Toxicity and biochemical effects of Neem Azal T/S, willow (*Salix aegyptiaca* L.) and Chasteberry (*Vitex agnus-castus* L.) on house fly, *Musca domestica* L. (Diptra : Muscidae)

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ABSTRACT

The house fly, *Musca domestica*, is a very significant pest due to transmitting of various human and animal pathogenic diseases. In a response to environmental threats of chemical insecticides, toxic and biochemical effects of a relatively new plant extracts of willow (*Salix aegyptiaca* L.) and chasteberry (*Vitex agnus-castus* L.) comparing with NeemAzal T/S were studied on 3rd larval instar of *M. domestica*. Results showed that NeemAzal T/S is highly toxic to 3rd larval instar with LC_{50} and LC_{90} of 0.009 and 0.098 µg mL^{-1}, respectively. Whereas, willow and chasteberry showed low toxic effects comparing with NeemAzal T/S with LC_{90} of 70.048 and 66.698 µg mL^{-1}, respectively. Concentrations of total protein markedly decreased in 3rd larval instar after 24 hours exposure to NeemAzal T/S, willow and chasteberry with no significant effects on total lipids compared with control. NeemAzal T/S, willow and chasteberry significantly decreased ALT activity, but NeemAzal T/S only markedly decreased AST activity. On the other hand, amylase (EC 3.2.1.1) significantly increased due to exposure to all tested substances with only significant increase in invertase (EC 3.2.1.26) activity due to exposure to chasteberry. Larval exposure to NeemAzal T/S, willow or chasteberry showed normal trehalase (EC 3.2.1.28) activity as control. These findings show that willow and chasteberry can cause marked toxic effects on larvae of *M. domestica* as well as NeemAzal T/S, which suggesting that more studies on insect development using these plant extracts could be useful.

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MS History: 20.09.2014 (Received)- 20.04.2015 (Revised)- 19.05.2015(Accepted)

Key words: *Musca domestica*, NeemAzal T/S, willow, chasteberry, bioassay, biochemical changes.

Citation: Gamila Sh. Selem and El-Sayed A. El-Sheikh**. 2015.** Toxicity and biochemical effects of NeemAzal T/S, willow (*Salix aegyptiaca* L.) and Chasteberry (*Vitex agnus-castus* L.) on house fly, *Musca domestica* L. (Diptra: Muscidae). *Journal of Biopesticides*, **8**(1):37-44.

INTRODUCTION

The house fly, *M. domestica* L., is a pest that breeds in a wide variety of organic matter particularly domestic garbage which affects animal husbandry and environment. House flies consider a significant pest associated with transmission of numerous human and animal pathogens (Malik *et al*., 2007; Palacios *et al*., 2009). They cause large number of diseases such as salmonellosis, polio, coxsackie, hepatitis, bacillary dysentery, cholera, typhoid, paratyphoid and amoebic dysentery (Graczyk *et al*., 2001; Ugbogu *et al*., 2006) as well as transmission of shigellosis and other diarrhea diseases (WHO, 2002). House flies are an effective vector of *Escherichia coli* O157:H7 among cattle and from cattle to humans, leading to possible outbreaks of enterohemorrhagic colitis (Sasaki *et al*., 2000; Ahmad *et al*., 2007). Sporadic burst of various pathogenic diseases and a stress pattern in livestock and fowls usually caused by high densities of houseflies, leading to physiological and behavioral changes in livestock (Kumar *et al*., 2012). Also, high population densities in poultry and livestock units cause irritation and annoyance to animals and employees with considerably reduction in egg and meat production (Miller, 1993).

Livestock pest management relies mainly on the intensive use of different groups of chemical insecticides (Mullen and Durden, 2002; Kozaki *et al*., 2009; El-Sheikh *et al*., 2014). A little of other

different strategies are used for pests of public health control such as bio-pesticides in terms of enomopathogenic bacteria and viruses (Rodrigues *et al*., 1998; Lietze *et al*., 2013; Zimmer *et al*., 2013), and plant-based extracts (Pavela, 2013). The intensive use of broad range of chemical insecticides to combat pest populations leads to develop high levels of resistance in a relatively short period (Chapman *et al*., 1993; Kaufman *et al*., 2001; Srinivasan *et al*., 2008; Scott *et al*., 2013).

