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EFFICIENCY OF *COLOCASIA ESCULENTA* LEAVES EXTRACT AND HISTOPATHOLOGICAL EFFECTS ON *CULEX PIPIENS* (DIPTERA: CULICIDAE)

By

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Abstract

This study evaluated the toxicity of *Colocasia esculenta* leaves extract on 3rd, 4th instars larvae and pupae of *Culex pipiens*. Bioassays showed that the 3rd instar larvae was the most susceptible to the different concentrations of extract, where the LC_{50} after 48 hr. post-exposure was 79.4l, 109.65 & 141.25 for the 3rd, 4th instars larvae and pupal stage respectively. The histo-pathological effects of *C. esculenta* leaves extract *o*n midgut regions and gastric caeca of the 3rd instar larvae were studied. When larvae were treated with 100 ppm of *C. esculenta e*xtract, all larvae developed dramatic pathological lesions especially Malpighian tubules were extensively affected. The midgut cells showed morphological deviation from normal ones, through slightly apical degenerated (lysis) of epithelial cells. The epithelial cells with extensive cellular microvilli were shrinkage, the nuclei showed pyknotic characteristic and the peritrophic mem-brane was appeared discontinuation in compared to control. When the 3rd larval instar was exposed to extract 400 ppm, the epithelial cells, adipose fabric and muscles were extensively affected. Also, the gastric caeca was affected obviously. These observation and alterations in cells of *Cx. pipiens* larvae are related to the dangerous effect of *C. esculent* leaves extract.

Key words: *Culex pipiens*, immature stages, *Colocasia esculent* leaves, Control, Histopathology

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Introduction

The taro plant, *Colocasia esculenta* Linnaeus (Family: Araceae) is an annual herbaceous plant. Taro was included in the high oxalate food group (Oscarsson and Savage, 2007). Taro has a long history of usage in traditional medicine in several countries across the world, especially in the tropical and subtropical regions. The herb has been known since ancient times for its curative properties and has been utilized for treatment of various ailments such as asthma, arthritis, diarrhea, internal hemorrhage, neurological disorders, and skin disorders. A wide range of chemical compounds including flavonoids, β-sitosterol, and steroids have been isolated from this species (Prajapati *et al,* 2011). Purification of lectin from *C. esculenta* corms has anti-insect potential towards *Bactrocera cucurbitae* (Thakur *et al,* 2013). *Colocasia esculenta* have been shown toxic effects to *Sitophilus zeamais* adults (Arannilewa and Odeyemi, 2007). *Colocasia esculenta* tuber agglutinin was effective against *Aphis gossypii* (Das *et al*, 2013). *C. esculenta* Plant has defense compound α-amylas inhibitors in corm that interact with insect α-amylases and subsequent insect mortality (Neeraj Wadhwa *et al,* 2013). Colocasia α-amylas inhibitors have been shown toxic effects to several insect pests such as *Corcyra cephalnica, Tribolium castaneum*, *Callosobruchus chinensis* and *Spodoptera littoralis* (Kumari *et al*, 2012). Therefore, it is necessary to exploit it to its maximum potential in the control mosquitoes. Vector-borne diseases affect two-thirds of the world's population and kill millions annually (Gubler, 1998). Mosquitoes of the genera *Aedes*, *Culex* and *Anopheles,* found in tropical and subtropical zones throughout the world, are the primary arthropod vectors for fevers and parasitic diseases. There are different types of mosquito vectors spread all over Qalyubiya Governorate (Ibrahim *et al,* 2011). It is well known that the use of broad spectrum chemical insecticides in the battle against insect

