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Insecticidal activities and mechanism of extracts from neem leaves against *Oxya chinensis*

[*Atividade e mecanismos inseticidas de extratos de folhas "neem" contra* Oxya chinesis]

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ABSTRACT

In this study, neem leaves were successively extracted with petroleum ether, 95% ethanol and water and the insecticidal activities of these extracts against *Oxya chinensis* larvae were measured. The results showed that 95% ethanol extract gave the highest extraction yield and insecticidal activity, and it was further extracted with five different solvents. The petroleum ether extract from the 95% ethanol extract possessed the highest insecticidal activity with median lethal concentration values ranging from 14.93 to 55.66mg/mL. The gas chromatography-mass spectrometer analysis showed that the petroleum ether extract mainly composed of alkanes, olefin, esters and amide. The pathological examination revealed that the prominent lesions, including reduced regenerative cells in midgut and swelled and degenerated cylindrical cells, were observed in the 5th instar *Oxya chinensis* after treatment. The ultrastructural features showed that the cylindrical cells, microvilli and mitochondria were seriously damaged. These results suggested that the petroleum ether extract from neem leaves had potent insecticidal activity and could be a candidate insecticide.

Keywords: neem leaves, *Oxya chinensis*, insecticidal activity

RESUMO

Nesse estudo, folhas "neem" foram extraídas com sucesso com éter de petróleo, 95% de etanol e água, e as atividades inseticidas desses extratos foram medidas contra larvas de Oxya chinesis*. Os resultados mostram que extrato com 95% de etanol deram o maior resultado de extração e atividade inseticida e foi então extraído utilizando mais cinco diferentes solventes. O éter de petróleo do extrato de 95% etanol apresentou maior atividade inseticida com concentração letal média variando de 14.93 a 55.66mg/mL. A análise por cromatografia de massa mostrou que o extrato de éter de petróleo está composto principalmente de alcanos, alcenos, ésteres e amidas. A avaliação patológica revelou que as lesões proeminentes, inclusive células regenerativas reduzidas no intestino e células cilíndricas edemaciadas e degeneradas foram observadas no quinto estágio de desenvolvimento da* Oxya chinesis *após tratamento. As características ultraestruturais mostraram que as células cilíndricas, microvilos e mitocôndrias apresentavam lesões graves. Esses resultados sugerem que o extrato de éter de petróleo de folhas de "neem" tem atividade inseticida potente e pode ser um candidato a inseticida.*

Palavras-chave: folhas nem, Oxya chinesis, *atividade inseticida*

INTRODUCTION

Oxya chinensis (Orthoptera: Acridoidae) mainly distributed in Africa, Oceania and Asia. In China, *O. chinensis* is distributed throughout the whole country except Tibet and Qinghai provinces (Yin *et al*., 2008). *O. chinensis* is a hazardous pest threatening the production of rice and other gramineous plants. The traditional control method relies on chemical pesticides. However, most of them have drawbacks such as

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resistance, toxicity and environmental contamination (Ba *et al*., 2013). At present, many bio-pesticides have been applied to control locusts, such as *Metarhizium anisopliae*, *Nosema locustae*, *Beauveria bassiana*, nematodes, entomopoxvirus and egg parasitic wasps (Tounou *et al*., 2008; Zha *et al*., 2011; Bitsadze *et al*., 2013). But it had been proved that these bio-pesticides were not effective enough to control locusts. For a long time, people have been looking for active insecticidal substances from natural products.