To overcome resistance problem and minimize negative health and environmental effects, searching for alternative and environmentally safe methods of pest control is encouraged (Zimmer *et al*., 2013). The insecticidal activity of different plant extracts against different pests has considered as attractive alternatives to synthetic chemical insecticides for pest management. As the plant extracts have little threats to environment and human health (Koul, *et al*., 2009), many of them have been reported as potential insecticides against house fly (Sukontason, *et al*., 2004). Efficacy of plant extracts against *M. domestica* has been reported which indicate that different essential oils and plant extracts (Sukontason, *et al*., 2004; Urzua *et al*., 2010) induced adverse effects on egg and larval stages, adult fecundity, emergence and life cycle of *M. domestica*. Also, combination effect of plant extracts with classical insecticides have been reported on housefly (Cakir, *et al*., 2008), which might possess economical and ecological benefits in terms of reducing the amount of chemical insecticides within environment and increasing the effect against insect pests. As plant extracts currently have high attention in scientific research, searching for new types as alternative to harmful chemical insecticides for controlling various stages of house fly is critical. In this regard, we investigated the toxicity and biochemical effects of a relatively new extracts of willow and chasteberry comparing with NeemAzal T/S on *M. domestica*.

MATERIALS AND METHODS

Insect rearing

The housefly used in this study was collected as adults from fields in Zagazig city, Sharkia Governorate, Egypt using traps. Adults have been reared under laboratory conditions of 25±2ºC, 14:10 (L:D) period, and 60±5% relative humidity. A diet consisted of wheat-bran, milk powder, brower's yeast and tap water in a ratio of (15: 5: 0.3: 15, respectively) was used for rearing the house fly larvae. After pupation, pupae were kept in cages (30x20x20 cm) where sufficient water and a mixture containing sugar and powdered milk were provided for adults as a media for egg laying.

NeemAzal T/S and ethanol extracts of willow and chasteberry

NeemAzal T/S® (1% Azadirachtin) formulation, the product of Trifolio-M GmbH-Germany, was kindly provided by Dr. I. Kelany. Willow (*Salix aegyptiaca* L.) and chasteberry (*Vitex agnus-castus* L.) leaves were used for extraction which has been collected from plants grown in the campus of Zagazig University. A weight of 250 gm fresh leaves of each plant was soaked in a liter of ethanol 95% (Algomhoria Co. for chemicals and medical supplies, Cairo) for a period of 2 weeks, then filtered using cheesecloth. Extracts were evaporated using a rotary evaporator (Büchi Labortechnik AG, Switzerland) at 105 rpm under vaccum 100 mpar. The extracted weight obtained from each plant was used for experiments after quantitatively transferring to clean glass vials. The resulted extracted powder considered as 100% when preparing concentrations. Chemicals used for biochemical determinations were of highest purity available which obtained from Algomhoria Co. for chemicals and medical supplies, Cairo.

Toxicity bioassays

Six concentrations of NeemAzal T/S (1%) formulation, willow or chasteberry extracts were used in toxicity determination. Concentrations' stocks of Neem Azal, willow and chasteberry were prepared in distilled water to give final concentrations range of 0.002 - 0.2 µg mL^{-1} (NeemAzal T/S), 0.01 - 100.0 µg mL^{-1} (willow) and 1.0 - 90.0 µg mL^{-1} (chasteberry) when mixing with diet. The diet was prepared as previously mentioned and 35 gm was thoroughly mixed with 2 mL of previously prepared concentration stocks to give the final concentrations of NeemAzal T/S, willow or chasteberry as mentioned above. Diets with the previously indicated concentrations were individually transferred into 175 mL plastic cups (4.5 cm base diameter, 7.0 cm top diameter, and 8.5 cm height) provided with perforated caps. The treated diets made a height of 4.5 cm in cups. Thirty of 3rd instar *M. domestica* larvae were exposed to each concentration after starvation for 2 hour. All

concentrations were replicated 3 times. Untreated diet was used as a control with the same number of larvae and replicates. Numbers of larvae died were recorded after 24 hours of exposure. Mortality percentages were corrected for each concentration with control according to Abbott (1925). Regression toxicity lines were established for the tested substances and the slope, LC_{50}, and LC_{90} values were estimated using Probit analyses (Finney, 1971).

