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Insecticidal activity of *Azadirachta indica* (Sapindales, Meliaceae) extracts against *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

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ABSTRACT

The potentiality of hexane, chloroform and methanol extracts of *Azadirachta indica* (Sapindales, Meliaceae) leaves against the fourth larval instar of the cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), were elucidated. Mortality response of the larvae was greater at the highest concentrations of the plant extracts than at lowest ones. Hexane is the most toxic plant extract causing the highest larval mortality due to contain the highest indication of secondary metabolites of steroids and other active substances that play an important role on the biological aspect of insect. Also, morphogenic abnormalities were detected when fourth larval instar of *S. littoralis* treated with different plant extracts, where all developmental stages of *S. littoralis* appeared varying degrees of deformities.

Key words: *Azadirachta indica*, plant extracts, *Spodoptera littoralis*, phytochemical screening, insecticidal activity.

Introduction

For centuries, agents derived from natural sources (Mother Nature), especially plants have been the primary source of medicine, manufacture and pests control. Environmental and human health concerns over excessive synthetic chemical insecticide use worldwide increasingly favor the development and marketing of alternative and safer methods for pest control in many countries (Cherry *et al.,* 1997). The development of insecticide resistance coupled with an increasing awareness of the possible detrimental effects of intensive insecticide use has stimulated interest in the development of integrated methods of pest control, which reduce pesticide inputs and produce a more sustainable farming system (Gautam *et al*., 2013).

The increased awareness of environmental pollution and the demand for safe food production have led to growing interests in use of natural products in plants' protection. Today about 200 plants with insecticidal activities are known (Singh *et al*., 2001). The Indian neem tree, *Azadirachta indica* A. Juss (Meliaceae), is a promising source of botanical insecticides. Due to their relative selectivity, neem products can be recommended for many integrated pest management programs (Biswas *et al*., 2002). It is generally believed that bioactivity of neem is due to the azadirachtin (AZA) (complex limonoids) content (Butterworth and Morgan, 1971).

Extensive work was done on the analysis of chemical constituents of neem crude extracts. Many bioactive components were isolated and identified from neem crude extracts such as azadirachtin, salannin, meliantriaol and nimbin. Among them, the most active ingredient reported is azadirachtin. These chemical constituents belong to the classes: beta-sitosterol, stigmasterol and limonoids. Also, the other tri and tetra cyclic compounds were isolated from this plant such as sulphides, flavonol glycosides, nimaton, quercetin, myrecetin, and kaempferol (Kokate *et al.,* 2010; Asif, 2012 and Hismath *et al.,* 2011). Different parts of the plant including flowers, leaves, seeds and bark have been used to treat both acute and chronic human diseases; and used as insecticide; antimicrobial, larvicidal, antibacterial, antiviral, and spermicidal (Gupta, *et al.,* 2017).

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Each part of neem tree has several medicinal values to treat a wide range of human disorders such as antiseptic, diuretic, cough, nausea, vomiting, fever and peptic ulcer. The juice of this plant is used traditionally for the treatment of gastrointestinal disease, where leaves were used by Indian for the treatment of chicken pox sleep (Iranmai *et al.,* 2012).

Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is an important key pest on many crops and covers over 40 families, containing at least 87 species of economic importance. (Albarrak, 2009 and Tiessen, 2012). In fact, the cotton leaf worm is a major limiting factor affecting crop and vegetable production in many other countries. *S. littoralis* is one of the most destructive agricultural lepidopterous pests within its subtropical and tropical regimes (Hosny *et al*., 1986). Extensive feeding by larvae, leading to complete stripping of the plants (Salama *et al*., 1970).

Thus, the current investigation was designed to determine the different active materials which present in *A. indica* leaves plant using different solvents and to elucidate their biological impact as mortality and malformation on *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) larvae, when the fourth larval instars were treated with different plant leaves extracts (chloroform, hexane, methanol and water extractions).

Materials and methods

Plant sample preparation:

The leaf samples were collected and washed carefully with water to remove dust and foreign materials. Then the washed leaves (200 gm) were dried under shade at temperature (25°C) for 7 days. After drying the leaf samples (150 gm) were ground into a powder form using a grinder for 30 s.

