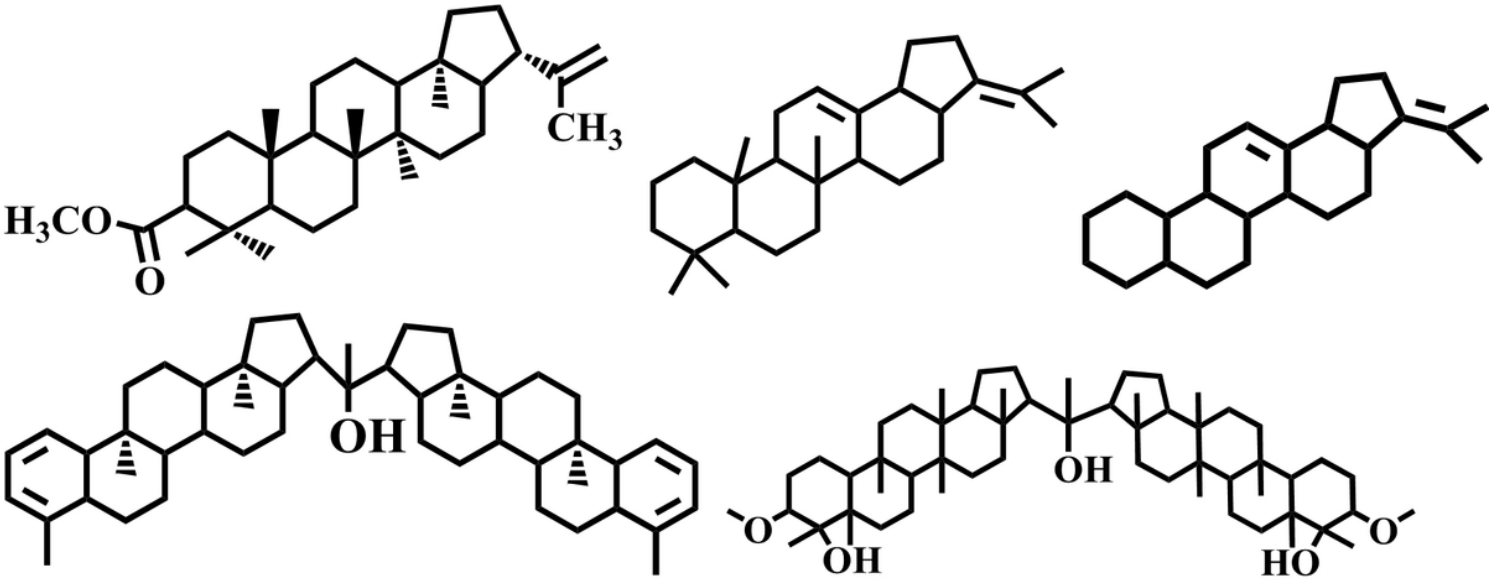

Figure 1

Triterpenoids characterized from the bioactive compound composition fraction of ethyl acetate extract of leaves of *A. latifolium*

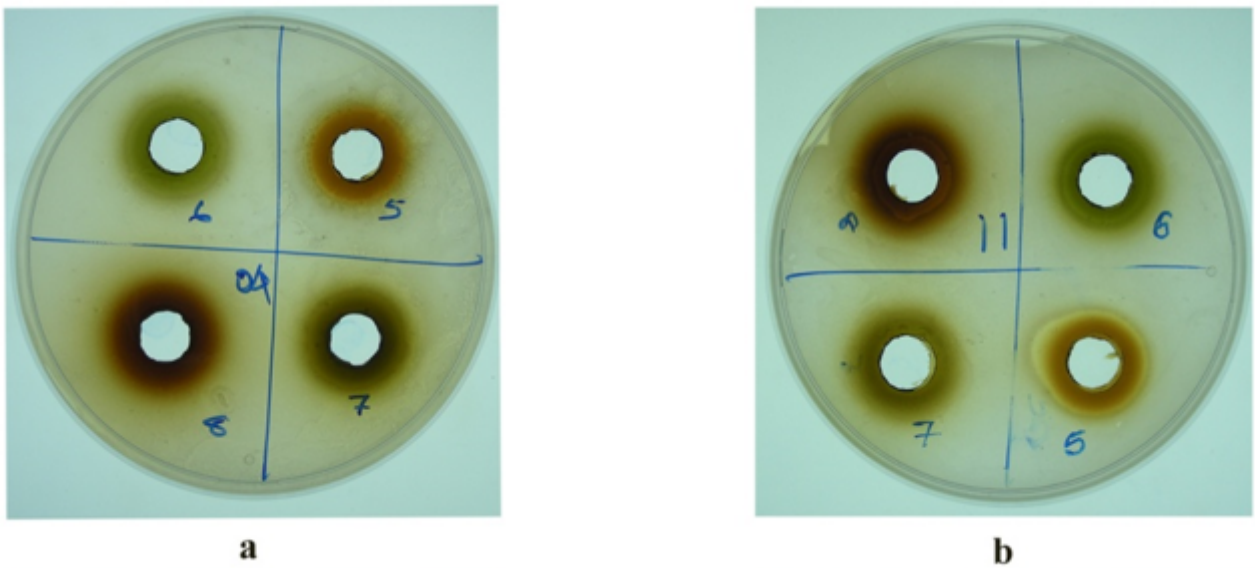

Figure 2

Antibacterial activity of sub fractions of *A. latifolium* leaves against gut bacteria isolated from *O. rhinoceros* larvae.

