Original Research Article

Toxic Effects of Methanolic Extracts of Plants Leaves on the Mortality and Enzymatic Parameters of *Callosobruchus maculatus* Fab. (Coleoptera: Bruchidae) Juveniles

ABSTRACT

This study was conducted in the laboratory to evaluate the effect of leaves extract of three botanicals on the biochemical parameters of insect pest of stored beans, *Callosobruchus maculatus*. The results of the qualitative phytochemicals revealed the presence of different metabolites such as tannins, saponins, cardiac glycosides, phenolic compounds, flavonoids, alkaloids etc. The toxic effect of methanolic leaves extracts of *Lasianthera africana, Hippocratea africana* and *Uvaria chamae* on the mortality and biochemical parameters after treatment against *C. maculatus* was evaluated. The result showed that treatment of the insects with these extracts significantly increased mortality in *C. maculatus*. There was a significant inhibition of the enzymatic activities of the digestive enzymes (such as amylase and invertase) in the treated larvae. The transaminase enzymes (AST and ALT) were found to be reduced in the insect after treatment (33.2 and 42.6) while the result of the phosphatase (ACP and ALP) enzyme activity showed a potent inhibitory effect of the leaves extract, which was more pronounced in ALP (75.8%) than ACP (31.8%).

Keywords: *Callosobruchus maculatus, Lasianthera africana, Hippocratea africana, Uvaria chamae,* Enzymatic**,** Toxicity

1. Introduction

Indiscriminate use of synthetic chemical pesticides to control pests has led to the development of insect's resistance and also affected non-target organisms, hence, an environmental-friendly alternative is needed [1]. "Recently, an intensive research has been carried out to control agricultural pests by using natural insecticides of plant origin to decrease hazards in the environment. Plant-based products had been used to control different pests by farmers for at least two millennia" [2]. "Research has revealed the availability of different varieties of phytochemicals in plant, the extracts of, which, their secondary metabolite have been used to control pests of various order including the Coleopterans" [3].

Based on literature, several botanicals possess pesticidal properties, but due to limited studies available on certain botanicals, our study was conducted to determine the toxicity of three (3) specific plants on the mortality and biochemical parameters of insect pest of stored beans, *Callosobruchus maculatus*. *Uvaria chamae*, one of our choice botanicals, possesses leaves alternately arranged, the leaf has simple structures, lanceolate in shape with entire lamina and net veined. Leaves are stipulate, leaf apex cuminate and the leaf vestiture is glabrous [4]. "*Lasianthera africana* is monospecific genus. It is a perennial glabrous shrub that reaches a height of 61 – 136 cm". [5]. "*Hippocratea africana* is a green forest perennial climber without hairs (glabrous), reproducing from seeds" [6].

The insect pest of stored beans, *C. maculatus*, is a cosmopolitan post-harvest pest that causes quantitative and qualitative losses of stored grains in West Africa and its infestation begins in the field at low levels and increases in stored population [7]. It is used as a model organism for research and education due to its rapid development in storage [8]. The adult weevils are short, stout-bodied beetles about 4.76 mm long with the wing covers shortened and not covering the tip of the abdomen [9]. Their antennae are usually conspicuous and the body is narrow toward the front [10]. This weevil lacks the 'snout' of a real weevil. It is reddish-brown overall, with two central black spots marked by black and grey elytra. The last abdominal segment extends below the short elytra, as well as having two black spots [11].

2. Materials and Methods

2.1 Collections and Identification of Plant Materials

The fresh leaves of *U. chamae, L. africana* and *H. africana* were obtained from the Faculty of Pharmacy Medicinal Farm of University of Uyo, Akwa Ibom State and validated by a taxonomist in the Department of Botany and Ecological Studies, University of Uyo. Voucher specimens with numbers: UUH/3687, UUH/3688 and UUH/3689 were deposited in their herbarium for further referencing.

2.2 Rearing of Test Organisms

To provide comparable age weevils for the experiment, *C. maculatus* cultures were established. A measured size of five (5) kg bean seeds were purchased and cleaned to remove any seeds with visible damage. To prevent potential field infestation, the clean seeds were kept in a sealed container in the refrigerator at 4°C for a month. Seeds were placed in soft bags and stored at room temperature for two weeks. The beetle is sexually dimorphic, hence easy to distinguish males from females. Sometimes the females are larger than the males, overall the females are darker while the males are brunette. The plate which covers the end of the abdomen is large and dark in female on the sides and smaller in male without the dark areas [12]. The insects were cultured on clean seeds, with 50 weevils per 200 g of seeds in each jar. To allow airing and prevent weevil from escaping, the jars were covered with muslin cloth and secured with a rubber band and kept at room temperature. All parent weevils were removed from each jar seven days after oviposition. The jars were placed in an insect rearing cage kept in the Entomology Laboratory, Department of Animal and Environmental Biology, University of Uyo, Uyo. Newly emerged two day old insects (juveniles) were used for the experiment.