pests had left both the soil and ground water contaminated with hazardous chemicals. Thus, the urgent need for a clean and safe environment has forced the majority of scientists to focus on the utilization of environmentally safe bio-control agents to manage mosquito vectors and to keep their numbers down the threading level. The biological control of immature stages now appears to be the most powerful means of reducing target populations of Culicidae and other dipteran pests. Muthukrishnan and Puspalatha (2001) evaluated the larvicidal activity of extracts from *Calophyllum inophyllum* (Clusiaceae), *Rhinacanthus nasutus* (Acanthaceae*), Solanum suratense* (Solanaceae) and *Samadera indica* (Simaroubaceae), *Myriophyllum spicatum* (Haloragaceae) against *Anopheles stephensi*. Several indigenous plants viz., *Ocimum basilicum*, *Ocimum santum*, *Azadirachta indica*, *Lantana camera*, *Vitex negundo* and *Cleome viscosa* were studied for their larvicidal action on the field which collected fourth instar larva of *Cx. quinquefasciatus* (Kalyanasundaram and Dos, 1985). Raveen *et al*. (2014) stated that *Nerium oleander* L (Apocynaceae) flower extracts has Larvicidal activity against *Culex quinquefasciatus.* Extracts from the Neem leaves have shown excellent insecticidal properties against fecundity and fertility of mosquito vector and were at the same time very eco-friendly (Schmutterer, 1990; Senthil Nathan *et al*, 2005). The Histopathological changes induced by different plant extracts on mosquitoes were documented by many authors (Jang *et al,* 2002; Silva-Filha and Peixoto, 2003).

This study aimed at the support of utilizing environmentally safe strategies for controlling mosquito vector *Cx. pipiens*. Thus the current study evaluated the efficiency of *Colocasia esculenta* leaf extract, as environmentally safe bio-control agents, against the larval and pupal stages of the filaria vector, *Cx. pipiens* in the laboratory. Larval susceptibility to *Colocasia esculenta* was also evaluated at the histopathological level to elucidate the effects of this potential bio-insecticide.

Materials and Methods

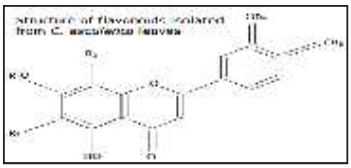
Rearing and maintenance of *Culex pipiens* stock culture in the laboratory. Egg rafts were collected from stagnant sea wage water of Benha. The hatched larvae were cultured and maintained in the laboratory at room temperature (27±2˚c) and 70 – 75 relative humidity. The larvae and pupae were fed on a mixture of brewer's yeast and dried powder bread in the ratio 1: 2. This Pupae and adults emerged from this larvae were reared in mosquito cage and the females were allowed to feed on pigeon blood and the males were provided with 10% glucose solution soaked in cotton. Eggs laid by these adults were culture in separate container and reared for more than three generations. The immature stages developed from these eggs were used for bioassay studies.

Collection and preparation of plant extract: *Colocasia esculenta* Linn belong to Family Araceae. It is commonly called as *taro*. Geographically, it occurs throughout India and is cultivated worldwide (Pullaiah, 2006). The leaves of *C. esculenta* were collected from cultivated areas at Benha, Qalyoubia Governorate. The leaves were washed with tap water and dried for 3 weeks in a shade at an environmental temperature. The dried leaves were powdered mechanically using commercial electrical stainless steel blender and extracted with ethanol in soxhlet apparatus (boiling point range 40-50˚c) for 8 h. the extract was filtered through Buchner funnel with Whatman No. 1 filter paper. The extract was concentrated in a rotatory vacuum evaporator and the residue obtained was stored at 4˚C (Arivoli *et al*, 2012).

Phytochemistry: Leaves contain calcium oxalate, fibers, minerals (calcium, phosphorus, etc.), and starch, vitamin A, B, C, etc. (Sheth, 2005). Phytochemically, these leaves also contain flavones, apigenin , luteolin and anthocyanins (Khare, 2007). Iwashina *et al*. (1999) carried out isolation and identification of the flavonoids in the leaves of *C. es-*

culenta plant. The flavonoids were identified by UV spectral analysis. They isolated eight flavonoids *viz*. orientin, isoorientin, isovitexin, vicenin-2, orientin 7-*O*-glucoside, isovitexin 3'-*O*-glucoside, vitexin X" -*O*-glucoside, luteolin 7-*O*-glucoside.