Extraction procedure for dry leaf powder samples:

The dry leaf powder samples (150 gm) were extracted with methanol solvent (350 ml) for 3 days using Soxhlet extractor until complete extraction. After extraction, the sample was filtered with filter paper (Whatmann No. 1). The methanol solvent was evaporated using a rotary evaporator (Yamato, Rotary Evaporator, model-RE 801, Japan) under pressure for 30 min resulting in a semi solid crude extract. The crude extracts was transferred into a separatory funnel and finally extracted by different solvents with increasing polarities followed the sequence of hexane, chloroform, methanol and water to give hexane, chloroform, methanol and water fractions, respectively. After extraction all crude extracts were put inside the fume hood for the solvents to evaporate. After the solvent was completely evaporated the hexane crude extracts, chloroform crude extracts, methanol crude extracts and water crude extracts of *A. indica* were obtained for preliminary phytochemical screening and estimate the effects of these extracts on larval survival.

Preliminary phytochemicals screening:

Hexane, chloroform, methanol and water crude extracts (1 g) was completely dissolved in 100 ml of its own mother solvents. It was prepared the stock solution. The obtained stock extracts from neem leaves were used for phytochemical screening according to well-established methods of Harborne (1998) and Kokate (1997).

Test for alkaloids:

One gram powder samples of *A. indica* plant taken in a conical flask and added ammonia solution (3 ml). It was allowed to stand for few minutes to evaluated free alkaloids. Chloroform (10 ml) was added to the conical flask shaken by hand and then filtered. The chloroform was evaporated from the crude extract by water bath and added Mayer's reagent (3 ml). A cream color precipitation was obtained immediately that showed the presence of alkaloids.

Test for flavonoids:

The stock solution (1 ml) was taken in a test tube and added few drop of dilute NaOH solution. An intense yellow color was appeared in the test tube. It became colorless when on addition of a few drop of dilute acid that indicated the presence of flavonoids.

Test for saponins:

The stock solution (1 ml) was taken in a test tube and diluted with 20 ml of distilled water. It was shaken by hand for 15 min. A foam layer was obtained on the top of the test tube. This foam layer indicated the presence of saponins.

Test for steroids:

The crude plant extracts (1 mg) was taken in a test tube and dissolved with chloroform (10 ml), then added equal volume of concentrated sulphuric acid to the test tube by sides. The upper layer in the test tube was turns into red and sulphuric acid layer showed yellow with green fluorescence. It showed the presence of steroids.

Test for tannins:

The stock solution (3 ml) was taken in a test tube and diluted with chloroform and added acetic anhydride (1 ml). Finally, sulphuric acid (1 ml) was added carefully by the side of test tube to the solution. A green colour was formed which showed the presence of tannins.

Test for triterpenoids:

The dry crude plant extract (5 mg) was dissolved in chloroform (2 ml) and then acetic anhydride (1 ml) was added to it. Concentrated sulphuric acid (1 ml) was added to the solution. Formation of reddish violet color shows the presence of triterpenoids.

Test for anthraquinones:

One milliliter of each crude stock extract solution was taken in a test tube and hydrolyzed with a diluted concentrated sulfuric acid. It was extracted with benzene. A dilute ammonia solution was added to the benzene layer. The appearance of a rose pink coloration suggested a positive response for anthraquinones.

Insect:

S. littoralis egg masses were obtained from Insecticide Center, Faculty of Agriculture, Cairo University. Larvae were reared in the laboratory on castor oil leaves (*Ricinus communis)* at 27 ± 2 °C, 65-70% relative humidity, with 12: 12 light: dark cycle. After pupation, emerging adult moths were transferred to cages (1 male 2 females per each cage) and fed on a 10% sucrose solution. For egg laying, cages were covered with muslin cloth. Eggs were surface sterilized in 10% formaldehyde for 1-3 min. (Hughes and Wood, 1981).

