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Inhibitory effect of leaf and root bark of *Calotropis procera* and *Parquetina nigrescens* on larval midgut enzyme activities of *Callosobruchus maculatus*

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Abstract
The study evaluated the protein content and effect of leaf and root bark of *Calotropis procera* and *Parquetina nigrescens* on midgut enzyme activities of the larvae of *Callosobruchus maculatus* with a view to solving the problem of cowpea infestation caused by *C. maculatus.* Evaluation of inhibitory effect of the leaf and root bark of *C. procera* and *P. nigrescens* on total protease, trypsin, chymotrypsin, α-amylase and lipase was carried out with 0.5% extract of each plant material as inhibitor source for the enzyme inhibitory assays. Midgut enzyme activities of protease, lipase and alpha amylase and protein content were also quantified. The results showed that the protein content in the midgut of the larvae of *C. maculatus* were 1.48±0.01, 1.51±0.01, 1.36±0.01 and 1.71±0.01 mg ml^{-1} in first, second, third, and fourth instar larvae respectively and were significantly ($P < 0.05$) different. Protease activity was higher than both alpha amylase and lipase activities in the insect midgut while inhibition of the extracts of leaf and root bark of *C. procera* and *P. nigrescens* on enzyme activities varied across plant materials and stages of instar larva of *C. maculatus*.

Keywords: midgut, larvae, protease, lipase and alpha amylase

Introduction
For an efficient management of pest control, it is imperative to know the type of enzymes present in the gut of insects and pests. Two major proteinase classes in the digestive systems of phytophagous insects are the serine and cysteine proteinases [1]. Lipase enzymes generally are defined as triacylglycerol hydrolases that break carboxylester bonds in triacylglycerols, diacylglycerols, galactolipids and phospholipids [2]. Triacylglycerols (TAGs) constitute a major lipid component in the diet of insects and their processes of digestion and absorption are very similar to those in mammals [3]. After feeding, TAGs are hydrolyzed in the midgut lumen and the products of digestion are absorbed and used for the synthesis of complex lipids [4]. Insects utilize enzymes to digest their food. One of these is alpa-amylase, which cleaves α-1, 4 glycosidic bonds in starch, glycogen, oligosaccharides, and polysaccharides and thereby promotes larval carbon assimilation from the diets [5]. Some plant serine proteinase inhibitors are bifunctional molecules and are able to inhibit trypsins as well as α-amylase [6]. Several functional proteins such as lectin-like, cereal-type and Kunitz-like which are often found in the seeds of leguminous and graminaceous plant, have been reported to inhibit a-amylase in insect midgut, thereby blocking the main energy source for insect growth and development [7]. The binding of these α-amylase inhibitors and amylase forms a stable enzyme–inhibitor complex. This makes insect midgut to secrete excessive digestive enzyme which often lead to insect death [8]. These inhibitors can affect the functional properties of a-amylase such as decreasing the binding of a-amylase with its substrates, resulting in reduction or loss of enzymatic activity [5].
The present research assessed inhibitory effect of leaf and root Bark of *Calotropis procera* and *Parquetina nigrescens* on midgut Enzyme activities of *Callosobruchus maculatus*

Materials and methods
Total protein determination of the midgut of *C. maculatus*
Preparation of midgut tissue and homogenate
Larvae were dissected under microscope and the midgut removed, placed in cold distilled water. A quantity of 0.1 g of midgut tissue was homogenized with 1 ml of phosphate buffer and centrifuged at 8000 g 4 °C for 10 minutes. The supernatant was collected into a new centrifuge tube and kept on ice until use.

Total protein determination
Protein concentration of gut larvae was determined by the method of Bradford [9] using Bovine Serum Albumin (BSA) as the standard (0.125, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 mg mL-1). Protein reagent was prepared by dissolving 100mg of coomossie brilliant blue G-250 in 50ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. The solution was filtered through Whatman No.4 filter paper and stored in a reagent bottle until use. An aliquot100 µl of the midgut homogenate were piptted into a test tube, 6 ml coomassie blue reagent was added and the absorbance read at 595nm after two minutes and before 1 hour against blank. For preparation of standard curve, varying concentrations of bovine serum albumin (BSA) were piptted into test tubes. The volume in the test tubes was adjusted to 1 ml with phosphate buffer (0.1M, pH6.6). A volume of 5 ml of protein reagent were added to the test tubes and the contents mixed. The absorbance at 595 nm was measured after 2 minutes and before 1hour against blank prepared from 1ml of phosphate buffer and 5ml protein reagent.