Compound	R_1	R_2	R_3	R_4
Vitexin	H	Glucose	H	H
Isovitexin	Glucose	H	H	H
Isoorientin	Glucose	H	H	H
Luteolin 7-O-glucoside	Glucose	H	Glucose	H



Mosquito-larvicidal bioassay: The concentrations of leaves extract were prepared as 20, 50, 100, 200 and 400 ppm for bioassay studies. Ten larvae and ten pupae were pipette into each of 100ml water with 1ml from each concentration (in three replicates). Larvae in distilled water were used as a control.

Statistical analysis: Data were subjected to one way ANOVA test using SPSS program version 21, the values of percentage mortality and their stander error (SE) were calculated. Moreover, the LC_{50} and LC_{90}, correlation coefficient (R^2) and slope were calculated using Probit analysis (Finney, 1971)

For the Histological studies untreated and treated 3rd larval instar were examined. Normal and treated 3rd instar *Cx. pipiens* larvae were used for investigating histological alterations at 48h post-treatment with 100 ppm and 400ppm of *C. esculenta* leaves extract. Then they were fixed in bouins solution for 24 h. After dehydration in a graded ethanol series, the material was embedded in paraffin wax and cut with glass knives in a rotary microtome. The sections (5mm thick) were stained with routine haematoxylin and eosin (H &E) according to Villalon *et al.* (2003) and examined with a photomicroscope Leica ICC 50 HD. Resulting images from control or treated larval preparations were imported into Adobe Illustrator Cs, 2003 Software and adjusted for contrast and suitable qualities.

Results

The results are shown in table (1) and figures (1 to 8)

Table 1: *C. esculenta* leaves extract effect on 3rd, 4th larval instars & pupal stage of *Cx. pipiens* after 48 hrs post treatment.

Concentration (ppm)	Mortality %		
	3rd instar larvae	4th instar larvae	Pupae
Control	0.0	0.0	0.0
20	16.4 ± 1.43	14.2 ± 1.92	12.4 ± 1.67
50	37.2 ± 1.56	31.0 ± 1.58	25.0 ± 1.58
100	54.4 ± 1.48	44.4 ± 1.81	36.4 ± 1.48
200	67.2 ± 1.58	65.4 ± 1.14	58.2 ± 1.14
400	97.0 ± 1.16	94.4 ± 1.60	87.4 ± 1.14
LC_{50} (ppm)	79.43	109.65	141.25
LC_{90} (ppm)	334.54	387.63	426.20
R^2	0.9186	0.9556	0.9754
Slope	0.1903	0.1983	0.1903

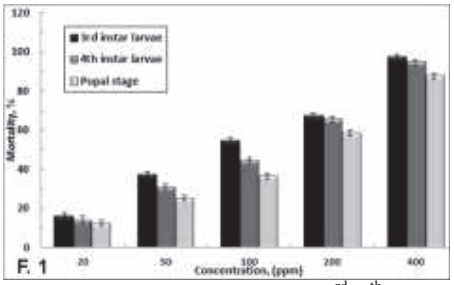


Fig. 1: Effect of different concentrations of *C. esculenta* extract against 3rd, 4th larval instars and pupal stage of *Cx. pipiens* after 48 hrs post treatment.

Discussion

In the present study, the mortalities percentage were increased by increasing the concentration of *C. esculenta* leaves extract on the 3rd, 4th larval instars and pupal stage. A linear relationship between concentrations and mortality percentages was made via a regression plot (Fig.1). Regression analysis showed a concentration-dependent significant correlation of the *C. esculenta* extract with larval and pupal mortality. Strong correlation between concentration and mortality where R^2 were 0.9186, 0.9556 and 0.9754 after 48 hrs post-treated 3rd, 4th larval instars and pupal stage (Tab. 1). The results agreed with Trabowesi *et al.* (2005), who found that Citrus oils and *Bacillus thuringensis* H-14 caused serious latent effect to adults and larval stages of *Cx. pipiens* and *Musca domestica*. Also, Amer and Mehlhom (2006) stated that thirteen oils (camphor, thyme, amyris lemon, cedar wood, Frankincense, dill, myrtle, juniper, black pepper, verbena, helichrysym and sandal wood) induced 100% mortality of mosquitoes larvae after 24hrs. Zayed *et al*. (2009) evaluated biological control of 4th instar *Cx. pipiens* larvae and found that 4th instar larvae was highly susceptible to *Bacillus thuringiensis* followed by *Citrus limon* and *Allium sativium* .

