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Inhibition of purified glutathione transferase from Hide Beetle (*Dermetes maculatus*) larva by ethanolic extract of cayenne pepper (*Capsium annum*)

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ABSTRACT

Glutathione transferase (GST) is an important enzyme involve in metabolizing many different classes of xenobiotics through conjugation with glutathione. The hide beetle (Dermetes maculatus) is an important insect pest ravaging agricultural stored products globally, thereby reducing their economic value. As such, the control of this insect is necessary to enhance both quality and quantity of stored products. This paper presents the inhibitory activity of the ethanolic extract of cayenne pepper (Capsium annum) on purified GST from the larvae of hide beetle. The larva were collected from decaying dried catfish (Clarias sp), demobilized by freezing at 4 °C and homogenized with 0.1 M phosphate buffer (pH 7.0) containing 1 mM each of ethelene diaminetetraacetic acid (EDTA) and β-mercaptoethanol on ice. The homogenate was subjected to cold centrifugation at 10,000 g for 20 min. The supernatant was used as the crude enzyme solution, which was purified by ion-exchange on DEAE-Sephacel and affinity Chromatography on glutathione-Sepharose 4B before inhibitory studies with C. annum ethanolic extract and known plant-based inhibitors. The concentration of C. annum extract causing fifty percent inhibition (IC50) of GST activity was similar to that of anisaldehyde but less potent when compared with α-terpeneol. The C. annum extract exert its inhibitory power on the glutathione transferase via mixed inhibition. These findings showed that GST is present in D. maculatus larvae, and the inhibitory properties of C. annum extract can be employed in designing inhibitors with enzymic target for pest control thereby improving quality and quantity of stored products.

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1. Introduction

Cayenne pepper (*Capsicum annum* L.) is one of the domesticated species of Solanaceae family (*C. annum, C. baccatum, C. chinense, C. pubescens and C. frutescens,*) is the most cultivated species worldwide (Kraft *et al.*, 2014; Bhutia *et al.*, 2015; Li *et al.*, 2019). In the placenta of the fruits, capsaicinoids known to be

present in the *Capsicum* genus and responsible for the hot and extremely burning sensation of chili peppers are synthesized through the condensation of medium-chain length fatty acids and vanillyl-amine (Lu *et al*., 2017; Li e*t al*., 2019). Capsaicinoids have five major components namely; capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin (Asnin and Park, 2015) of which in cayenne peppers, 80-90% of the total pungency are contributed by capsaicin and dihydrocapsaicin (Zewdie and Bosland, 2001).

Dermestes maculatus (DeGeer 1774), a black dull and usually hairy species, belongs to the commonly referred to as the hide beetle (McNamara *et al.*, 2008; Amadi and Dimkpa, 2018). It feeds on carrion and dry animal products such as dog meats, bacon, cheese, dried fish and produce from poultry (McNamara *et al*., 2008; Muftau, 2012; Shuaibu and Yahaya, 2018). In India and Italy, these beetles have been identified as a major pest of the silk industry too. They aggregate on their host where individual organism feed and mate in response to pheromones from the males. The aggregations vary in size with mostly one to thirteen beetles per small piece of food (McNamara *et al*., 2008).

The enzyme, glutathione transferases (GSTs; EC: 2.5.1.18) are endogenous compounds involved in the removal of xenobiotics through glutathione conjugation reaction. Using glutathione (GSH), they catalyze the nucleophilic attack of variety of electrophilic compounds containing carbon, sulphur, nitrogen or oxygen atom to form conjugates of glutathione (GS conjugate) (Hayes *et al.*, 2005). These enzymes partake in many biological reactions, including the detoxification of xenobiotics, transport of proteins, cell proliferation, clearance of oxidative stress products and in the induction of apoptosis signaling pathway. In the cytosol, active GSTs exist as a dimer of approximately 25 kDa protein subunits with the binding sites of reduced glutathione (GSH) and the hydrophobic substrates known as the G-site and H-site, respectively (Sheehan *et al.*, 2001; Suthar, 2017). GST universal substrate is 1-chloro-2,4-dinitrobenzene (CDNB) but they also conjugate GSH with bromosulfophthalein and 1,2-dichloro-4-nitrobenzene (Booth *et al*., 1961).