Bioassay tests:

Different extracts of *A. indica* were completely dried and then dissolved separately in DEMSO (Dimethyl sulfoxide) and the mother stock solutions were stored in 4 °C till use. Bioassays were performed with fourth instars of *S. littoralis* larvae using concentrations 5, 15, 25 and 30 % of each plant extract, using leaf-dip bioassay method as described by Tabashnik *et al*. (1991). Castor oil leaves were firstly washed with distilled water and dipped in solutions of different plant extracts for 5-10 seconds and allowed to air dry in room temperature. Treated leaves were placed individually into

Petri dishes (15 cm diameter), then twenty five newly molted of the 4th larval instar were applied after starvation for about four to six h (25 larvae / replicate). Larvae were allowed to feed on treated leaves for 24 h, then these leaves removed and replaced by other untreated. In control experiment, larvae were fed on castor oil leaves dipped in distilled water only. All experiments were carried out under controlled conditions, 25 ± 2 °C and 65 ± 5 % RH. Larval mortality was recorded after 72 h, and the mortality percentage was corrected according to Abbott's formula (Abbott, 1925). Probit analysis (Finney, 1971) was determined to calculate the lethal concentration values using a software computer program (SAS, 2008).

Statistical analysis:

The laboratory bioassays were conducted in a completely randomized design with 3 replications. ANOVA and a post hoc Duncan test were performed with PASW Statistics 18 for Windows program (www.spss.com) to analyze treatment differences. The means were separated on the basis of least significant differences at the 0.05 probability level.

Results and Discussion

Neem extracts are toxic to insects but not to humans or plants and have various biological effects on insects (Srivastava, 2001), also repel insects without damaging the crop itself (Isman, 1993). Neem extracts have also been reported to be toxic to all insect life-stages (Reddy and Miller, 2014; Leng and Reddy, 2012), causing either mortality or physical abnormalities (Reddy *et al.,* 2014).

The results recorded in table (1) showed that alkaloids, flavonoids, saponins, steroids and tannins were present in different polarities of crude neem leaf extracts. However, none of the crude extracts showed any color change for the anthraquinone and triterpenoids test. Alkaloid and flavonoids had presented in all four organic leaf extracts, but saponnins and tannins were presented only in methanol and water extracts. Moreover, steroids were presented high indication color (yellow with green fluorescence color) in hexane extract, and with lowest indication in other solvents. Similar results were investigated by Hashmat *et al.* (2012). Different biological activities depend on the plant secondary metabolites and the regular consumption of them by human being may have serious consequences for health, both positive and negative (Mossini *et al.,* 2009).

Table 1: Phytochemical Analysis of *Azadirachta indica* Extracts.

Phytochemical Test	Hexane	Chloroform	Methanol	Water
Alkaloids	+	+	+	+
Flavonoids	+	+	+	+
Saponins	-	-	+	+
Steroids	++	+	+	-
Tannins	-	-	+	+
Triterpenoids	-	-	-	-
Anthraquinones	-	-	-	-

150 of the 350 known limonoids are found in *A. indica*, and many of these have insecticidal properties. The insecticidal effect of *A. indica* extracts has been reported for over 350 species of arthropods. Azadirachtin, a limonoid derived from *A. indica*, is a strong antifeedant and interrupts growth and reproduction for many insect species (Srivastava, 2001).

The present study found that different extracts of *A. indica* showed a potent effect and a larvicidal activity on *S. littoralis* 4th larval instar. The bioassay test revealed that the mortality of the fourth larval instar increased progressively with the increase of the plant extract concentrations, with LC_{50} values of 14.3, 18.8, 25.2 and 27.1 % for hexane, chloroform, methanol and water, respectively. In other words, when 4th larval instar of *S. littoralis* were treated with different plant extraction, hexane proved to be the most toxic plant extraction causing the highest larval mortality (67.89 %) which was recorded at 20 % hexane extract, followed by chloroform and finally methanol. By comparison, water extraction showed low activity recording toxicity index value, in other words, the lowest number of dead larvae (36.7%) was reported at 20 % water extract (Fig. 1, 2, 3 & 4). This may

be due to these extractions contain steroids which is considered an oily substances that has a high impact on insects (Hossain *et al*., 2013).

