

**BIOACTIVITY OF AQUEOUS AND N-HEXANE NEEM LEAF EXTRACTS AGAINST THE COWPEA WEEVIL, *Callosobruchus maculatus* (F)**

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*Corresponding author: jlekan2001@yahoo.co.uk**Abstract**

The incessant problems encountered with overreliance on synthetic insecticides have necessitated the search for alternative control strategies which are safe, effective and affordable. This study investigated the bioactivity of neem (*Azadirachta indica* A. Juss.) leaf extracts against the storage pest of cowpea, *Callosobruchus maculatus* (F.), under laboratory conditions. The weevils were reared on cowpea seeds treated with aqueous and n-hexane leaf extracts at concentrations 0.5, 1.0, 2.0 and 3.0 mg/ml to compare toxicity, anti-oviposition effect, adult emergence and adult longevity. After 24 h of exposure, observed mortality was 63%, 73% and 97% for weevils poisoned with 3.0 mg/ml aqueous, 2.0 mg/ml n-hexane and 3.0 mg/ml n-hexane leaf extracts, respectively. The 24-h median lethal concentrations (LC_{50}) obtained for aqueous and n-hexane extracts were 5.28 ± 1.25 mg/ml and 2.27 ± 0.13 mg/ml, respectively with corresponding median lethal time (LT_{50}) values of 36.14 ± 3.73 h and 21.69 ± 3.17 h. Female weevils reared on treated cowpea seeds laid significantly fewer eggs and adult longevity was significantly shortened. Adult emergence was also reduced significantly in a concentration-dependent manner and there was no offspring emergence from seeds treated with high concentrations of n-hexane extract. However, leaf extracts did not alter the natural sex ratio of the insect pest. The results from this study showed that neem leaf extract is effective in controlling *C. maculatus* and could, therefore, serve as an alternative to synthetic insecticides in controlling the storage pest.

Keywords: Adult emergence, Anti-oviposition, *Azadirachta indica*, *Callosobruchus maculatus*, Mortality**Introduction**

Cowpea, *Vigna unguiculata* (L.) Walp., is a staple food for many people in several parts of Africa and is valued as a nutritional supplement to cereals. It consists of 23-25% protein, 1.9% fat, 6.3% fibre and 67% carbohydrate (Hall *et al.*, 1997; Islam *et al.*, 2008). It is also a good source of calcium, iron, vitamins and carotene. In 2017, Africa accounted for about 96% of the world's cowpea production, with Nigeria (46%) and Niger (26%) predominating world's production (FAO, 2019).

The major storage constraints in Nigeria and other tropical countries are the insect and vertebrate pests (Adedire, 2011). Among these, the cowpea weevil, *Callosobruchus maculatus* (F.), is the most important pest and it is capable of consuming 50-90% yield in storage annually (IITA, 1989). Farm storage for 3 or 4 months (Caswell, 1981) and six months (Singh and Jackai, 1985) resulted in 50% and 70% weevil infestation, respectively with at least 30% reduction in seed weight. In Nigeria, the dry weight loss due to *C. maculatus* exceed 2,900 t annually (Ajayi, 2012) and the concomitant damage renders the seeds nutritionally deficient and unsuitable for both commercial and private consumption (Oke and Akintunde, 2013).

Various physical and cultural control measures have been applied against this pest but the dominant method has been the chemical control strategy using phosphine and methyl bromide

fumigation. Inappropriate use of fumigants has resulted in cases of food poisoning in Nigeria with records of mortality among consumers (Gwary *et al.*, 2012). Illiteracy, non-observation of the regulatory safety period before seeds are sold for consumption and incessant urge for profit among traders have been identified as some of the factors perpetuating this problem. This has necessitated a search for alternative control measures which are effective while at the same time safe, cheap and sustainable.