Azadirachta indica A. Juss (syn Melia azadirachta L.) commonly known as neem, possessed biocidal activities such as insecticidal and fungicidal, and neem-based pesticides have been recognized as one of the best bio-pesticides all over the world (Montes-Molina *et al*., 2008; Javed *et al*., 2008; Anjorin *et al*., 2008). Azadirachtin isolated from neem was found to have biocidal activity against nearly 400 species of pests (Srinivasan *et al*., 2011). Neem-based pesticides with excellent insecticidal activity against locusts are labeled as ideal pesticides (Zhang *et al*., 2004). Extracts of neem with more than 300 known components affect the physiology and behavior of a wide range of insects, mites and nematodes (Wakil *et al*., 2014; Ebadollahi *et al*., 2013; Adebayo and Krettli, 2011; Okafor *et al*., 2010; Du *et al*., 2008; Tan and Luo, 2011; Simpson *et al*., 2011). Many compounds with diverse chemical structures and different modes of action are classified as botanical insecticides. In Orthoptera (such as grasshoppers, crickets, locusts), the antifeedant effect seems especially important. A number of species refuse to feed on neem-treated plants for several days, sometimes several weeks (Montes-Molina *et al*., 2008; Lehman *et al*., 2007; Sharma *et al*., 2008).

Currently, some compounds that isolated from neem showed insecticidal activity against O. chinensis, such as saponins and azadirachtin (Amtul and Shakoori, 2014). In this paper, our objectives were to isolating the insecticidal active fraction from extracts of neem leaves by column chromatography and studying their insecticidal activity against the O. chinensis nymph in vitro and the mechanism of action by the light microscope and transmission electron microscopic.

MATERIALS AND METHODS

Neem (Azadirachta indica A. Juss) leaves were collected from Panzhihua city in Sichuan Province of China, which was identified by the Pharmacy Laboratory of Sichuan Agricultural University.

Oxya chinensis nymph were collected from the farm of Sichuan Agricultural University, reared in breeding boxes at the condition of 25±2℃, 16:8 light duration and 70±10% relative humidity, and fed with fresh corn leaves for 2d. The 5^{th} instar Oxya chinensis were used in the experiments.

Neem leaves were successively extracted with petroleum ether, 95% ethanol and water. Each extract was taken to dryness under vacuum at 40℃ and used for the assay of their insecticidal activity. The extraction rate (%) was calculated as follows:

Extraction rate (%) = [weight of extract (g) / weight of neem leaves (g)] × 100%

The insecticidal activity was evaluated by leaf-soaking and larva-soaking methods. Three extracts were diluted to 25mg/mL with water and a small amount of Tween-80 (1%). Tested insects were dipped into three extractions for 10s respectively, and then fed with the corn leaves which were dipped by the same diluted extraction for 3s. The insecticidal activity was measured by the mortality after 24h, 48h, and 72h. Water and tween-80 was used as negative control, and the whole procedure was repeated thrice. The mortality was calculated by the formulas below:

Percentage of mortality= (number of the dead nymph/number of nymph introduced) × 100%
Corrected percentage of mortality= [1- (n in T after treatment / n in C after treatment)] × 100%

Where “n” is the number of nymph, “T” is the treated group and “C” is the control group.

The 95% ethanol extract suspended in water was successively extracted with petroleum ether, chloroform, ethyl acetate and n-butanol, respectively. Then, the petroleum ether extract was fractionated by column chromatography over silica gel G (100~200 mesh) eluted with a-

hexane/ethyl acetate/acetone (8.5:1:0.5, v/v/v) mixture to give three fractions (F1-F3) (Du *et al*., 2009). The five extractions and three fractions (F1-F3) were diluted to 50, 25, 12.5, 6.25 and 3.12 mg/mL with water and Tween-80, respectively, and their insecticidal activities were measured as described above.

The main components of F1 were analyzed by GC-MS (Agilent 6890-5973N). The Agilent HP-5 column was used and the oven temperature program was as follows: 100℃ for 3 min; 100℃ to 120℃, 5℃/min; 120℃ to 280℃, 10℃/min; 280℃ for 10min. The injector and detector temperatures were 270℃ and 280℃, respectively. The Full-scan molecular weight ranged from 45 to 750.

The midguts tissue of the nymph from post-treatment by the petroleum ether extract after 24h, 48h and 72h were subjected to histological examination. They were pressed in a fixation medium of 10% solution of buffered formalin (pH 7.4) and enclose in paraffin-intended subsequent histopathological examination. A 5μm section of each organ was stained with hematoxylin and eosin. Each section was examined under an optical microscope.