2.3 Preparation of plant powder and extract

After collection, the plant leaves were washed, chopped into pieces and room-dried to a constant weight. Using an electrical power-driven blender (Braum Multiquick Immersion Hand Blender, B White Mixer MR 5550CA, Germany), the dry plants were melded into fine powder and then kept in an airtight container pending use. The crude leaf extracts were then prepared using standard procedures as outlined by Santana [13]; Mukhtar and Huda [14] and Fatope [15]. This involved soaking 50 g of the powder for 48 – 72 hours at room temperature in 95 percent methanol. This was followed by filtrate evaporation using a rotatory evaporator to obtain the crude extract.

2.4 Phytochemical Analysis of the Plants

The initial phytochemical screening of the different plants was carried out in Pharmacognosy Laboratory of University of Uyo, Akwa Ibom State using the standard procedures as described by Prashant [16]; Kokate [17]; Evans [18] and Harbone [19].

2.5 Determination of LC_{50}

The acute toxicity (LC_{50}) of the extracts used in this study were established using the method of Ousman [20] and Abbott [21] where LC_{50} were obtained from a concentration that will have a 50% mortality effects on the test organism after 24 hours.

2.6 Toxicity Experiment

"Healthy grains were kept in a freezer for one week to control hidden infestation, then left to equilibrate to room temperature. Twenty (20 g) of the grains was measured into 200 ml plastic cups and treated with different unitary and binary formulations of the botanicals at 5 and 10 percent concentrations. A conventional insecticide at 0.25 g concentration was used as standard, while the experimental control was set up without any treatment. An hour after the addition of the botanicals, 10 pairs of sexed juvenile *C. maculatus* were introduced into treated and untreated grains within the plastic containers. The plastic cups were covered with white muslin cloth held in place with rubber bands. The experiment was laid out using a completely randomized design and replicated four times. Mortality was recorded after 7, 14, 21 and 28 days of treatment. Insects were considered dead on failure to respond to three probes using a blunt dissecting probe" [22, 23,].

2.7 Determination of the Digestive Enzyme

Amylase and invertase activities were assayed calorimetrically according to the methods described by Ishayaa and Swirski [24]. The activities of the enzymes were based on the digestion of starch and sucrose, respectively. The free aldehyde groups of glucose formed after starch and sucrose digestion were allowed to react with 3, 5-dinitro salicylic acid reagent. The reduced dinitro salicylic acid was measured spectrophotometrically at 550 nm.

2.8 Determination of Transaminase Enzymes (AST and ALT)

Aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) were determined calorimetrically according to the method of Adel *et al.* [25] and Reitman and Frankle [26]. AST transfers the amino group from L-aspartate to Ketoglutaric acid, producing a new amino acid (L-glutamic) and a new keto acid (Oxaloacetate). ALT transfers the amino group from D, L-alanine to ketoglutaric acid, resulting in L-glutamic acid and pyruvic acid. The oxaloacetate and pyruvate formed from both reactions react with 2, 4-dinitrophenylhydrazine, forming oxaloacetate or pyruvate hydrazine, which in alkaline medium form a brown colour, which was measured spectrophotometrically at 546 nm.

2.9 Phosphatase Enzymes

Acid phosphatase (ACP) and alkaline phosphatase (ALP) were determined according to the method described by Powell and Smith [27]. In this method, the phenol released by enzymatic hydrolysis of disodium phenyl phosphate was measured spectrophotometrically.

2.10 Data Analysis

Data collected on the toxicity experiment were analyzed using percentages, while that of biochemical assay were subjected to univariate analysis of variance (ANOVA). Significant means were separated using New Duncan Multiple Range Test. T- test was used to compare the differences between the enzymes at the same treatment group. Results were presented as means ± standard deviation of mean and significant means were accepted at $p<0.05$. All analyses were done using Statistical Packages for Social Sciences (SPSS) version, 23.0 (IBM Corporation, Armonk USA).