The values of LC_{50} after 48 hrs were 79.4l, 109.65 & 141.25 for the 3rd, 4th larval instars and pupal stage respectively. Thus, the 3rdlarval instar was the most susceptible to *C. esculenta* leaves extract. Govindarajan *et al*. (2008) found that methanol leaf extract of *C. fistule* was lethal to the larvae of *Cx. quinquefaciatus* and *Anopheles stephenis* with LD_{50} values of 17.97 and 20.57 mg/L, respectively. Mathew *et al.* (2009) reported that leaf chloroform extracts of *Nyctanthes arbortristis* showed lethal values (LC_{50} after 48 hrs with 303.2 & 518.2ppm) against *Aedes aegypti* and *An. stephensi*, respectively.

In the present study, the control 3rd instar larvae of *Cx. pipiens,* midgut and gastric caecum showed a well preserved layer of epithelial cells (EP). The ovoid nuclei (N) located centrally. Microvilli border (MV) in the midgut and gastric caecum (GC) were normal. The peritrophic membrane (PM) surrounds the food bolus (FB) and the adipose fabric (AF) has a loose meshwork of lobes invested in connective tissue strands. The fat cells are closely adhered the cytoplasm is stuffed with big nuclei. The muscle (M) is composed of striated fibers, each fiber consists of a number of parallel fibrillae. The Malpighian tubules (MT) are rounded, thin, tubes that have large nucleus and a well defined brush border surrounding a relatively narrow lumen (Fig. 2).

The histopathological effects of *C. esculenta* leaves extract *o*n Midgut regions and gastric caeca of 3rd instar larvae were studied. The choice of these regions is justified by the fact that they are directly in contact with toxic element (calcium oxalate) in the taro extract. When treated larvae with 100 ppm. of *C. esculenta e*xtract, all larvae developed dramatic lesions. The larvae treated with 100 ppm. Malpighian tubules were enlarged with necrosis and degeneration in nuclei of the cells. The most noticeable effect was an increase in the lumen of the tubules (Fig.3). This effect may be related to oxalate content and other phytochemicals component in *C. esculenta* extract. Bradbury and Nixon (1998) reported that, most taro cultivars taste acrid and can cause swelling of lips, mouth and throat if eaten raw. This acridity is caused by needle–like calcium oxalate crystals raphids penetrated soft skin. One case of systemic toxicity attributed to taro leaf ingestion was identified with muscle spasms and renal insufficiency (Oscarsson and Savage 2007). When treated larvae with 100 ppm. extract, midgut showed morphological deviation from normal ones, through slightly apical degeneration (lysis) of epithelial cells. These cells with extensive cellular microvilli were shrinkage the nuclei are showing pyknotic characteristic (PN) and the peritrophic membrane was appeared discontinuation in Compared to control (Figs. 4a & b). The epithelial cells destruc-