Botanical insecticides have been reported to be environment-friendly and a number of plant extracts have been assessed against *C. maculatus* and other insect pests with diverse results. Extracts of cashew kernel were found to be effective against *C. maculatus* (Adedire *et al.*, 2011); leaf extracts of *Morinda lucida* (Benth) were found to possess effective toxicity, ovicidal and repellent effects against *C. maculatus* (Ajayi, 2012); essential oils from leaves of *Ageratum conyzoides* L., *Chromolaena odorata* (L.) R. M. King and H. Rob. and *Lantana camara* L. were found to be effective against the maize weevil, *Sitophilus zeamais* (Motsch.) (Bouda *et al.*, 2001); while Ogunsina *et al.* (2011) reported that extracts of *L. camara*, *Monodora myristica* (Gaertn.) Dunal and *Euphorbia lateriflora* (Schum. and Thonn.) were more effective against *C. maculatus* compared to *S. zeamais*.

Azadirachta indica (Rutales: Meliaceae) commonly known as neem plant and locally known as *Dongoyaro* in Nigeria is an evergreen plant that is endemic to southern and south-eastern Asia, but now found in Africa, the United States, and Australia (Ogbuewu *et al.*, 2011). Neem plant contains several chemical constituents, the most active and well-studied compound being azadirachtin (Lokanadhan *et al.*, 2012; Chaudhary *et al.*, 2017). The neem seed oil has toxic, antifeeding and anti-oviposition properties against insects (Paneru and Shivakoti, 2001; Rajapakse and Ratnasekera, 2008) but non-availability of seeds during some months of the year has constituted a limitation to its use. In addition, utilization of seeds for this purpose is not generally considered acceptable. On the contrary, neem leaves are available all year-round and their utilization for insecticidal purposes is less burdensome to the plant compared to when seeds are used. Nevertheless, there is a dearth of information on the effectiveness of neem leaf extracts against cosmopolitan pests like cowpea weevil.

The present study was, therefore, designed to determine insecticidal efficacy of aqueous and n-hexane neem leaf extracts against *C. Maculatus* with respect to toxicity, anti-oviposition activity, adult emergence and longevity on treated seeds.

Materials and Methods

Insect Rearing

Adult cowpea weevils were collected from naturally infested cowpea seeds bought at the Bodija market (7°43'28" N and 3°91'52" E), in Ibadan, Nigeria. The insects were reared on uninfested cowpea seeds in the Insect Physiology Laboratory of the Department of Crop Production and Protection, Faculty of Agriculture, Obafemi Awolowo University (OAU) at 25.7±1°C, 67.7±2% RH and 12:12 light: dark photoperiod. Weevil rearing continued to F₃ generation to ensure a pure culture before newly emerged adults were used for experiments.

Preparation of plant samples and leaf extraction

Young neem leaves were collected within OAU, Ile-Ife and air-dried on a shaded platform to prevent degradation by direct sunlight. The dried leaves were chopped into small bits and milled mechanically into fine powder. The volatile oil was extracted from 500 g of the powdered material by distillation using n-hexane in a Clevenger apparatus

(Papachristos and Stamopoulos, 2004) and water was removed from the oil using Na₂SO₄. Aqueous extraction was also made from another 500 g of the material by boiling the leaf powder in water for a few seconds. The filtrate was then transferred to a rotary evaporator to remove the solvent, the concentrated extract was air-dried and kept in a fridge until needed for experiments. Both aqueous and n-hexane leaf extractions were carried out at the Drug Research and Production Unit, Faculty of Pharmacy, OAU, Ile-Ife.

Toxicity Test

Prior to the test, healthy but susceptible cowpea seeds were stored in a deep freezer for 72 h to ensure elimination of arthropods present in the pack. Disinfested cowpea seeds (10 g) were treated in triplicated Petri dishes with 2 ml each of 0.5, 1.0, 2.0, and 3.0 mg/ml aqueous extract. A similarly arranged set was treated with n-hexane extract in a similar manner while application of 2 ml n-hexane (extraction solvent) and untreated seeds served as positive and negative control experiments, respectively. The cowpea seeds were thoroughly mixed with the extracts to ensure uniform coating and air-dried, allowing the solvent to evaporate completely, before ten 36 h old adult *C. maculatus* were introduced into each Petri dish. All treatments were arranged using a completely randomized design and each Petri dish was monitored for insect mortality at 1, 6, 12, 18 and 24 h post-exposure.