Determination of protease, lipase and alpha amylase enzymes activity of larval midgut of *C. maculatus*.

Quantification of the total protease, trypsin, chymotrypsin, lipase, and alpha amylase activities of the midgut enzyme of *C. maculatus* were determined according to Cohesion Bioscience assay kits

Effects of leaf and root bark of *Calotropis procera* and *Parquetina nigrescens* on the enzyme activity of the four instars larvae of *C. maculatus*

For measurement of inhibitory effect of leaf and root bark of *C. procera* and *P. nigrescens* on total protease, trypsin, chymotrypsin, α-amylase and lipase, 0.5% extract of each plant material was prepared as inhibitor source and used for the enzyme inhibitory assays [10, 11], 70 µl of each extract of leaf and root bark of *C. procera* and *P. nigrescens* were incubated at 70^0C for 20 mins to inactivates endogeneous enzymes and added to the reaction mixtures of total protease, trypsin, chymotrypsin, α-amylase and lipase activities as described by Cohesion Biosciences assay kit.

$$\% \text{ Inhibition} = \frac{\text{Activity with plant extract}}{\text{Activity without plant extract}} X\ 100$$

Data analysis

Data obtained were subjected to analysis of variance (ANOVA) procedure of Minitab 16.1 [12]. Tukey's Test at P = 0.05 was used to compare means.

Results

The results of the enzyme activity and protein content of the first, second, third and fourth instar larval midgut of *C. maculatus* are represented in the Table 1. Total protease activity of the instars ranged between 1.21± 0.1 – 1.68± 0.01 µmol min^{-1} mg^{-1}. Fourth instar larval midgut had the highest (0.83±0.01 µmol min^{-1} mg^{-1}) trypsin activity while the lowest (0.54±0.01 µmol min^{-1} mg^{-1}) was in third instar larval midgut and were significantly different (P < 0.05). Chymotrypsin activity had 0.43±0.01, 0.52±0.01, 0.31±0.01 and 0.66±0.02 µmol min^{-1} mg^{-1} in first, second, third, and fourth instar larval midgut respectively and they were significantly different (P < 0.05). However, alpha amylase activity was 0.24±0.01, 0.31±0.01, 0.12±0.01 and 0.44±0.01 µmol min^{-1} mg^{-1},in first, second, third, and fourth instar larval midgut respectively and they were significantly (P < 0.05) different. Lipase activity had 0.35±0.01, 0.41±0.01, 0.21±0.01 and 0.59±0.01 µmol min^{-1} mg^{-1} in first, second, third, and fourth instar larval midgut respectively and they were significantly (P < 0.05) different. The protein content in the midgut of the larvae of *C. maculatus* were 1.48±0.01, 1.51±0.01, 1.36±0.01 and 1.71±0.01 mg ml^{-1} in first, second, third, and fourth instar larvae respectively and were significantly (P < 0.05) different.

Inhibitory effect of crude extracts of leaf and root bark of *C. procera* and *P. nigrescens* on proteolytic activity of the larval midgut of *C. maculatus* are shown in Table 2. The percentage inhibition on proteolytic activity of the first instar larval midgut of *C. maculatus* was highest (75.6±0.10) in the plant extract from the leaf of *P. nigrescens* while that of *C. procera* leaf had the lowest (34.2±0.10). Percentage inhibition on proteolytic activity of the second instar larval midgut of *C. maculatus* had the highest (82.7±0.20) in the plant extract from *P. nigrescens* leaf and the lowest (42.3±0.10) in the root bark of *C. procera*. The percentage inhibition on proteolytic activity of the third instar larval midgut of *C. maculatus* had the highest (85.6±0.10) in the plant extract from *P. nigrescens* leaf, followed by *C. procera* leaf which recorded 74.6±0.10 and the lowest (41.3±0.10) in *C. procera* root bark. The percentage inhibition on proteolytic activity in the midgut of the fourth instar larvae of *C. maculatus* had the highest (86.4±0.10) in the plant extract from *P. nigrescens* leaf, followed by *C. procera* leaf (85.7±0.20) and the lowest (34.2±0.10) in the root bark of *C. procera*. The percentage inhibition on the proteolytic activity of the first, second, third and fourth instar larval midgut of *C. maculatus* was significantly different (P<0.05).