3. Results

3.1 Phytochemical Composition of the plants extracts

The results of the qualitative phytochemicals revealed the presence of different metabolites as shown in Table 1. Steroids and terpenes were strongly present in *H. africana* but *U. chamae* and *L. africana* had it in trace. Anthraquinones were in trace in *U. chamae* but absent in both *L. africana* and *H. africana*. Cardiac glycosides were strongly present in *U. chamae* but moderately present in both *L. africana* and *H. africana*. Saponins was detected to be strongly present in *L. africana* but moderately present in both *H. africana* and *U. chamae*. Tannins and phenols were strongly present in *L. africana* but moderately present in both *U. chamae* and *H. africana*. Flavonoids were strongly present in both *U. chamae* and *H. africana* but moderately present in *L. africana*. Alkaloids were strongly present in *U. chamae* but moderately present in both *H. africana* and *L. africana*. Also, phlobatannins was moderately present in both *U. chamae* and *H. africana* but absent in *L. africana*.

Table 1: Qualitative phytochemical analysis of the different extracts

	U. chamae	*L. africana*	*H. africana*	Test
Anthraquinones	+	-	-	Borntrager
Steroids/terpenes	++	+	+++	Liebermann-Burchard
Cardiac glycoside	+++	++	++	Keller-kiliani, Salkowsiki
Saponin	++	+++	++	Frothing, Fehling solution, Na_2Co_3
Tannins & Phenols	++	+++	++	Ferric Chloride, Pb acetate
Flavonoids	+++	++	+++	NaOH, Mayer, Wagner
Alkaloids	+++	++	++	NaOH, Shinda
Phlobatannins	++	-	++	Dragendoff, Mayer, Wagner

+++ = strongly present; ++ = moderately present; + = trace; - = absent

3.2 Toxicity (Mortality) Assessment

Results on the toxicity assessment after the application of the unitary and binary formulations of different plant extracts at 5 and 10 percent concentrations are shown in Table 2. Significant difference ($p<0.05$) in mortality was observed among different treatments depending on the type of extract combinations, concentration and time after treatment. Significantly higher mortality was recorded under binary formulations as compared to unitary formulations at both concentration levels of treatment. The highest mean mortality of *C. maculatus* was recorded as the result of the combinations of *U. chamae* + *L. africana*, *U. chamae* + *H. africana* and *L. africana* + *H. africana* treatment at both concentrations. The result also showed that binary

formulations where *U. chamae* was added had the highest mortality of 90 percent. The toxicity effect of the binary formulations was comparable with the synthetic insecticide (Aluminium phosphide). Increased mortality of weevils was observed after the treatment with higher concentration. Among the unitary formulations, the highest mortality was recorded at higher concentration (10%) after treatment with *U. chamae* followed by *H. africana* and *L. africana.*

Table 2: Mortality of *C. maculatus* (mean ± SD) on beans seeds admixed with unitary and binary formulations (5% and 10%) of different botanical extracts

Conc. (%)	Groups	Duration (Day)			
		7	14	21	28
10					
	U. chamae	0.75 ± 0.50^{ab1}	1.75 ± 0.50^{b2}	2.75 ± 0.50^{b2}	4.00 ± 0.00^{b3}
	L. africana	0.25 ± 0.50^{b1}	0.75 ± 0.50^{bc1}	1.25 ± 0.50^{b12}	2.00 ± 0.00^{b2}
	H. africana	0.50 ± 0.58^{ab1}	1.25 ± 0.96^{b12}	2.00 ± 0.82^{b2}	3.00 ± 0.82^{b2}
	U. chamae + *L. africana*	1.00 ± 0.00^{ab1}	2.25 ± 0.50^{b2}	3.25 ± 0.50^{b2}	4.50 ± 1.00^{b3}
	U. chamae + *H. africana*	1.00 ± 0.00^{ab1}	2.25 ± 0.50^{b2}	3.50 ± 0.58^{b3}	5.00 ± 0.82^{b4}
	L. africana + *H. africana*	1.00 ± 0.00^{ab1}	1.75 ± 0.50^{b2}	2.75 ± 0.50^{b2}	4.00 ± 0.00^{b3}
5	*U. chamae*	0.50 ± 0.58^{b1}	1.25 ± 0.50^{b1}	1.75 ± 0.96^{b1}	2.50 ± 1.29^{b1}
	L. africana	0.50 ± 0.58^{b1}	0.75 ± 0.96^{bc1}	1.00 ± 0.82^{bc1}	1.50 ± 1.29^{b1}
	H. africana	0.75 ± 0.50^{b1}	1.00 ± 0.82^{bc1}	1.50 ± 0.58^{b1}	2.50 ± 0.58^{b1}
	U. chamae + *L. africana*	0.50 ± 0.58^{b2}	1.00 ± 0.82^{bc2}	1.75 ± 0.96^{b12}	2.75 ± 0.96^{b2}
	U. chamae + *H. africana*	0.25 ± 0.50^{b1}	1.00 ± 0.82^{bc1}	1.50 ± 1.29^{b1}	2.50 ± 1.29^{b1}
	L. africana + *H. africana*	0.75 ± 0.50^{b1}	1.25 ± 0.50^{b1}	1.75 ± 0.96^{b1}	2.50 ± 1.00^{b1}
	Standard control	2.00 ± 0.00^{a1}	4.00 ± 0.00^{a2}	6.00 ± 0.00^{a3}	8.00 ± 0.00^{a4}
	Experimental control	0.00 ± 0.00^{b1}	0.00 ± 0.00^{c1}	0.00 ± 0.00^{c1}	0.00 ± 0.00^{c1}