Adult longevity and anti-oviposition test

Ten grams of disinfested cowpea seeds were subjected to different treatments as described for toxicity test and ten (2 : 8) 36 h old adult weevils were introduced into each Petri dish. The weight of treated seeds inside each Petri dish (i.e. weight of seeds + weight of coating) was determined before weevils were introduced. All treatments were arranged in Completely Randomized Block Design and replicated five times. The Petri dishes were monitored daily and insect mortality was recorded. Adult longevity was measured as time taken (days) from emergence to death of an individual insect. Ovipositing females glue eggs on the surface of cowpea seeds and the number of eggs laid on each seed as well as total number of eggs per Petri dish were counted carefully using a magnifying lens. Percent oviposition deterrence (OD) (Singh, 2011) and oviposition activity index (OAI) (Kramer and Mulla, 1979) were calculated thus

$$\% \text{ OD} = \frac{\text{No. of eggs laid on control seeds} - \text{Number of eggs laid on treated seeds}}{\text{No. of eggs laid on control seeds}} \times 100$$

$$\text{OAI} = \frac{\text{Number of eggs laid on treated seeds} - \text{No. of eggs laid on control seeds}}{\text{Number of eggs laid on treated seeds} + \text{No. of eggs laid on control seeds}}$$

Oviposition activity index ranges from -1 to +1; negative and positive values indicate oviposition deterrence and attraction for oviposition, respectively.

Offspring emergence from treated cowpea and concomitant seed damage

After the number of eggs laid on cowpea seeds had been counted, the experimental set up was left for additional days till all offspring emerged. The Petri

dishes were monitored daily and adult emergence was recorded per treatment. The sex of each emerging adult was determined using established distinguishing features (Bandara and Saxena, 1995); the plate covering the end of the abdomen is large and dark in colour along the sides in females, and smaller without the dark areas in males. At the cessation of adult emergence, treated seeds per Petri dish were weighed and percent weight loss was determined as

$$\% \text{ Weight loss} = \frac{\text{Initial weight of content of Petri dish} - \text{Final weight of content after adult emergence}}{\text{Initial weight of content of Petri dish}} \times 100$$

Data Analysis

Percent data obtained for cowpea seed weight loss, mortality and anti-oviposition tests were square root transformed before being subjected to analysis of variance (ANOVA) using SAS v. 9.13. Offspring emergence counts were also natural log transformed before analysis while longevity data were subjected to ANOVA without transformation. Each ANOVA was followed by the Tukey's multiple comparison post-test for statistical significance. The median lethal concentration (LC₅₀) and lethal time (LT₅₀) were determined from mortality data using the predictive function of Microsoft® Office Excel. Adult emergence data were subjected to *t*-test procedure to determine significant difference in proportion of male to female offspring.

Results

The mortality of exposed cowpea weevils was evidently concentration- and time-dependent (Table 1); the first record (16.7% mortality) occurred within 1 h of exposure to 3.0 mg/ml hexane leaf extract and by the 24th hour, a maximum of 96.7% mortality was attained. The estimated 24-h median

lethal concentration and time were 5.28 ± 1.25 mg/ml; 36.14 ± 3.73 h and 2.27 ± 0.13 mg/ml; 21.69 ± 3.17 h for aqueous and hexane extracts, respectively. The influence of neem leaf extracts on oviposition activity of *C. maculatus* and adult weevil emergence is presented in Table 2. A significantly lower number of eggs were laid on cowpea seeds coated with leaf extracts, resulting in 52-87% reduction in egg laying. The leaf extracts produced negative oviposition index values in female weevils and the values reduced with increasing concentration. Generally, fewer offspring emerged from coated cowpea seeds especially at higher extract concentration but the insect sex ratio was not affected. There was no weevil emergence from cowpea seeds coated with 2 mg/ml and 3 mg/ml hexane leaf extracts. The longevity of emerging adult weevils as well as concomitant reduction in weight of infested cowpea seeds are shown in Table 3. The leaf extracts reduced longevity of emerging offspring and weight loss in infested seeds significantly. The reduction was more evident when hexane extract was used. There was a positive linear relationship between adult weevil emergence and weight loss in infested cowpea seeds with a coefficient of determination (*R*²) of 0.94 (Fig. 1).