Biochemical determination

For biochemical investigations, calculated lethal concentrations of 50% of NeemAzal T/S, willow or chasteberry were used for larval treatments. Stocks of NeemAzal T/S, willow or chasteberry were prepared in distilled water and mixed with larval diet as mentioned before to give the required concentration. Thirty larvae of 3rd instar *M. domestica* were used for every treatment and the experiment was replicated 3 times. Samples of live larvae (0.5 gm) were randomly collected for biochemical determination at 24 hours. Larvae (0.5 gm) were homogenized in 10 mL cold phosphate buffer (0.1M, pH 7.4) using Universal Laboratory Aid homogenizer, Type MPW-309 (Mechanika Precyzyjna, Warsaw, Poland) on ice at 1000 rpm for 60 Sec. Larval homogenates were centrifuged at 4000 rpm for 10 minutes at 4ºC. Supernatants were filtered using filter paper of Whatmann 1, and then kept at -20 until determination within 2 weeks. Total protein in larval homogenates was determined according to Gornall *et al*. (1949). Total lipids were estimated according to the method described by Frings and Dunn (1970). Aspartate aminotransferase (AST) and Alanine aminotransferse (ALT) were determined according to Reitman and Frankel (1957). Carbohydrates hydrolyzing enzymes of amylase, invertase, and trehalase were determined according to the method described by Ishaaya and Swirski (1976). Supernatant was spectrophotometrically analyzed for the previously mentioned biochemical parameters with an UV-VIS Digital Spectrophotometer, model S104D/WPA (Cambridge, UK).

Statistical analysis

Probit analysis (Finney, 1971) was used for lethal concentrations and obtaining slope values using Polo-PC Plus v.3.1 statistical software. Data of lethal concentrations was considered significantly different when their corresponding confidence limits (CLs) didn't overlap (El-Sheikh, 2015). SPSS 14 for windows software package was used for statistical analysis of biochemical changes using least significant difference (LSD) of One-Way ANOVA.

RESULTS AND DISCUSSION

Toxic effects of willow and chasteberry compared with NeemAzal T/S are shown in Table 1. Data indicates that NeemAzal T/S has markedly toxic effects on 3rd instar of house fly compared with willow and chasteberry extracts (as no interaction among their corresponding CLs) on both levels of LC_{50} and LC_{90}. Willow extract is more effective than chasteberry on LC_{50} level with no significant differences at LC_{90} level as overlapping between their CLs exists. Results of relative potency shows that NeemAzal T/S has a toxic effect on 3rd larval instar of house fly higher than both willow and chasteberry extracts with 38.3- and 954.0-times (at LC_{50} level), and 714.8- and 680.6-times (at LC_{90} level), respectively. At the same time, Table 1 shows that the response of house fly larvae to the toxic effects of NeemAzal T/S and chasteberry is higher than willow as indicated from their regression line slopes which estimated to be 1.26, 0.56 and 1.44 for Neem Azal T/S, willow and chasteberry, respectively.

M. domestica is a highly reproductive potential pest insect that requires good control practices to protect the health of humans and animals, as well as optimizing animal reproduction. Control programs are usually based on the use of chemical insecticides for targeting larvae and adults. However, these methods carry potential risks for both the environment and human health (Zimmer, 2013). Plant-based materials are attracting more attention to pest control for minimizing environmental threats of chemical insecticides. Willow, originates from the Middle East especially Egypt and other countries of this origin, has important pharmacological activities (Asgarpanah, 2012). Chasteberry, grows in the Mediterranean countries and central Asia, used to treat ovarian insufficiency and uterine bleeding (Newall *et al*., 1996). Neem tree, *Azadirachta indica*, is one among natural

Table 1. Toxicity of NeemAzal T/S, willow and chasteberry on third instar larvae of *M. domestica*.

Treatment	n*	Lethal concentrations**		Slope ± SE	X^2	RP***	
		LC_{50} (95% CLs)	LC_{90} (95% CLs)			LC_{50}	LC_{90}
NeemAzal T/S	85	0.009 (0.007-0.012)	0.098 (0.066-0.167)	1.26±0.12	5.25	-	-
Willow	90	0.345 (0.17-0.64)	70.048 (30.23-217.05)	0.56±0.05	0.74	38.3	714.8
Chasteberry	85	8.586 (6.60-11.02)	66.698 (46.97-105.39)	1.44±0.12	1.13	954.0	680.6