Values as mean ± standard deviation. Values with different alphabet superscripts along a column were significantly different; while values with different numeric superscripts across a row were significantly different ($p < 0.05$).

3.3 Digestive Enzymes

The results of the activity of amylase revealed a significant decrease ($p<0.05$) in the *C. maculatus* treated with the *L. africana*, *H. africana* and *U. chamae* (47.70 ± 5.3; 43.23 ± 3.5 and 40.35±2.5 µm/min/g, respectively) when compared to controls (78.97 ± 1.3 µm/min/g). Similarly, there was a significant decrease ($p<0.05$) in invertases (46.27 ± 3.9; 42.85 ± 3.8 and 38.75 ± 3.9 µm/min/g, respectively) when compared with control (72.32 ± 4.4). These values represent 51.3 % (amylase) inhibition and 54.7% (invertase) respectively as shown in table 3. These enzymes are secreted to play role in digestion and utilization of starch and sucrose. Therefore, the impairment of these substrates' availability might have inhibited the digestive enzymes activity in the tested weevil. It therefore shows that *U. chamae, L. africana* and *H. africana* delays feeding initiation and the actual movement of food bolus through the digestive tract.

3.4 Transaminase Activities (AST and ALT)

In table 3, it is observed that treatment with different plant extracts reduces the activity of aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT), as the percentages inhibition were 33.2% and 42.6%, respectively when compared to the controls. The mean average of ALT concentrations of different extract of *L. africana, H. africana* and *U. chamae* were 4.35 ± 0.25, 4.23 ± 0.22 and 4.15 ± 0.11, respectively when compared to the control (6.15 ± 0.05). There was a significant difference ($p<0.05$) between the treated and untreated groups. The activity of AST are found to be decreasing when treated with extracts of *L. africana, H. africana* and *U. chamae* as compared to control. There was a significant difference between the treated and untreated larvae. Therefore, it is clear that the observed reduction in the enzymatic activities of AST was higher than that of ALT.

3.5 Phosphatase enzymes (ACP and ALP)

Table 3 showed the effect of the tested plant extracts on the enzymatic activity of acid and alkaline phosphatases (ACP and ALP). The result showed a potent inhibitory effect on both enzymes, ACP (31.8%) and ALP (75.8%) when compared to the control. The mean average of ACP activities of different extract were 3.04 ± 0.16, 3.04 ± 0.13 and 4.01 ± 0.25 U/100mg for *L. africana, H. africana* and *U. chamae*, respectively, compared to 4.2 ± 0.02 U/100mg of the control. There was a significant difference between the treated and untreated groups. The mean average of ALP activities of different extract were 72.6 ± 9.8, 70.5 ± 8.7 and 68.4 ± 6.7 U/100mg for *L. africana, H. africana* and *U. chamae*, respectively, compared to 245.8 ± 8.5 U/100mg of the control. There was a significant decrease in ALP than ACP in the treated groups when compared with the control.

Table 3: Enzymatic activity of larvae of *C. maculatus* treated with different extracts.

Enzymes Sample	Enzymes activities				
	L. africana	*H. africana*	*U. chamae*	Control	% inhibition
Digestive enzymes (µm/min/g tissues)					
Amylase	47.7 ± 5.3	43.23 ± 3.5	40.35 ± 2.5	78.77 ± 1.3	51.3
Invertase	46.27 ± 3.9**	42.85 ± 3.8	38.75 ± 3.9	72.32 ± 4.4	54.7
Transaminase enzymes(µm/min/g tissues)					
AST	3.0 ± 0.25**	3.1 ± 0.14	2.9 ± 0.38	5.4 ± 0.39	33.2
ALT	4.35 ± 0.25**	4.32 ± 0.22	4.15 ± 0.11	6.15 ± 0.5	42.6
Phosphate enzymes(µm/min/g tissues)					
Acid Phosphate	3.04 ± 0.16	3.04 ± 0.13	4.01 ± 0.25	4.02 ± 0.2	31.8
Alkaline Phosphate	72.9 ± 9.8	70.5 ± 8.7	68.4 ± 6.7	245.8 ± 8.5	75.8