Table 1. Mortality of cowpea weevils after exposure to different concentrations of aqueous and n-hexane neem leaf extracts

Concentration of leaf extract (mg/ml)	Period (h) postexposure to leaf extracts									
	1 h		6 h		12 h		18 h		24 h	
	Aqueous	Hexane	Aqueous	Hexane	Aqueous	Hexane	Aqueous	Hexane	Aqueous	Hexane
0.5	0.00 ^a	0.00 ^b	0.00 ^b	3.33 ^b	10.00 ^a	10.00 ^{bc}	13.33 ^{ab}	16.67 ^{bc}	23.33 ^{abc}	20.00 ^b
1.0	0.00 ^a	0.00 ^b	0.00 ^b	0.00 ^b	0.00 ^a	3.33 ^c	16.67 ^{ab}	6.67 ^c	26.67 ^{ab}	16.67 ^b
2.0	0.00 ^a	0.00 ^b	3.33 ^b	30.00 ^a	6.67 ^a	43.33 ^{ab}	10.00 ^{ab}	56.67 ^{ab}	23.33 ^{abc}	73.33 ^a
3.0	0.00 ^a	16.67 ^a	10.00 ^a	60.00 ^a	23.33 ^a	83.33 ^a	43.33 ^a	90.00 ^a	63.33 ^a	96.67 ^a
C ₁	0.00 ^a	0.00 ^b	0.00 ^b	0.00 ^b	3.33 ^a	3.33 ^c	6.67 ^{ab}	6.67 ^c	6.67 ^{bc}	6.67 ^b
C ₂	0.00 ^a	0.00 ^b	3.33 ^b	0.00 ^b	3.33 ^a	0.00 ^c	3.33 ^b	6.67 ^c	3.33 ^c	13.33 ^b

Mean values with similar letter(s) within the same column are not significantly different at probability level of 0.05. C₁ and C₂ represent positive (hexane-treated seeds) and negative (untreated seeds) control experiments, respectively.

Table 2. Effect of neem leaf extracts on oviposition performance of exposed cowpea weevils and offspring emergence

Leaf extracts	Conc. (mg/ml)	Anti-oviposition test			Offspring emergence			
		Average number of eggs laid	Percent reduction in egg laying	Oviposition index	Number of F ₁ offspring	Male offspring	Female offspring	Sex ratio (m: f)
Aqueous	0.5	28.33 ^b	65.80	-0.66	11.67 ^{ab}	3.33 ^{bc}	5.67 ^{ab}	1:1.7; <i>t</i> = 0.42, <i>P</i> = 0.351
	1.0	39.67 ^b	52.11	-0.52	48.67 ^a	20.00 ^a	26.33 ^a	1:1.3; <i>t</i> = 0.42, <i>P</i> = 0.347
	2.0	15.33 ^b	81.49	-0.81	1.00 ^b	0.67 ^{bc}	0.33 ^b	2:1; <i>t</i> = -0.45, <i>P</i> = 0.343
	3.0	11.00 ^{bc}	86.72	-0.87	0.33 ^b	0.00 ^c	0.33 ^b	-
Hexane	0.5	13.67 ^{bc}	76.70	-0.77	3.67 ^{ab}	1.67 ^{bc}	2.67 ^{ab}	1:1.6; <i>t</i> = 0.42, <i>P</i> = 0.347
	1.0	9.33 ^c	77.84	-0.78	0.67 ^b	0.00 ^c	0.00 ^b	-
	2.0	10.67 ^{bc}	81.81	-0.82	0.00 ^b	0.00 ^c	0.00 ^b	-
	3.0	20.00 ^b	84.10	-0.84	0.00 ^b	0.00 ^c	0.00 ^b	-
Control	C ₁	108.67 ^a	-	-	31.00 ^a	14.67 ^a	18.33 ^a	1:1.2; <i>t</i> = 0.43, <i>P</i> = 0.344
	C ₂	102.59 ^a	-	-	23.67 ^a	13.33 ^a	13.67 ^a	1:1; <i>t</i> = 0.09, <i>P</i> = 0.469

Mean values with similar letter(s) within the same column are not significantly different at probability level of 0.05. C₁ and C₂ represent positive (hexane-treated seeds) and negative (untreated seeds) control experiments, respectively.