*Total number of larvae used; **Estimated lethal concentrations of LC_{50}s and LC_{90}s resulted from Probit analysis according to Finney (1971). Lethal concentrations are indicated with 95% confidence limits (CLs). Data of lethal concentrations considered significant when their CLs are not overlapping; ***Relative potency (RP) was determined by dividing LC_{50}s and LC_{90}s of willow or chasteberry by the correspondence of NeemAzal T/S.

insecticides that have demonstrated high potential control of different noxious insects (Isman, 2006). In this regard, the toxicity of willow and chasteberry extracts on 3rd larval instar of house fly, *M. domestica*, showed clear effect with high comparable toxic effect of NeemAzal T/S. Azadirachtin is considered the most important active ingredient contained in neem seeds. This triterpenoid compound shows variable effects on insect pests including oviposition and feeding deterrence, growth regulation, fecundity and fitness reduction (Schmutterer, 1990; Ruiu *et al*., 2008). Kumar *et al*. (2012) evaluated the insecticidal effect of essential oil, *Eucalyptus globules* (Myrtales: Myrtaceae), against the house fly. Their contact toxicity results showed median lethal concentration between 2.73 and 0.60 µL cm^{-2} for different observation days, while median lethal time varied between 6.0 and 1.7 days. Kumar *et al*. (2012) concluded their findings as *E. globulus* oil has considerable activity against larvae and pupae of house fly that demonstrates its potentiality as a viable option for the development of eco-friendly product for house fly control.

The effects of exposure to LC_{50} of NeemAzal T/S, willow and chasteberry for 24 hours on some biochemical parameters in 3rd larval instar of house fly are presented in Tables 2, 3 and 4. For total protein and total lipids concentrations in larval homogenate, chasteberry treatment significantly (p<0.05) reduced the total protein concentration comparing with NeemAzal T/S and willow. In the same way, NeemAzal T/S and willow significantly (p<0.05) reduced total protein concentration compared with control. Treatments of NeemAzal T/S, willow and chasteberry did not show significant effect on total lipid comparing with control (Table 2).

Table 2. The effect of median lethal concentrations (LC_{50}) of NeemAzal T/S, willow and chasteberry on total protein and total lipids concentrations in 3rd instar larvae of *M. domestica* after exposure for 24 hours.

Treatment	Concentration (µg mL^{-1})	Concentrations (mg/mL)±SD	
		total protein*	total lipid*
Control	0	18.74±0.79[a]	270±15[a]
NeemAzal	0.009	11.77±0.16[b]	220±53[a]
Willow	0.345	12.45±0.48[b]	240±40[a]
Chaste berry	8.586	8.82±0.91[c]	284±15[a]

*Data in the same column followed by different letters are significantly different at p<0.05, when analyzed using LSD of One-Way ANOVA.

NeemAzal T/S markedly (p<0.05) decreased AST comparing with plant extracts and control, whereas, NeemAzal T/S, willow and chasteberry significantly (p<0.05) decreased ALT activity compared with control (Table 3).

Table 3. The effect of median lethal concentrations (LC_{50}) of NeemAzal T/S, willow and chasteberry on AST and ALT activities in 3rd instar larvae of *M. domestica* after exposure for 24 hours.

Treatment	Concentration (µg mL^{-1})	Activity (µg pyruvate mL^{-1} min^{-1})±SD	
		AST*	ALT*
Control	0	0.259±0.002[a]	0.189±0.029[a]
NeemAzal	0.009	0.188±0.008[b]	0.081±0.049[b]
Willow	0.345	0.252±0.046[a]	0.037±0.001[b]
Chaste berry	8.586	0.262±0.029[a]	0.087±0.051[b]

* Data in the same column followed by different letters are significantly different at $p<0.05$, when analyzed using LSD of One-Way ANOVA.