** significantly different ($p<0.05$)

4.0 Discussion

The qualitative phytochemical screening of all the plants extract (*U. chamae*, *H. africana* and *L. africana*) revealed the presence of alkaloids, saponins, tannins, flavonoids, phenols and cardiac glycosides. This result was in agreement with the report of [28, 29, 30] who carried out "a phytochemical screening of some methanolic plants extracts and found tannins, flavonoids, alkaloids and saponins to be the most abundant phytochemical present". Anthraquinone was not present in the botanicals used in our study, while Phlobotannins was moderately present. The report of this study was in agreement with the findings of [31, 32, 33] who carried out the phytochemical screening of extract of *L. africana and H. africana* and reported no trace of anthraquinone, but moderate presence of Phlobatannins. The strong presence of cardiac glycosides observed in this study was in agreement with the findings of [34, 32, 30] who observed heavy presence of the same metabolites in *L. africana* and *H. africana* but disagreed with the findings of Rajeswari *et al.* [35] who observed no trace of cardiac glycosides when screening the extract of *H. africana.*

Our findings on the transaminases showed that ALT and AST were less than that observed in untreated control. From the present findings it is very clear that there was inhibition of AST on the treated seed grains. This is in agreement with [36] who reported that "as far as the activity of transaminase is concerned, pyrethroid and Neem formulation compounds were found to have inhibitory effects and the decrease of AST was more than in ALT". Hassen [37] denoted that the activity of tissue-specific enzymes was used to diagnose the harm caused by chemical toxicity to particular organs and tissues. Accordingly, the two most diagnostic-potential enzymes are aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Since transaminase enzymes help to produce energy, ALT and AST assist in the transition of amino groups and play an important role in the process of the Krebs or the process of high energy output i.e. the amino acid, lipid, and carbohydrate metabolism [38, 39]. Since AST and ALT are essential anaplerotic enzymes that provide oxaloacetate and pyruvate as critical precursors of Krebs' cycle respectively, inhibition of these enzymes caused impairment of this process that could affect the normal reproduction and growth rate of the treated insects. This assertion is in accordance with [40] who concluded that plant extracts induced inhibition of AST and ALT enzymes, thus decreasing the reproductive capacity of *Aphis craccivora*. Therefore, the use of plant extracts, which have been found to suppress these enzymes, can help combat pests of insects.

According to our findings, alkaline phosphatase activity in treated groups was lower than in control when using *L. africana,* while its activities increased when using *H. africana* and *U. chamae* extract. Such increase is in agreement with El-Gindi [41] who found that topical treatment of *Parasarcophaga argyrostoma* with juvenile hormone, pyriproxyfen at 1 % leads to highly significant increases in ALP activity. Acid phosphatase activity in the treated groups was found to be lower than that in control; hence in agreement with the findings of Mostafa [42, 43] who recorded significant reduction in the acid phosphatases activity at all times intervals when treatment having formulation of plant extracts (*Margason*) were administered to 4^{th} and 6^{th} instar larvae of *Spodoptera littoralis.* Since both enzymes (ALP and ACP) are closely related to insect development, nutrition, egg maturation and metamorphosis, their inhibition could affect the transport of nutrients which in turn may impair the normal development of the insect. This is in agreement with the findings of [44, 45, 46] who reported that botanical extracts caused a reduction in ACP and ALP activities. The extracts was found to have more effects on the weevils as juvenile hormone on larvae. The findings here are in accordance with [47] who found that s-alp (Soluble alkaline phosphatase) activity was increased in all tissue whereas m-alp (membrane alkaline phosphatase) was increased in the midgut and hindgut by juvenile hormone analog (JHA) treatment, and also the larval duration was increased. This is also in agreement with [48] who observed that the plant oils may have a regulatory impact on juvenile or insect development. The rise in alkaline phosphatase may also be due to the *U. chamae's* juvenile hormone effect because juvenile hormone contributes to increased alkaline phosphatase.

5.0 Conclusion

Our study showed that botanicals, used in this study, are viable potent pesticide in the control of *C. maculatus*. They are environmentally friendly and biodegradable, since it is a biological method of pest control. These plants are harmless for mammals at the dosages reported, so efforts should be increased to cultivate, package, and apply them on a large scale as botanical insecticides.

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