a - *S. maltophilia,* b - *B. cereus,* 5,6,7,8- sub fractions of ethyl acetate fraction

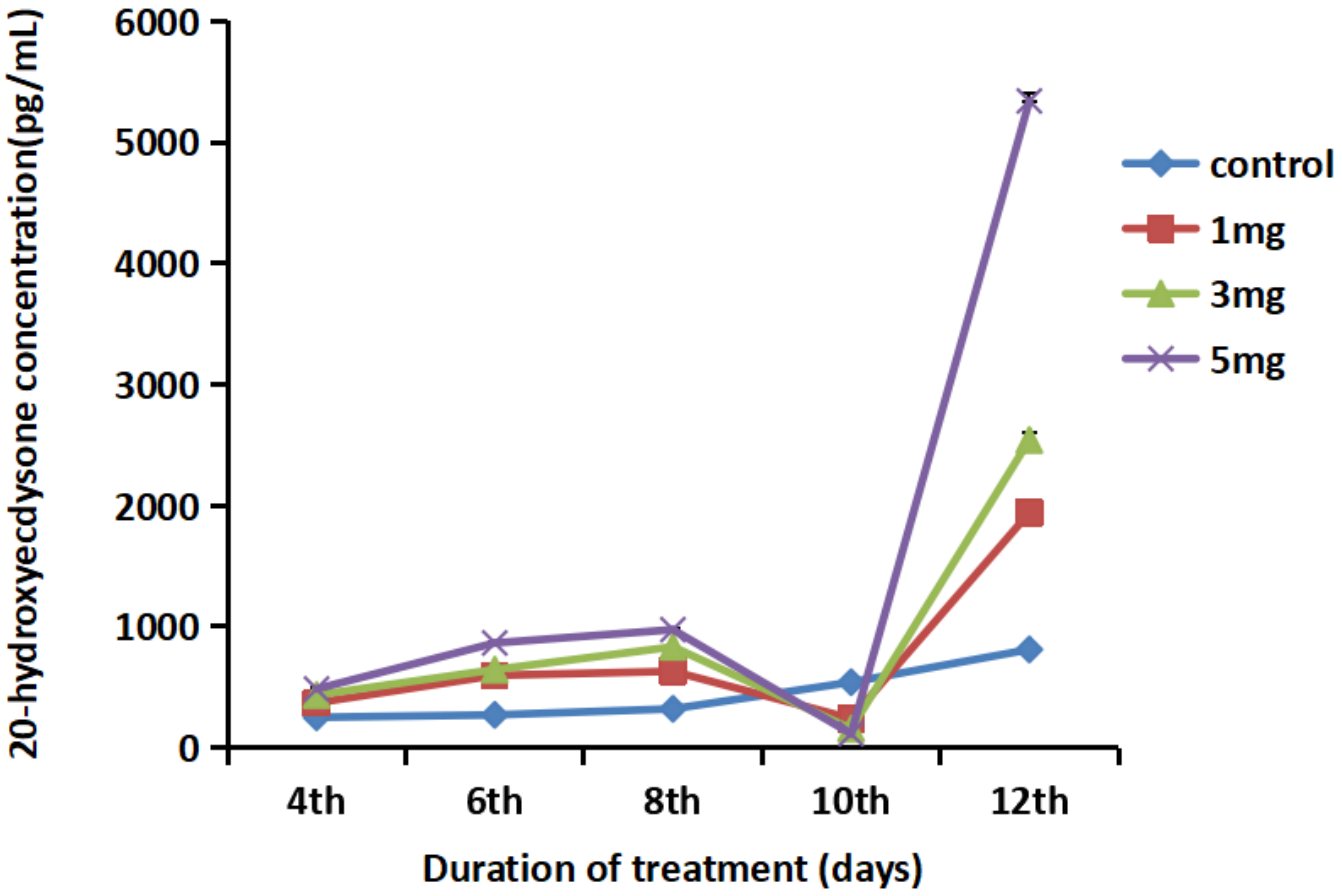

Figure 3

Effect of compound mixture from leaves of *A. latifolium* on 20-hydroxyecdysone titre in the haemolymph of third instar larvae of *O. rhinoceros*.

Each value is a mean of three separate determinations with error bar.