tion of lining the midgut was associated with paralysis and cessation as in *B. thurengiensis* (Clark *et al*, 2005). When 3rd larval instar was treated with 400 ppm. of *C. esculenta* extract, an acceleration of the lysis of epithelial cells and destruction of the peritrophic membrane were perceptible. The epithelial cells with microvilli, nucleus, and cytoplasmic organelles, were bursting into the gut lumen. The degeneration of the midgut epithelial cells (DEP) were also extensively affected as disorganized, some cells hypertrophy through swollen of some epithelial cells and the most of the epithelial cells evacuated from their content and finally sloughed off as scattered groups into the lumen. The adipose fabric become vaculated, less eosinophilic with a notable degeneration (Fig.5). This agreed with Hamouda *et al*. (1996) who found that *Cx. pipiens* midgut of treated with *Artemisia judaica* epithelial layer was affected, vacuolated, swollen cells, masses of cellular material appeared in the lumen and epithelium lost normal picture. The muscles were also hypertrophied, with the marked degeneration of muscles (DM) by fissures, vacuoles and disruption of sarcolemma (Fig. 6). On the other hand, Ngai and Ng (2007) found normal muscles, nuclei and microvilli of brush as well as adiposis fabric. The untreated larvae of *Cx. pipiens* gastric caecum showed epithelial columnar cells with ovoid nuclei are located in the center of the cell and a regularly microvilli border (Fig. 7).

The larvae treated with 400 ppm. of *C. esculenta* extract showed on the level of this region morphological and serious damage of the epithelial columnar cells, some cells appear slightly hypertrophied with a perceptible beginning of vacuolization at the apical level. These vacuoles invaded the cells. Sometimes, we noted an enlargement of intercellular spaces, the epithelial cells start to burst, cells degenerated and we noted a cytoplasmic rejection of cells material that mixed with food bolus (Fig. 8). David *et al.* (2000) found that phytochemicals primarily affect the midgut epithelium and secondarily affect the gastric caecum and the Malpighian tubules in mosquito larvae. This study gave symptoms similar data (Hamouda *et al,* 1996; Hussein and Shoukry, 1997; Assar and El-Sobky, 2003; Zayed *et al,* 2009) on *Cx. pipiens* that may indicate high toxicity of *C. esculenta* leaves extract against mosquitoes, being an effective biocontrol agent and the present study may encourage further researches on using simple and inexpensive application methods for controlling mosquitoes in their breeding sites.

In Egypt, the occurrence of *Culex pipiens* and other culicine mosquitoes contributes to the risk of mosquito borne disease transmission in the Arab Countries as well. Apart, filariasis (El Bahnasawy *et al*, 2013b), *Culex pipiens* transmits viral hemorrhagic fevers as the West Nile Fever (El Bahnasawy *et al*, 2013a) and the Rift Valley Fever (El Bahnasawy *et al*, 2013c). The re-emerging of *Aedes aegypti* in Southern Aswan and Toshka District is another disaster problem (Heikal *et al*, 2011)

Conclusion

Among arthropods, mosquitoes from medical, veterinary and economic point of view top all groups. Apart from diseases transmission, mosquitoes can make human life miserable. The results showed that *Colocasia esculenta* leaves extract considered as an eco-friendly insecticide and available in Egypt.