The transmission electron microscopy assay was conducted using previously established methods (Fichi *et al*., 2007). The midguts tissue of the nymph was cut off after post-treatment by the petroleum ether extract for 24h, 48h and 72h and fixed by glutaraldehyde precooling (2.5%, pH 7.2). The tissue was then further fixed with 1.0% osmic acid followed by dehydration with an acetone gradient prior to embedding. Ultra-thin longitudinal sections were cut and stained with uranyl acetate. The sections were then observed using a transmission electron microscope (JEM-1010, Joel, Japan) (Hu *et al*., 2015).

All results were expressed as a mean±standard deviation (S.D.) for the indicated number of experiments. Groups were compared using variance analysis and the Duncan's multiple comparison test (DMRT) by using DPS data processing system. A value of $P < 0.05$ was considered significant.

RESULTS

The extraction rates of the crude extracts of neem leaves were shown in Table 1. The extraction rates of water, 95% ethanol and petroleum ether extract were 46.80%, 25.93% and 2.20%, respectively. The insecticidal activities of the crude extracts of neem leaves against 5^{th} instar Oxya chinensis was shown in Table 2. The insecticidal activities of the three extracts were 0, 10% and 10%, respectively in 24h, 0, 16.67% and 20%, respectively in 48h and 0, 26.67% and 33.33%, respectively in 72h. The petroleum ether extract was found to have the highest insecticidal activity, and the water extract had no insecticidal activity. The 95% ethanol extract had a little lower insecticidal activity, but much higher extraction rate than the petroleum ether extract. Thus, the 95% ethanol extract was further studied.

The 95% ethanol extract are further purified with petroleum ether, chloroform, ethyl acetate and n-butanol in sequence extracted method, respectively. Mortalities of the Oxya chinensis nymph treated with the five extracts was shown in Table 3. At a concentration of 25mg/mL, the mortalities of the petroleum ether extract were 23.33%, 73.33%, 80%; and the mortalities of the chloroform extract were 23.33%, 30%, 43.33%, in 24h, 48h, and 72h, respectively. The ethyl acetate, n-butanol and water extracts were found to have no significant insecticidal activity in all portions. These results indicated that the petroleum ether extract showed the highest insecticidal activity than other extracts in different time. Therefore, it was necessary to conduct a further study on the petroleum ether extract.

Table 1. The extraction rates of the crude extracts of neem leaves

Extraction solvent	Solid-liquid ratio	Dry matter (g)	Extraction rate (%)
Petroleum ether	1:10	0.44	2.20
95% ethanol	1:10	3.66	25.93
Water	1:10	9.36	46.80

Table 2. Insecticidal activities of the crude extracts of neem leaves against 5rd instar *O. chinensis*

The tested samples	Mortalities (mean±S.E. %)		
	24h	48h	72h
The petroleum ether extract	10±0cd	20±5.77bc	33.33±3.33a
The 95% ethanol extract	10±5.77cd	16.67±6.67bc	26.67±3.33ab
The water extract	0±0d	0±0d	0±0d
Control	0±0d	0±0d	0±0d

The difference between data with the different small letter within a column is significant (P＜0.05).

Table 3. Insecticidal activities of five extracts of neem leaves against 5rd instar *O. chinensis*

The tested samples	Mortalities (mean±S.E. %)		
	24h	48h	72h
The petroleum ether extract	23.33±3.33c	73.33±8.82a	80±5.77a
The chloroform extract	23.33±3.34c	30±10c	43.33±8.82b
The ethyl acetate extract	0±0d	0±0d	0±0d
The n-butanol extract	0±0d	0±0d	0±0d
The water residue	0±0d	0±0d	0±0d
Control	0±0d	0±0d	0±0d

The difference between data with the different small letter within a column is significant (P＜0.05).

The toxicity results of the petroleum ether extract are shown in Table 4, the median lethal concentration (LC_{50}) value of the petroleum ether extract to 5th instar Oxya chinensis are 55.66mg/mL, 20.07mg/mL and 14.93mg/mL in 24h, 48h and 72h, respectively. These results indicated that the insecticidal activity of petroleum ether extract showed the relation of time-and concentration-dependent.