Table 3. **Weight reduction in infested cowpea seeds and longevity of cowpea weevils on seeds coated with neem leaf extracts**

Leaf extracts	Conc. (mg/ml)	Percent loss in seed weight	Adult longevity (day)
Aqueous	0.5	2.78 ^{bc}	4.03 ^b
	1.0	4.75 ^{ab}	4.87 ^b
	2.0	0.92 ^c	5.33 ^b
	3.0	0.60 ^c	4.93 ^b
Hexane	0.5	1.89 ^{bc}	4.97 ^b
	1.0	0.84 ^c	2.77 ^c
	2.0	0.28 ^c	2.03 ^c
	3.0	0.52 ^c	1.90 ^c
Control	C ₁	7.07 ^a	10.40 ^a
	C ₂	7.86 ^a	10.80 ^a

Mean values with similar letter(s) within the same column are not significantly different at probability level of 0.05. C₁ and C₂ represent positive (hexane-treated seeds) and negative (untreated seeds) control experiments, respectively

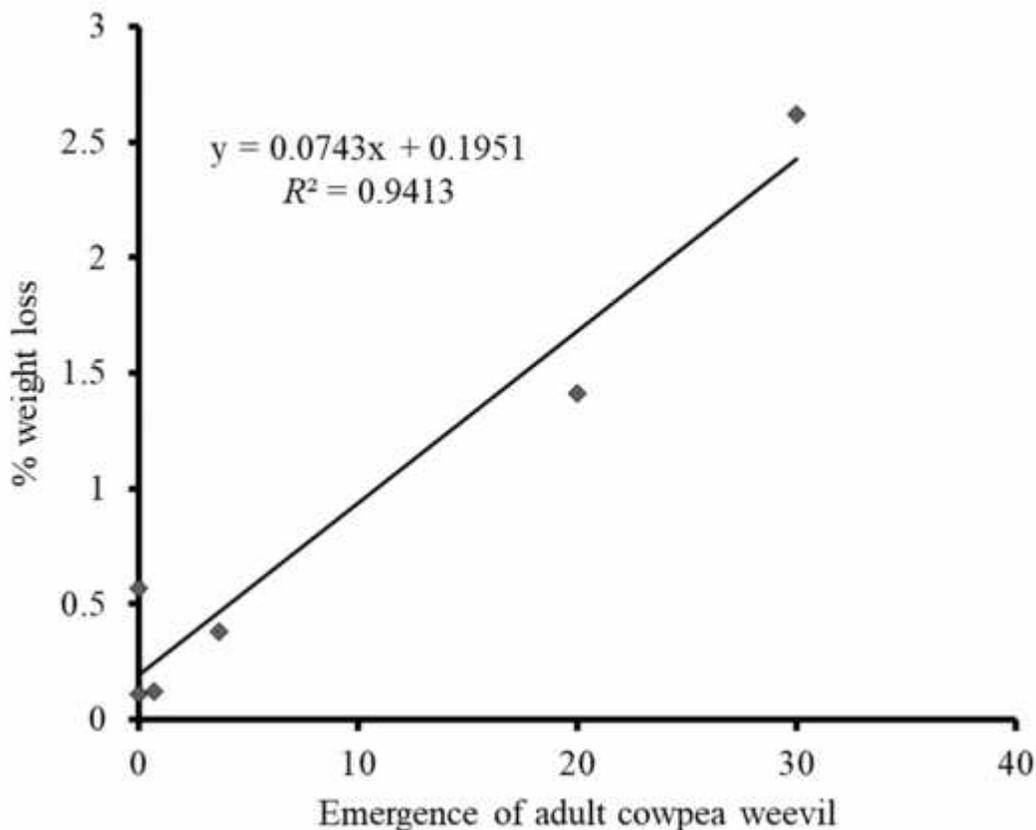


Figure 1. The regression relationship between adult weevil emergence and weight loss in infested cowpea seeds

Discussion

The results obtained in this study have shown that neem leaf extracts could be used to protect cowpea seeds in storage with a significant reduction in yield loss. This outcome is lending credence to previous numerous workers (e.g., Adedire *et al.*, 2011; Ileke and Oni, 2011; Chaudhary *et al.*, 2017; Edwin and Jacob, 2017) who established insecticidal properties of different plants and advocated the use of botanical insecticides in place of synthetic chemicals. The hexane extract was more effective in controlling the weevils and this was consistent with the significantly lower LC₅₀ and LT₅₀ values compared to those of aqueous extract. The higher efficacy of hexane leaf extract can be attributed to the fact that the volatile solvent was able to extract more active ingredient such as azadirachtin from the neem leaves than water could do.