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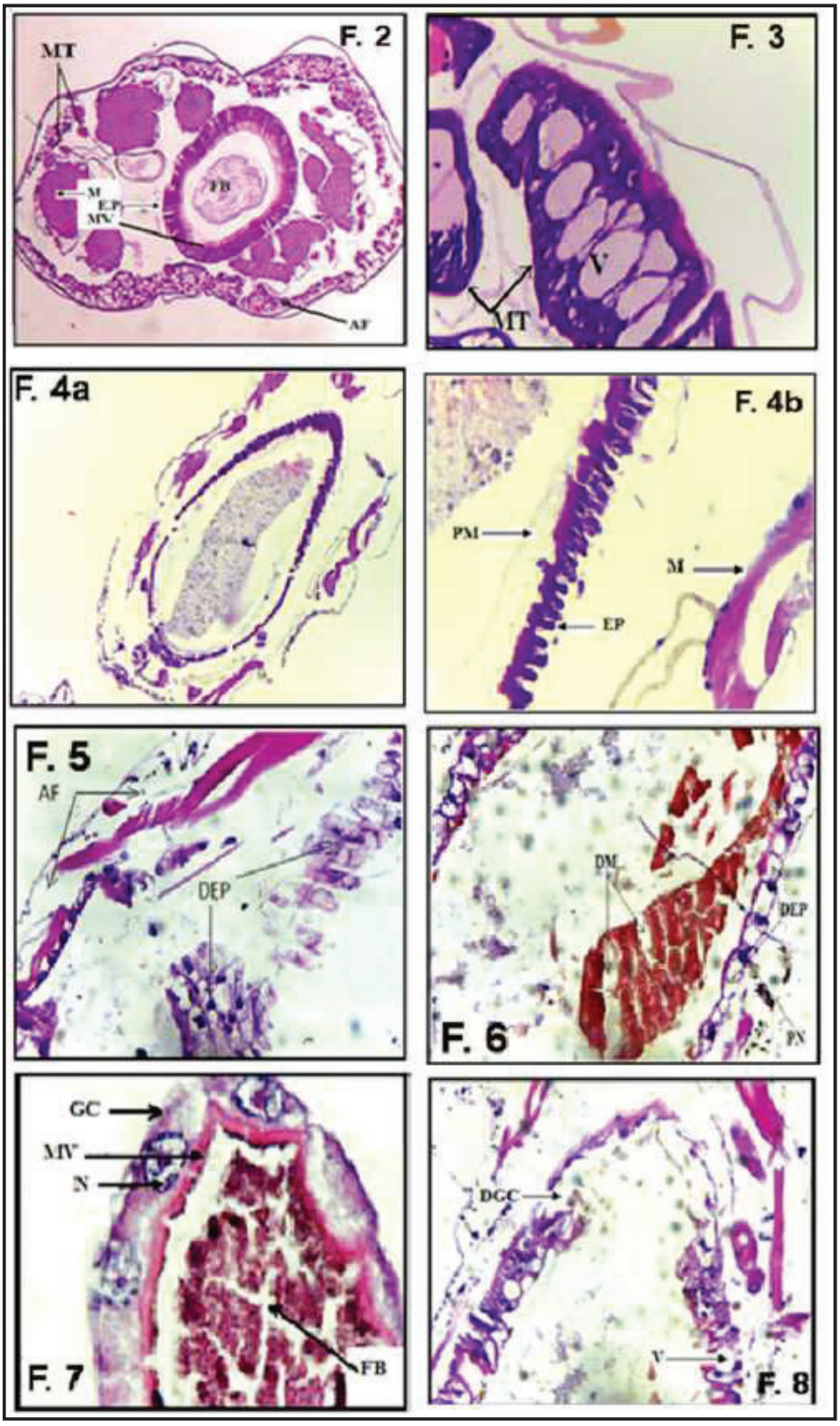
Explanation of figures

Fig. 2: Cross section in midgut of untreated *Culex pipiens* 3rd instar larva (H&E, x200).
Fig. 3: Malpigian tubules of treated (100ppm) *Cx. pipiens* 3rd instar larva (H&E, x400).
Fig. 4a: Cross section in midgut of treated (100ppm) *Cx. pipiens* 3rd instar larva (H&E, x100).
Fig. 4b: Cross section (part) in midgut of treated (100ppm) *Cx. pipiens* 3rd instar larva (H&E, x400).
Fig. 5: Longitudinal section (part) in midgut of treated (400ppm) *Cx. pipiens* 3rd instar larva showing scattered groups of DEP cells in lumen and disappearance of AF (H&E, x400).
Fig. 6: Longitudinal section (part) in midgut of treated (400ppm) *Cx. pipiens* 3rd instar larva showing degenerated muscles (DM) and degenerate with swollen epithelial cells (DEP), (H&E, x400).
Fig. 7: Longitudinal section of gastric caeca (GC) of untreated *Cx. pipiens* 3rd instar larva (H&E, x400).
Fig. 8: Longitudinal section of gastric caeca (GC) of treated (400ppm) *Cx. pipiens* 3rd instar larva showing vacuoles (V) and degenerated GC (H&E, x400).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