The inhibitory effect of crude extract of leaf and root bark of *C. procera* and *P. nigrescens* on trypsin activity in the midgut of the larvae of *C. maculatus* as presented in the Table 3 showed that inhibition on trypsin activity in the first instar of *C. maculatus* had the highest (56.7±1.00) in *P. nigrescens* root bark and the lowest (36.4±0.20) was in leaf of *P. nigrescens*. Inhibition percentage on trypsin activity in the second instar larvae of *C. maculatus* were in the range of 33.80±0.10 to 53.1±0.1. The leaf of *C. procera* had the highest inhibition while the root bark of *C. procera* had the lowest inhibitory effects on trypsin activity. The percentage inhibition on trypsin activity in the third instar larvae of *C. maculatus* had the highest (72.6±0.20) in *C. Procera* leaf, followed by the leaf of *P. nigrescens* (63.9±0.10) and the lowest (19.20±0.10) in *C. Procera* root bark. Similarly, the percentage inhibition on trypsin activity in the fourth instar larvae of *C. maculatus* had the highest (74.3±0.10) in *C. Procera* leaf and the lowest (11.7±0.10) was in root bark of *C. procera*. The percentage inhibition on trypsin activity of the first, second, third and fourth instar larvae of *C. maculatus* was significantly different (P<0.05).

The inhibitory effect of the crude extract of leaf and root bark of *C. procera* and *P. nigrescens* on chymotrypsin activity of the larval midgut of *C. maculatus* as presented in the Table 4 showed that the inhibition on chymotrypsin activity in the first instar had the highest (47.6±0.06) in root bark of *P. nigrescens* and the lowest (35.3±0.30) in root bark of *C. procera*. The percentage inhibition on chymotrypsin activity in the second instar larval midgut of *C. maculatus* was highest (56.2±0.20) in *C. procera* leaf, followed by the root bark of *P. nigrescens* (44.30±0.10), and the lowest (36.5±0.10) in root bark of *C. procera*. The percentage inhibition on chymotrypsin activity in the third instar larval midgut of *C. maculatus* had the highest (62.5±0.50) in *C. procera* leaf, followed by the leaf of *P. nigrescens* (53.1±0.00) and the lowest (22.3±0.17) in root bark of *C. procera.* In the fourth instar larval midgut of *C. maculatus*, percentage inhibition on chymotrypsin activity had the highest (77.3±0.20) in *C. procera* leaf and the lowest (17.5±0.20) in root bark of *C. procera*. The percentage inhibition on chymotrypsin activity in the first, second, third and fourth instar larval midgut of *C. maculatus* was significantly different (P<0.05).

The inhibitory effect of crude extract of leaf and root bark of *C. procera* and *P. nigrescens*on α- amylase activity of the larval midgut of *C. maculatus* as presented in the Table 5 showed that inhibition on α- amylase activity in the first the highest (86.5±0.01) in *C. procera* leaf, followed by the root bark of *P. nigrescens* (80.2±0.10) and the lowest (71±0.20) in root bark of *C. procera*. However, the percentage inhibition on α- amylase

activity in the second instar larval midgut of *C. maculatus* was highest (84±2.00) in *P. nigrescens* root bark and the lowest (73.4±0.02) in the leaf of *C. procera.* The leaf of *P. nigrescens* had the highest (87.4±0.02) inhibition percentage on α- amylase activity in the third instar larvae of *C. maculatus* while *P. nigrescens* root bark showed the highest (95.45±0.01) inhibition on α- amylase activity in the fourth instar larval midgut of *C. maculatus.* The percentage inhibition on the activity of α-amylase in the first, second third and fourth instar larval midgut of *C. maculatus* was significantly different (P<0.05).

The percentage inhibition of the crude extract of leaf and root bark of *C. procera* and *P. nigrescens* on lipase activity of the larval midgut of *C, maculatus* as shown in the Table 6 revealed that inhibition on lipase activity was high and ranged between 76.45±0.01 to 96.00±1.00. The inhibitor from the leaf of *P. nigrescens* had highest percentage inhibition on lipase activity while that of *C. procera* leaf was the lowest. The percentage inhibition on lipase activity in all the four instars was significantly different (P<0.05).