Table 4**.** Insecticidal activity of the petroleum ether extract of neem leaves against 5rd instar *O. chinensis*

Concentrations (mg/mL)	Mortalities (mean±S.E. %)		
	24h	48h	72h
50	43.33±13.33b	83.33±8.82a	96.67±3.33a
25	23.33±3.33bc	73.33±8.82a	80±5.77a
12.5	3.70±3.70c	18.15±13.46c	21.48±11.48bc
6.25	0±0c	6.67±6.67c	16.67±16.67c
3.12	0±0c	3.70±3.70c	7.41±7.41c
Control	0±0c	0±0c	0±0c

The difference between data with the different small letter within a column is significant (P＜0.05).

Three fractions (F1-F3) obtained from the petroleum ether extract by column chromatography were tested for insecticidal activities against 5th instar Oxya chinensis. The results were shown in Table 5 to 7. F1 showed the highest insecticidal activity. It has also shown that the three fractions (F1-F3) displayed insecticidal activity against 5th instar Oxya chinensis in all test time and concentrations.

The identified compounds and retention times have been given in Table 8. The petroleum ether extract consisted mainly of 6 alkanes (pentacosane, hexacosane, heptacosane, nonacosane, triacontane and hentriacontane), 1 olefin (squalene), 2 esters (hexadecanoic acid, ethyl ester and 9, 12, 15-octadecatrienoic acid, ethyl ester, (Z, Z, Z)-) and 1 amide (erucyl amide).

Table 5. Insecticidal activity of F1 of the petroleum ether extract of neem leaves against 5rd instar *Oxya chinensis*

Concentrations (mg/mL)	Mortalities (mean±S.E. %)		
	24h	48h	72h
50	86.67±8.82abc	100±0a	—
25	60±5.77bcd	90±10ab	96.67±3.33a
12.5	43.33±8.82de	76.67±14.53abc	93.33±3.33a
6.25	26.67±3.33ef	60±5.77bcd	90±10ab
3.12	16.67±3.33ef	56.67±24.04cd	73.33±17.64abc
Control	0±0f	0±0f	0±0f

The difference between data with the different small letter within a column is significant ($P<0.05$).

Table 6. Insecticidal activity of F2 of the petroleum ether extract of neem leaves against 5rd instar *Oxya chinensis*

Concentrations (mg/mL)	Mortalities (mean±S.E. %)		
	24h	48h	72h
50	70±15.28bc	93.33±3.33a	100±0a
25	60±5.77c	86.67±6.67ab	96.67±3.33a
12.5	20±5.77efg	53.33±13.33cd	93.33±6.67a
6.25	13.33±6.67fg	36.67±8.82de	63.33±6.67c
3.12	3.33±3.33fg	6.67±3.33fg	23.33±8.82ef
Control	0±0f	0±0f	0±0f

The difference between data with the different small letter within a column is significant ($P<0.05$).

Table 7. Insecticidal activity of F3 of the petroleum ether extract of neem leaves against 5rd instar *Oxya chinensis*

Concentrations (mg/mL)	Mortalities (mean±S.E. %)		
	24h	48h	72h
50	86.67±3.33ab	100±0a	—
25	43.33±12.02cd	80±11.55ab	90±10a
12.5	16.67±16.67efg	63.33±3.33bc	80±5.77ab
6.25	13.33±3.33efg	36.67±14.53de	46.67±8.82cd
3.12	3.33±3.33fg	3.33±3.33fg	26.67±3.33def
Control	0±0g	0±0g	0±0g

The difference between data with the different small letter within a column is significant ($P<0.05$)

Table 8. Chemical composition of F1 by GC-MS

Number	Retention time (min)	Compound name
1	18.310	Hexadecanoic acid,ethyl ester
2	19.996	9,12,15-Octadecatrienoic acid,ethyl ester,(Z,Z,Z)-
3	22.735	Pentacosane
4	23.539	Hexacosane
5	24.438	Heptacosane
6	25.380	Erucyl amide
7	25.906	Squalene
8	26.772	Nonacosane
9	28.165	Triacontane
10	30.098	Hentriacontane