Also, the active ingredients of neem have low solubility in water but they are highly soluble in organic solvents (Chaudhary *et al.*, 2017). The

increase in insecticidal efficacy with increasing concentration and time of insect exposure is in agreement with the report of Ketoh *et al.* (2000), Rajapakse and Ratnasekera (2008) and Achio *et al.* (2012). Toxicity is determined by the proportion of the active fraction in a given volume of plant extract and this would invariably increase with extract concentration; a longer period of exposure would also increase the risk of poisoning. In the presence of these two factors, insecticidal efficacy of a potent active ingredient would be enhanced.

The neem leaf extracts showed good oviposition deterrence against the cowpea weevil, reducing egg laying efficacy by as much as 87%. This could be due to the ability of azadirachtin to inhibit oogenesis and synthesis of ovarian ecdysteroid in females and interrupt sperm production in males (Chaudhary *et al.*, 2017). These are very important physiological processes needed for reproductive success in insects. A similar observation was made by Ahmad *et al.* (2015) and he reported that neem leaf dose of 1.5 mg/100 g seed deterred oviposition effectively. The

negative oviposition index values indicated that the weevils were deterred from laying eggs at all extract concentrations tested in this study. The effectiveness of aqueous extract in discouraging oviposition, even at low concentration, provides an avenue for a cheap, safe and renewable alternative to synthetic pesticides, especially, for resource-poor local farmers.

A number of factors could be responsible for the significant reduction in offspring emergence from extract-treated cowpea seeds. The inability of laid eggs to stick to the coated cowpea seeds may reduce egg survival and adult emergence. The inability of young adult insects to chew through the already treated seed coats impregnated with constituents of the extract such as phenolic compounds can also contribute to eventual reduction in number of emerging offspring (Derkyi *et al.*, 2010). Neem extracts possess immense antifeedant properties due to its efficacy in suppressing the feeding sensation in insects and this may also hinder the growing larvae from utilizing treated seeds for necessary growth and development (Isman *et al.*, 1991). Laying of eggs on cowpea seeds with direct contact with toxic extracts may also have adverse effect on viability of the embryo. Ovicidal activity had been reported for neem extracts against *C. maculatus* (Makanjuola, 1989; Ivbijaro, 1990; Lale and Abdulrahman, 1999; Lale and Mustapha, 2000) and this could also be responsible for the poor offspring emergence from cowpea seeds treated with the extracts. However, the plant extracts did not alter sex ratio of *C. maculatus*, a property that is considered desirable. The 1:1 sex ratio observed in *C. maculatus* agrees with the conventional theory of evolutionary stable strategy which held that sex ratios typically fluctuated around 1:1 (Fisher, 1930). Sex ratio distortion can have adverse effects on the equilibrium frequency distribution of alleles.

There was an excellent fit between the number of offspring emerging from experimental cowpea seeds and the rate at which seeds depreciated in weight. The growing larvae undergo a series of moults within the seeds, consuming the endosperm and embryo. They eventually burrow to a position just underneath the seed coat where new adults chew through the seed coats during the process of eclosion. Higher number of emerging offspring translated to higher reduction in seed weight and in the present study, offspring emergence accounted for 94% of the reduction in seed weight. Inability of the weevils to utilize treated seeds, in a concentration-dependent manner, was one of the factors that determined adult longevity in the experimental weevils. Azadirachtin, along with other related active ingredients in neem

such as triterpenoids azadirachtin B, salannin and nimbin, have biocidal efficacy and they act by disrupting growth and development in insects and by deterring their feeding (Morgan, 2009).

Conclusion

The results obtained from the present study confirmed that neem leaf extracts, especially that extracted with n-hexane, are effective in controlling the storage pest of cowpea. This could serve as an alternative to synthetic insecticides thereby reducing the environmental risks associated with the commercial products. The effectiveness of the aqueous extract would also be of immense help to local farmers as they would not need to purchase expensive solvents such as n-hexane and it makes the use of the botanical insecticide sustainable.

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