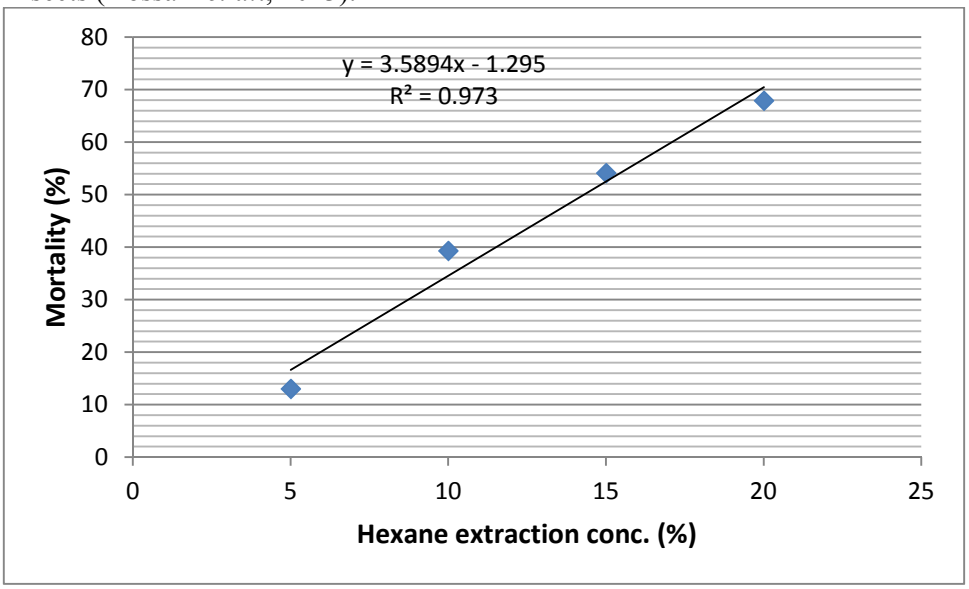

Fig. 1: Mortality responds of *S. littoralis* 4th larval instar to different concentration of *A. indica* hexane extract.

Similarly, Shaurub *et al.* (2014) investigated that the mortality of *S. littoralis* 4th larval instars increased progressively with the increase of the neem extract (azadrachtin) concentrations. It is generally believed that bioactivity of neem is due to the azadirachtin (complex limonoids) content, which is considered as bitter compounds found in the plant oil which have the principle toxic effect on insects (Butterworth and Morgan, 1971).

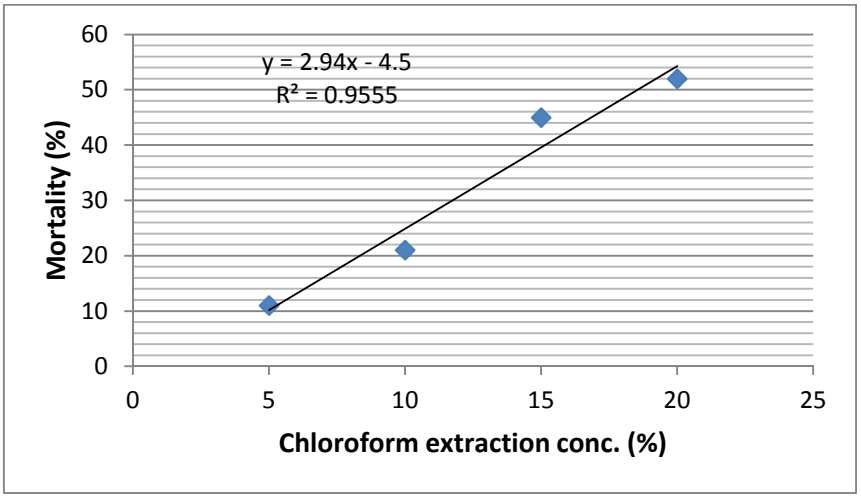

Fig.2: Mortality responds of *S.littoralis* 4th larval instar to different concentration of *Azadirachta indica* chloroform extract