Under light microscope, the tissue showed normal structure in the control group (Figure 1A). In the experiment group, after 24h treatment, muscle layer of midgut tissue was complete; layer of intestinal wall cells thickened and part of intestinal wall cells fell off; many nuclei of regenerative cells became pyknotic; the regenerative cells were filled with red mesh-like

material; cylindrical cells swelled and part of them fell off (Figure 1B). After 48h of treatment, muscle layer of midgut tissue was complete; large area of the layer of intestinal wall cells fell off; microvilli disappeared absolutely; regenerative cells of midget were filled with red mesh-like material and nuclei became smaller; cylindrical cells swelled and lots of them occurred fatty degeneration (Figure 1C). After 72h treatment, layer of intestinal wall cells almost fell off; leaving only the circular muscle layer which became thinner and stained lighter; circular muscle layer of midget occurred vacuolar degeneration (Figure 1D).

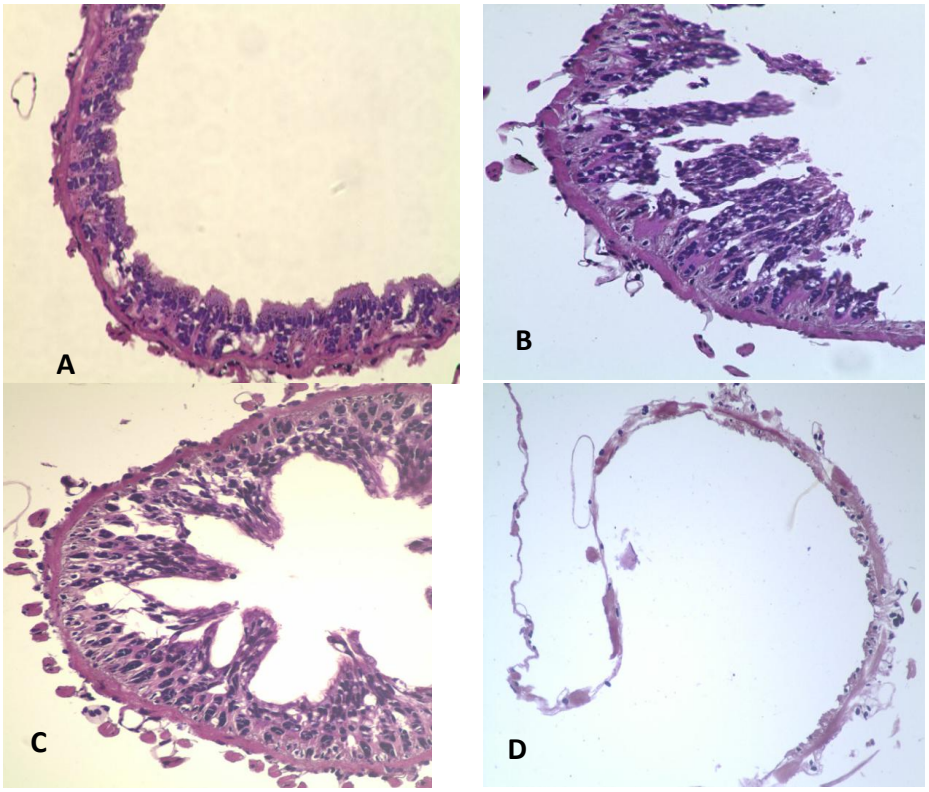

Figure 1 Histopathological changes in the midgut of the *Oxya chinensis* larvae treated with the petroleum ether extract for different times. A: Control group (H.E. ×400), the tissue showed normal structure. B: After 24h treatment (H.E. ×400), layer of intestinal wall cells thickened and part of intestinal wall cells fell off; many nuclei of regenerative cells became pyknotic; the regenerative cells were filled with red mesh-like material; cylindrical cells swelled and part of them fell off. C :After 48h treatment (H.E. ×400), large area of the layer of intestinal wall cells fell off; microvilli disappeared absolutely; regenerative cells of midget were filled with red mesh-like material and nuclei became smaller; cylindrical cells swelled and lots of them occurred fatty degeneration. D:After 72h treatment (H.E. ×200), layer of intestinal wall cells almost fell off; leaving only the circular muscle layer which became thinner and stained lighter; circular muscle layer of midget occurred vacuolar degeneration.