Data of Table 4 shows the effect of NeemAzal T/S, willow and chasteberry extracts on amylase, invertase and trehalase of 3rd larval instar of house fly. All tested substances markedly ($p<0.05$) increased amylase activity comparing with control, whereas, the significant effect on invertase activity was occurred due to chasteberry extract treatment with no significant effects on trehalase when 3rd instar larvae treated with any of the tested agents. The effect of exposure to different plant-based extracts and oils on biochemical changes were investigated on different insects (Abdel-Rahman and Al-Mozini, 2007; Borzoui *et al*., 2013). Reduction in total protein of the current study due to larval treatment with NeemAzal T/S, willow and chasteberry for 24 hours indicates the potential of the tested agents in disrupting the protein balance. NeemAzal T/S markedly reduced the activity of both AST and ALT, while willow and chasteberry significantly reduced ALT activity. This results show the effect on both total protein and transaminase enzymes as they considered key enzymes in the formation of non-essential amino acids (Mordue and Goldsworthy, 1973), which the changes in their levels could be correlated with anabolism or catabolism of protein. Maintenance of the balanced amino acid pool in insects is the result of various biochemical reactions carried out by a group of amino-transferase enzymes (Meister, 1957). As ALT activity markedly reduced due to exposure to the tested agents, this may be attributed to the reduction in total protein concentration, which the level of transaminases can be varied according to their amount of synthesized protein (Gilbert, 1967).

A better understanding of how digestive enzymes function is essential in order to develop methods for insect control (Maqbool *et al*., 2001). As digestion is a phase of insect physiology on which little research has been performed, despite the economic importance of the food of insects and the fact that the most important control measures involve the action of digestive juices on poisons taken into the digestive tract (Swingle, 1925). We determined the effect of NeemAzal T/S, willow, and chasteberry on digestive enzymes of amylase, invertase, and trehalase. Results indicate that all tested materials significantly increased amylase, and only chasteberry markedly increased invertase, with no effect on trehalase compared with control. Significant effects on amylase, a hydrolytic enzyme that found in microorganisms, plants and animals, could affect catalyze of carbohydrates (Franco *et al*., 2000). Only chasteberry significantly increased invertase activity that cleaves sucrose into the monosacccharides, glucose, and fructose. Invertases play a central role in carbohydrate metabolism of plants and animals (Heil *et al*., 2005), however, a limited number of studies have tried to quantify invertase activity in animals (Zhang *et al*., 1993). This might be due to the particular methodological problems arising from the quantification of invertase in animals whose carbohydrate metabolism is highly active. No effects on trehalase activity, an enzyme that hydrolyzes trehalose to yield two glucose molecules, was noted due to 3rd larval instar treatment. This mean that the tested compounds may do not have significant effects on the function role of trehalase such as physiological processes, including flight metabolism (Clegg and Evans,1961), and chitin synthesis during molting (Tatun *et al*., 2008). Trehalase proteins have been purified from several insect species and are divided into soluble (Tre-1) and membrane-bound (Tre-2) trehalases. However, no functions of the two trehalases in chitin biosynthesis in insects have yet been reported (Chen *et al*., 2010). In insects, all these function of trehalase are achieved through the hydrolysis of trehalose, the principal hemolymph sugar in insects that acts as an indispensable substrate for energy production and macromolecular biosynthesis (Friedman, 1978).

Table 4. The effect of median lethal concentrations (LC_{50}) of NeemAzal T/S, willow and chasteberry on amylase, invertase and trehalase activities in third instar larvae of *M. domestica* after exposure for 24 hours.

Treatment	Concentration (µg ml^{-1})	Activity (µg glucose ml^{-1} min^{-1})±SD		
		Amylase*	Invertase*	Trehalase*
Control	0	0.112±0.027[b]	0.315±0.014[b]	0.206±0.014[a]
NeemAzal	0.009	0.212±0.018[a]	0.341±0.044[b]	0.193±0.015[a]
Willow	0.345	0.191±0.032[a]	0.355±0.021[b]	0.162±0.032[a]
Chaste berry	8.586	0.232±0.044[a]	0.407±0.047[a]	0.208±0.096[a]

* Data in the same column followed by different letters are significantly different at $p<0.05$, when analyzed using LSD of One-Way ANOVA.

In conclusion, the effect of 3rd instar larvae of *M. domestica* treatment with some relatively new extracts and a commercial formulation of NeemAzal T/S showed toxic effects. The toxicity was high with NeemAzal T/S on both LC_{50} and LC_{90} levels comparing with willow or chasteberry extracts. For plant extracts, willow showed high toxic effects than chasteberry on LC_{50} level with no significant differences at LC_{90}. All the tested materials caused changes in biochemical parameters tested in this study, suggesting that these materials can be given more attention for more studies on the biological effects and development of *M. domestica* for possible use in control programs.

ACKNOWLEDGMENTS

Authors thank Dr. I. Kelany for providing the formulation of NeemAzal T/S®.

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