Table 1: Enzyme activity and protein content of the first, second, third and fourth instar larval midgut of *C. maculatus*.

Instar larva	Enzyme activity(µmol min^{-1} mg^{-1}) and protein content (mg ml^{-1})					
	PA	TrPA	CTPA	AA	LA	PC
First	1.42±0.01^{c}	0.62±0.02^{c}	0.43±0.01^{c}	0.24±0.01^{c}	0.35±0.01^{c}	1.48±0.01^{c}
Second	1.49±0.01^{b}	0.71±0.01^{b}	0.52±0.01^{b}	0.31±0.01^{b}	0.41±0.01^{b}	1.51±0.01^{bc}
Third	1.21±0.01^{d}	0.54±0.01^{d}	0.31±0.01^{d}	0.12±0.01^{d}	0.21±0.01^{d}	1.56±0.01^{b}
Fourth	1.68±0.01^{a}	0.83±0.01^{a}	0.66±0.02^{a}	0.44±0.01^{a}	0.59±0.01^{a}	1.71±0.01^{a}

Means in the same column with the same alphabets are not significantly different (P<0.05).
PA = Proteolytic activity, TrPA = Trypsin proteolytic activity, CTPA=Chymotryapsin proteolytic activity, AA = Amylase activity, LA = Lipase activity, PC = Protein content

Table 2: Inhibitory effect of crude extract of leaf and root bark of *C. procera* and *P. nigrescens* on proteolytic activity of the larval midgut of *C. maculatus*

Plant materials	% inhibition			
	First instar larvae	Second instar larvae	Third instar larvae	Fourth instar larvae
C. procera leaf	34.20±0.10^{d}	45.30±0.10^{c}	74.60±0.10^{b}	85.70±0.20^{b}
C. procera root bark	56.40±0.10^{c}	42.30±0.10^{d}	41.30±0.20^{d}	34.20±0.10^{d}
P. nigrescens leaf	75.60±0.10^{a}	82.70±0.20^{a}	85.60±0.10^{a}	86.40±0.10^{a}
P. nigrescens root bark	66.50±0.10^{b}	53.30±0.00^{b}	51.20±0.10^{c}	44.10±0.10^{c}

Means in the same column with the same alphabets are not significantly different (P<0.05).

Table 3: Inhibitory effect of crude extract of leaf and root bark of *C. procera* and *P. nigrescens* on trypsin activity of the larval midgut of *C. maculatus*

Plant materials	% inhibition			
	First instar larvae	Second instar larvae	Third instar larvae	Fourth instar larvae
C. procera leaf	41.20±0.10^{c}	53.10±0.10^{a}	72.60±0.20^{a}	74.30±0.10^{a}
C. procera root bark	45.40±0.10^{b}	33.80±0.10^{d}	19.20±0.10^{d}	11.70±0.10^{c}
P. nigrescens leaf	36.40±0.20^{d}	45.70±0.20^{b}	63.90±0.10^{b}	68.80±0.10^{b}
P. nigrescens root bark	56.70±0.10^{a}	42.10±0.10^{c}	25.40±0.10^{c}	12.20±0.10^{c}

Means in the same column with the same alphabets are not significantly different (P<0.05).

Table 4: Inhibitory effect of crude extract of leaf and root bark of *C. procera* and *P. nigrescens* on chymotrypsin activity of the larval midgut of *C. maculatus*

Plant materials	% inhibition			
	First instar larvae	Second instar larvae	Third instar larvae	Fourth instar larvae
C. procera leaf	44.10±0.10^{b}	56.20±0.20^{a}	62.50±0.50^{a}	77.30±0.20^{a}
C. procera root bark	35.30±0.10^{d}	36.50±0.10^{c}	22.30±0.00^{d}	17.50±0.20^{d}
P. nigrescens leaf	41.70±0.10^{c}	44.20±0.10^{b}	53.10±0.00^{b}	64.00±1.00^{b}
P. nigrescens root bark	47.60±0.30^{a}	44.30±0.10^{b}	33.60±0.20^{c}	29.00±1.00^{c}

Means in the same column with the same alphabets are not significantly different (P<0.05).