Under electron microscopic transmission, in the control group, rough endoplasmic reticulums were orderly with a lot of ribosomes (Figure 2A). After 24 h of treatment, rough endo-plasmic reticulum decreased in the cytoplasm and the ribosomes began to fall (Figure 2B). After 48 h of treatment, rough endoplasmic reticulums were disorganized and ribosomes fell off (Figure 2C). After72 h treatment, rough endoplasmic reticulums expanded extremely, fractured and vacuolated (Figure 2D).

In the control group, the microvilli of midgut of the Oxya chinensis nymph were orderly (Figure 3A). After 24 h of treatment, microvilli arranged neatly; the pathologic changes were not obvious (Figure 3B). After 48 h of treatment, microvilli fractured and fell off (Figure 3C). After 72 h of treatment, microvilli disappeared and a lot of vacuoles appeared (Figure 3D).

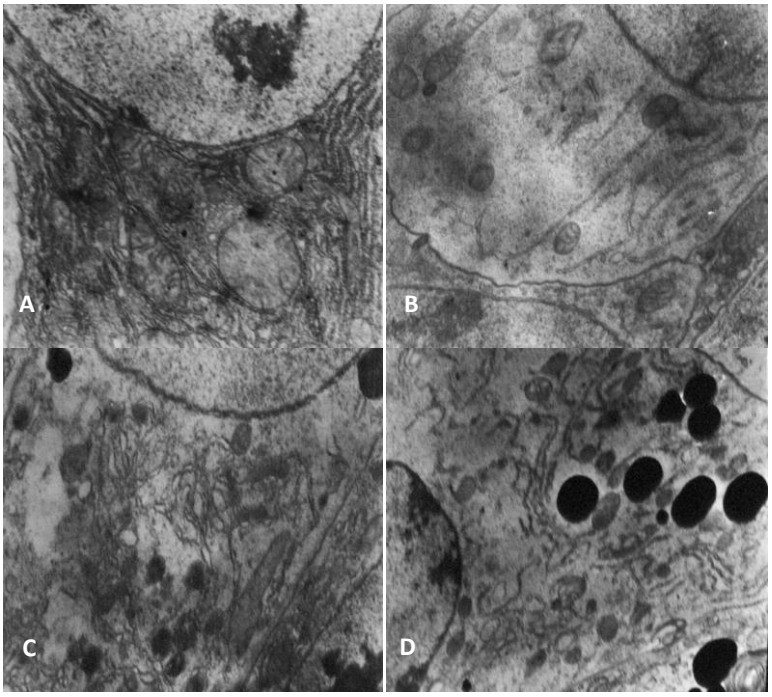

Figure 2 Effects of the petroleum ether extract on the midgut of Oxya chinensis larvae. Control group (A): rough endoplasmic reticulums were orderly with a lot of ribosomes (×15000). Experiment group: after 24h treatment (B), rough endo-plasmic reticulum decreased in the cytoplasm and the ribosomes began to fall (×12000); after 48h treatment(C), rough endoplasmic reticulums were disorganized, expanded, and ribosomes fell off (×17000); after 72h treatment(D), rough endoplasmic reticulums expanded extremely, fractured and vacuolated (×10000).