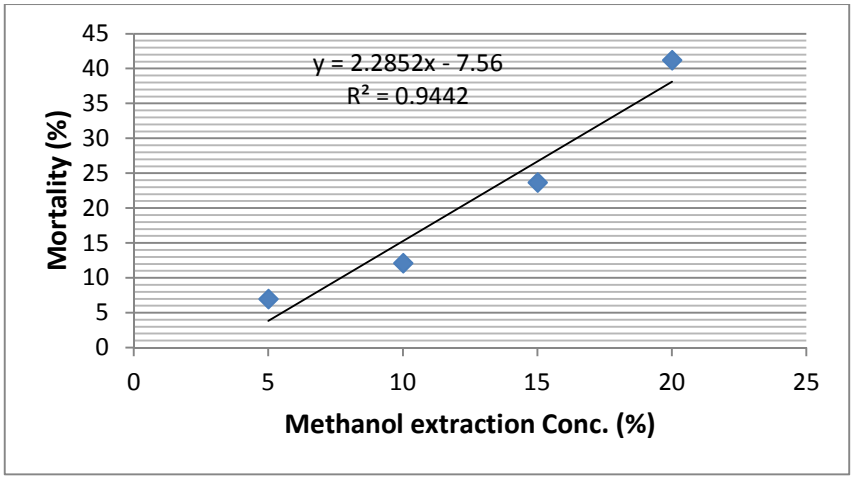

Fig. 3: Mortality responds of *S. littoralis* 4th larval instar to different concentration of *A. indica* methanol extract

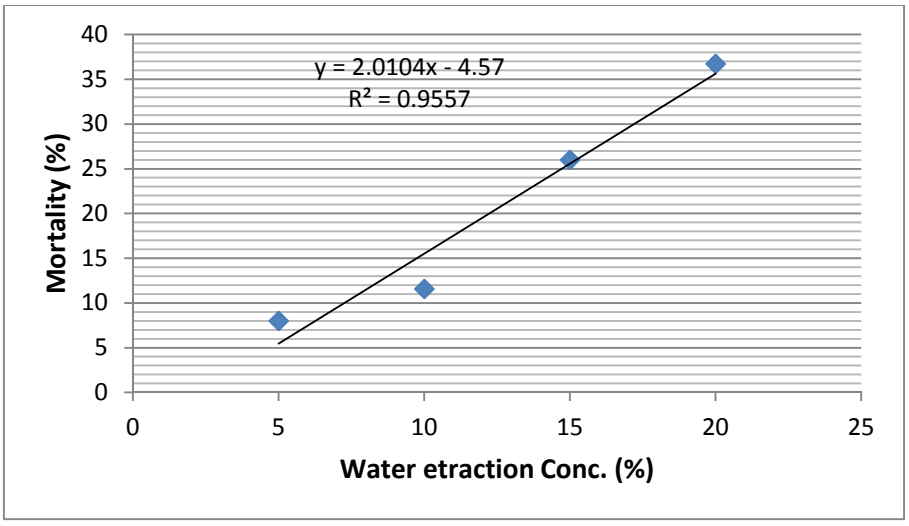

Fig. 4: Mortality responds of *S.littoralis* 4th larval instar to different concentration of *Azadirachta indica* water extract.

Results reported in this study showed the morphogenic effect of the tested plant extracts on *S. littoralis*. Treated larvae are smaller in size than untreated control one, failed to molt and finally, could not shed the old exuviae (fig. 5a & b). Malformed pupae are smaller in size than normal and generally missed the normal pupal features (fig. 5 c & d). Adult abnormalities showed widely separated folded wings. Moreover, malformed moths with cropped, shorter and undeveloped wings were detected.

It is clear that treatment of *S. littoralis* with different neem extracts showed similar morphogenic abnormalities, where all developmental stages of *S. littoralis* appeared varying degrees of deformities. Similar results were detected by Gaaboub *et al*. (2005) who reported that the highest number of malformed pupae resulted after exposure of 4th larval instar of *S. littoralis* with different extracts of *Neotorularia aculeolata*.