Table 5: Inhibitory effect of crude extract of leaf and root bark of *C. procera* and *P. nigrescens* on α- amylase activity of the larval midgut of *C. maculatus*

Plant materials	% inhibition			
	First instar larvae	Second instar larvae	Third instar larvae	Fourth instar larvae
C. procera leaf	86.45±0.01^{a}	73.35±0.02^{d}	65.00±2.00^{d}	60.15±0.01^{d}
C. procera root bark	71.00±1.00^{d}	76.10±0.00^{c}	67.10±0.10^{c}	86.30±0.10^{c}
P. nigrescens leaf	75.25±0.01^{c}	81.75±0.01^{b}	87.35±0.02^{a}	92.25±0.01^{b}
P. nigrescens root bark	80.20±0.10^{b}	84.00±2.00^{a}	78.00±1.00^{b}	95.45±0.01^{a}

Means in the same column with the same alphabets are not significantly different (P<0.05).

Table 6: Inhibitory effect of crude extract of leaf and root bark of *C. procera* and *P. nigrescens* on lipase activity of the larval midgut of *C. maculatus*

Plant materials	% inhibition			
	First instar larvae	Second instar larvae	Third instar larvae	Fourth instar larvae
C. procera leaf	84.35±0.02[c]	92.30±0.10[a]	95.45±0.01[a]	96.00±1.00[a]
C. procera root bark	83.30±0.20[b]	84.20±0.10[c]	87.30±0.10[c]	90.00±1.00[c]
P. nigrescens leaf	76.45±0.01[d]	82.15±0.03[d]	86.25±0.02[d]	88.15±0.02[d]
P. nigrescens root bark	91.15±0.03[a]	91.10±0.00[b]	93.10±0.10[b]	94.10±0.17[b]

Means in the same column with the same alphabets are not significantly different (P<0.05).

Discussion

The differentiation of midgut epithelial cells occurs in *C. maculatus* larvae during postembryonic development [13, 14], while in the successive development, there is replacement of larval midgut epithelial cells to adult midgut epithelial by the formation of new epithelial cells in insects [15]. In all the larval instars, the midgut epithelial cells produce various digestive enzymes that help in food digestion in the midgut larval of *C. maculatus.* The midgut epithelial cells of *C. maculatus* larvae contains protein synthesizing biomolecules such as RNA and DNA that are actively engaged in the production of proteins for various digestive enzymes during larval development [14]. Presence of food in the midgut larvae of *C. maculatus* initiates progressively the concentration of protein [16]. The present investigation reveals that the concentration of total protein in the midgut larval of *C. maculatus* shows an initial rise from first to second instar larvae and a significant depletion in third instar larvae. This depletion observed in the third instar larvae probably due to degradation of protein to release energy [17] and less consumption of food at the third instar stage which may cause the midgut cells to produce less digestive enzyme. However, the need for more protein by the fourth instar larvae to metamorphose to pupal stage may be responsible for more protein in the midgut of fourth instar which is required for the development of other structural organs needed for the pupal stage [18].

The results of the current study demonstrated the presence of serine proteases lipase and α-amylase in the larval midgut of *C. maculatus*, and their tendency to reduce or increase in activity during developmental stages [19]. It is generally accepted that the midgut regulates the release of digestive enzymes in response to the quantity of food consumed by insects [20]. Increase in proteolytic activity of larvae reared on cowpea may be attributed to the high protein content of the host [19]. Trypsin-like, and chymotrypsin-like proteases were active in the midgut extract, but trypsin-like proteinase had significant activity in the midgut compared with the chymotrypsin-like proteases, which is supported by higher trypsin activity compared with the chymotrypsin-like proteases activity [21] as observed in this study. Alpha amylase hydrolyses alpha 1, 4-linkages of starch molecules to produce the needed energy of insects [22]. Lipids are an important component of insect diet that is degraded in the lumen, and used for the synthesis of complex lipids [23].

The inhibitory effect of protease inhibitors confirms the presence of trypsin-like and chymotrypsin-like activities in gut extracts of *C. maculatus* [21]. The percentage inhibition on α- amylase and lipase activity of *Passifloraedulis*, *Coccinia grandis* and *Ribesrubrum* as reported by Joseph and George [22] is similar to the results of this study. High percentage inhibitory effect on α-amylase and lipase activity shows that plant materials such as *C. procera* and *P. nigrescens* may have compound rich in anti-diabetic and antilipidemic activity.

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