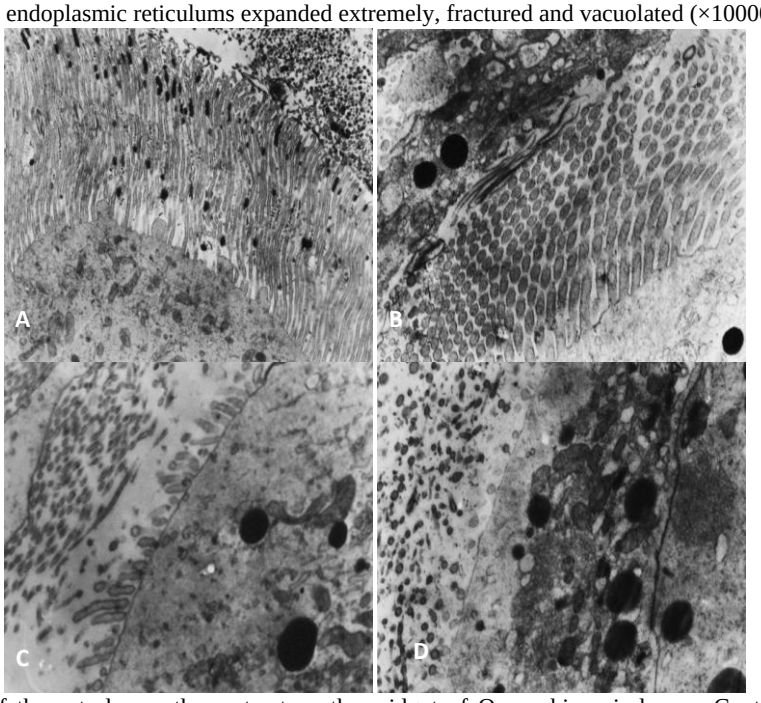

Figure 3. Effects of the petroleum ether extract on the midgut of Oxya chinensis larvae. Control group (A), the microvilli of midgut of the Oxya chinensis larvae was orderly (×6000). Experiment group: after 24h treatment (B), microvilli arranged neatly, the pathologic changes were not obvious (×12000); after 48h of treatment (C), microvilli fractured and fell off (×15000); after 72h of treatment (D), microvilli disappeared and a lot of vacuoles appeared (×10000).

In the control group, the mitochondria of the dermis cells were distributed evenly; crest clarity was regularly arranged in the plate layer; the surrounding membrane was clearly observed (Figure 4A). After 24 h of treatment, mitochondria swelled in different degrees; cristae shortened and decreased (Figure 4B). After 48 h of treatment, mitochondria swelled obviously; a matrix of mitochondria occurred at electronic clear area; cristae almost disappeared (Figure 4C). After 72 h of treatment, mitochondria swelled extremely; many vacuoles occurred and broke; myelin figures were observed (Figure 4D).