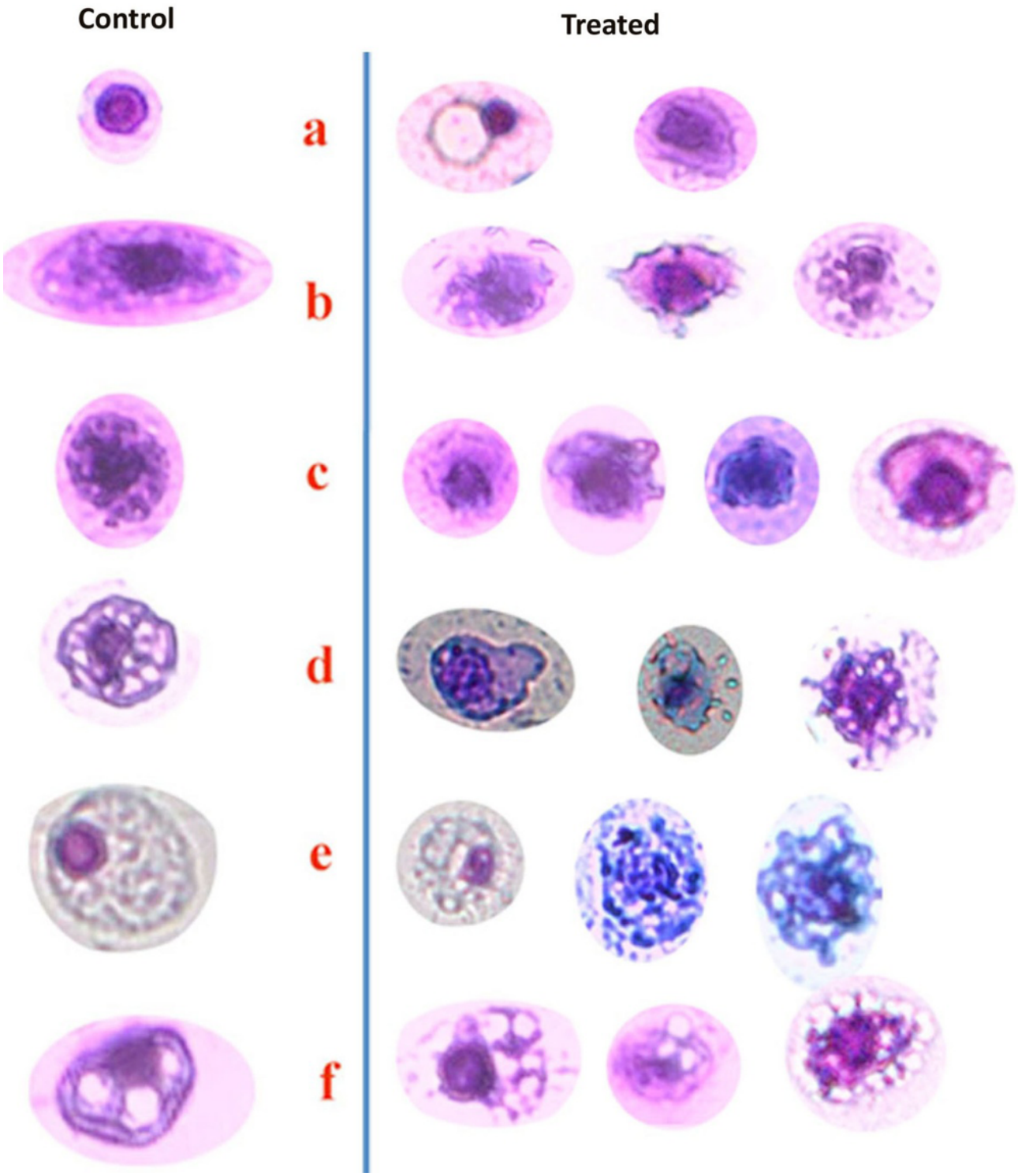

Figure 4

Cellular changes caused by compound composition from leaves of

A. latifolium on haemocytes in the third instar larvae of *O. rhinoceros*

a- prohemocyte, b- plasmatocyte, c - granulocyte,

d -adipohaemocyte, e- oenocytoid, f- spherulocyte

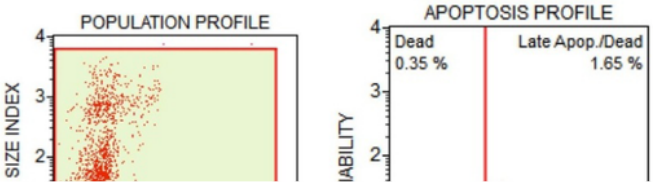

Figure 5

Flow cytometric analysis of haemocytes of larvae of O*. rhinoceros* after staining by annexin V-FITC and propidium iodide.

a- Untreated cells, b, c, d- cells treated with 1 , 3 and 5 mg compound mixture respectively.

Figure 6

Cell cycle profile of haemocytes of third instar larvae of *O.rhinoceros* stained with propidium iodide.

a-untreated cells, b-cells treated with compound mixture