High score of adult malformation was noticed in the wings led to fly failure, which may be due to the inhibition of LDH and lipid (Kitto and Briggs, 1962). Similar results were reported by Bakr *et al.* (2012) when *S. littoralis* larvae were treated with neem oil. Also, Abdel-Aziz *et al.* (2013) recorded that the maximum value of the total malformation during generation when 2nd larval instar of *S. littoralis* were trated with neem oil. Riba *et al.,* (2003) investigated that azadirachtin (neem oil) has growth regulatory effect causing greatly extended instar lengths, delayed molts, mortality at ecdysis of *Nezara viridula.*

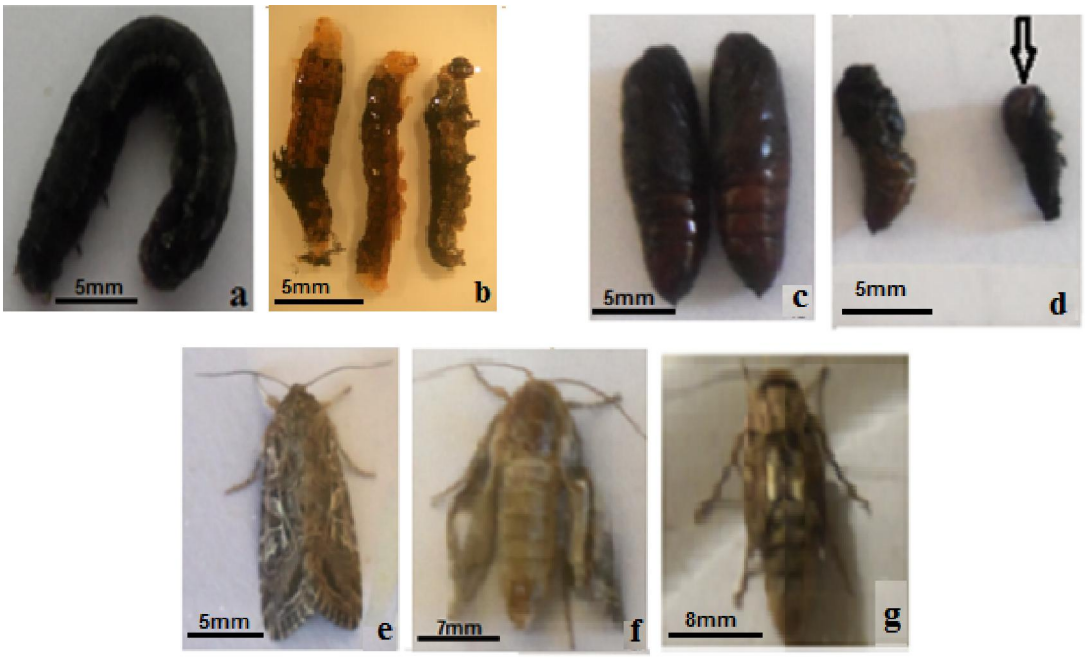

Fig. 5: Developmental stages of *S. littoralis* a) Normal larva, b) plant extracts treated larvae, failed in shedding off old exoskeleton (exuvium) and small in size. c) Normal pupae. d) Pupae which treated as larvae, shrink body with flattened head. e) Normal adult, f) Adults which treated as larvae, wings are folded and widely separated g) Adult with undeveloped wings.

Increasing doses of azadirachtin in larval stages resulted several abnormalities and deformities on *S. exigua* (Elumalai *et al.,* 2010). Korrat *et al.* (2012) explained that the metamorphosis of the larvae of *S. littoralis* treated with three newly insecticides were failed to pupate; deformed pre-pupae and pupae can't complete the molting process and died.

Conclusion

While, *A. indica* derivatives do not have a quick mortality effect, their safety for most non-target organisms makes such extracts more suitable for use than traditional broad spectrum synthetic pesticides. Furthermore, the present results suggest an interesting opportunity to develop bio-insecticides based on extracts from *A. indica* for use in integrated pest management of insect pests that may affect crop production. Further studies are needed to examine the influence of neem extracts on the feeding physiology, life table parameters, oviposition, and mating behavior of *S. littoralis*. Also, further studies are needed to determine the percentages of all these active materials and isolate pure compounds from the crude extract.

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