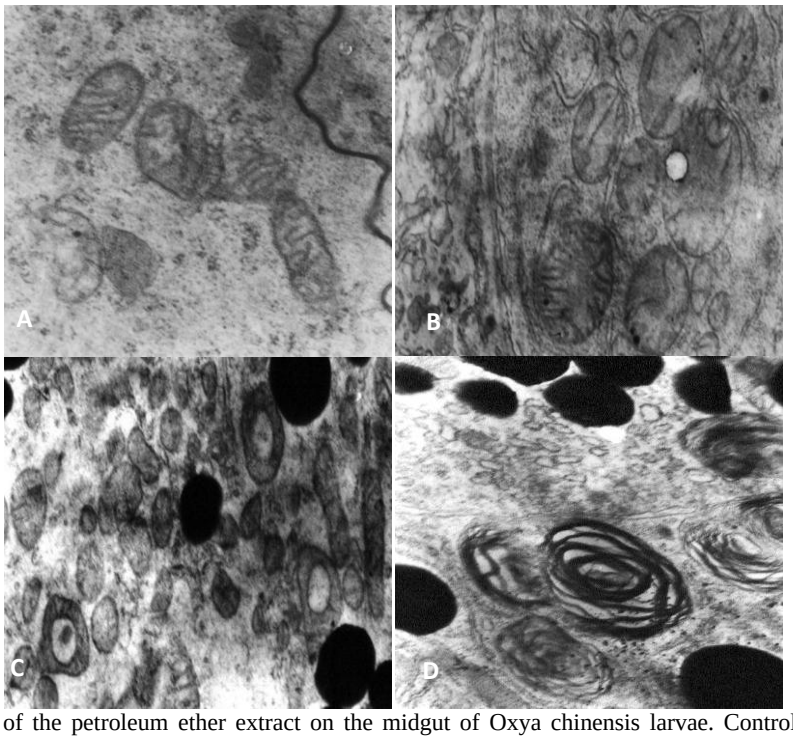

Figure 4. Effects of the petroleum ether extract on the midgut of Oxya chinensis larvae. Control groups (A), the mitochondria of the dermis cells were distributed evenly, crest clarity was regularly arranged in the plate layer, and the surrounding membrane was clearly observed (×30000). Experiment group: after 24h treatment (B), mitochondria swelled in different degrees; cristae shortened and decreased (×17000); after 48h treatment (C), mitochondria swelled obviously; a matrix of mitochondria occurred at electronic clear area; cristae almost disappeared (×17000); after 72h treatment (D), mitochondria swelled extremely; many vacuoles occurred and broke; myelin figures were observed (×20000).

DISCUSSION

Neem belonging to the Meliaceae family has been investigated intensively for its potential as a bio-insecticide (Verma *et al*., 2007; Farooq *et al*., 2011). The insecticidal activity of neem generally attributes to azadirachtin which is a well-known potent insecticide (Zheng *et al*., 2011; Kumar *et al*., 2010). 0.3% azadirachtin EC (150mL/hm^2 and 210 hm^2) was sprayed in the grassland to investigate the control effect on grasshoppers. The results showed that the effects of controlling *Calliptamus abbreviate* ikovnnikov and *Oedaleus decorus* asiaticas after 3d were 90.6% and 92.5% (Yang *et al*., 2006). In this study, the results showed that the petroleum ether extract from neem leaves had obvious stomach toxicity against *O. chinensis* nymph from the toxic symptoms and displayed insecticidal activity against 5th instar *O. chinensis* in all test time and concentrations in a time-and concentration-dependent manner. The present study may encourage further researches on using simple and inexpensive application methods for controlling grasshoppers.

The main components of the petroleum ether extract of neem leaves were 6 alkanes, 1 olefin, 2 esters and 1 amide. However, the phytochemical analysis did not reveal the presence of

Azadirachtin, which has been considered as one of the main active compounds of neem. These results suggested that Azadirachtin was excluded from the petroleum ether extract during our separation process and other compounds from the extract of neem leaves also possessed potent insecticidal activity and could be used as insecticide. In the previous study, we found that octadecanoic acid-tetrahydrofuran-3, 4-diyl ester (ODA-THF) exhibited acaricidal activity against *Sarcoptes scabiei* var. cuniculi nymph (Du *et al*., 2009), which will be subject of more studies in further experiments.

The petroleum ether extracts damaged the pivotal organs of the digestive system which was also one of the causes of death. The important reason was the peritrophic membranes disappearance and the midgut cells destruction. The peritrophic membranes are the first obstacle through the foods pass through the digestive tract wall and are an important target for many pesticides (Hegedus *et al*., 2009). The observed histopathological effects of the petroleum ether extract on the midgut tissue of *O. chinensis* nymph were similar to the results on Mythimna separate and Plutella xylostella (Lü *et al*., 2010). It stated that the midgut of *O. chinensis* was affected after treatment with the petroleum ether extract and the midgut membrane system was damaged seriously. All the above observations showed that damaging the normal structure of midgut and endomembrane may be one of the primary mechanisms.

Transmission electron microscopy results showed that the autolysis of midgut cells was the main cause of the changes. After 48h of treatment, the results showed that midgut cells began to change. The microvilli showed fractures, fell off, and disappeared; partial vacuolation formed. The lysosome increased and organelle started autolysis. Based on the above results, it could be concluded that one of the main reasons was that the midgut cells fell off. But the protease activity of midgut, such as acid phosphatase, needs to be further studied to confirm the results.

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