In Africa, *Clarias gariepinus*, also called the African catfish is the most-farmed species (Dauda *et al*., 2018) with *Dermestes maculatus* (DeGeer, 1774) known as pest of the dried fish (Zakka *et al*., 2013; Shuaibu and Yahaya, 2018). It has been reported that both larvae and adults of *D. maculatus* feed on dried *C. gariepinus* resulting in a quantitative loss of about 50% of the edible part due to fragmentation (Amadi and Dimkpa, 2018) and also the nutritive value of the stored fish (Shuaibu and Yahaya, 2018, Amadi and Dimkpa, 2018). Notably, the beetle at the larvae developmental stage has a prolonged life time and very destructive to stored agricultural products, particularly to dried fishes, hides and skins. At this stage, compared with many other insect pests of stored product, *D. maculatus* are able to withstand the effect of most synthetic insecticides and adverse environmental conditions (Amadi and Dimkpa, 2018; Shuaibu and Yahaya, 2018).

The use of chemical insecticide has being the conventional method of pest control both on the field and for stored products. Chemical insecticides can be detriment to both the target and non-target organisms and for this reason, efforts are being put into investigating plant materials, in part or whole, as bio-pesticides and as alternative to chemical pesticide for the control of pest. The aim of this study is to evaluate the inhibitory potential of ethanolic extract of *C. annum* on glutathione transferase of *D. maculatus* using biochemical and enzymological approaches. This study might be a step in the development of plant-based inhibitors for the control of stored product pests and enhancing product quality and quantity.

3.0 Methodology

3.1 Reagents and Chemicals

Sodium chloride, sodium acetate, 2-mercaptoethanol, 1-chloro-2,4-dinitrobenzene (CDNB), ethylenediaminetetra-acetic acid (EDTA) reduced glutathione (GSH), the GSTrap 4B (1 × 1 cm) affinity column and bovine serum albumin (BSA), were all purchased from Sigma (St. Louis, USA). Sucrose, ammonium persulfate, N, N, N, N-tetramethyl ethylenediamine (TEMED), acrylamide, Tris-HCl, Tris-Glycine, sodium dodecyl sulfate (SDS), potassium dihydrogen phosphate, ethanol, dipotassium hydrogen phosphate and DEAE-Sephacel were products of Pharmacia Fine Chemicals (Uppsala, Sweden). The other reagent used were of analytical grade.

3.2 Methods

3.2.1 Preparation of fruits for extraction process

Fresh *C. annum* fruits used in this study was purchased from Akure Main Market, Ondo State, Nigeria. The fruits were sorted from dirt or any contaminations and sun-dried for three weeks, before they were pulverized into fine powder using a high-speed electric blender for 5 min. The *C. annum* powder was sieved with a 2 mm mesh-sized sieve and stored in an air-tight sample bottle.

3.2.2 Preparation of ethanolic extract of *C. annum* fruit extract

According to the method of Sadek *et al*. (2003), 100 g of *C. annum* powder was dissolved in 400 mL of absolute ethanol mixing in ratio 1g of powder to 4 mL of ethanol (w/v) for extraction of the bioactive components. The mixture was kept at room temperature and shaken at intervals for 72 h followed by filtration of the extract using Whatman filter paper No. 42. After filtration, the excess ethanol was removed by rotary evaporator heating at 60°C, to obtain solid extract and dried in vacuum desiccator until required for further analysis. The yield of dry granules was used for the preparation of stock solution using ethanol.

3.2.3 Collection and culturing of Hide beetle (*Dermestes maculatus*) Larvae

Adult *D. maculatus* was collected by hand picking from an infested dried catfish (*Clarias gariepinus*) purchased from Akure Main Market, Ondo State, Nigeria and was kept in the laboratory to acclimatize for 32 days before they were used for artificial infestation in this study. These adults cultured and maintained on a large quantity of fresh and dried *Clarias gariepinus* in a plastic bucket covered with wire mesh. The late instar of *D. maculatus* larvae were harvested used for the experiments.

3.0 Preparation of Crude Enzyme from Whole Larva

Surviving larvae from each group after 48 h exposure to *C. annum* extract were demobilized by freezing. After removing the hard wings, the whole tissue was stored in the freezer until use. At a ratio of 1:3, the larva were homogenized using mortar and pestle in ice-cold phosphate buffer (0.1 M, pH 7.0) containing 1 mM each of EDTA and β-mercaptoethanol. The homogenate was subjected to cold centrifugation at 10, 000 *g* at 4 °C for 30 min. The supernatant was collected and stored in aliquots at −4 °C as crude enzyme.

3.1 Assay of Glutathione Transferase Activity

The assay of GST activity was carried out according to method of Habig *et al*. (1974). The reaction initiated by the addition of enzyme solution (30 µL) to the reaction tube containing of 25 mM CDNB and 10 mM GSH as substrates in phosphate buffer (100 mM, pH 6.5). The change in absorbance of product formed after 3 min was measured at 340 nm with UV/Visible-spectrophotometer (Jenway 6280, USA), using 9.6 mM^{-1} cm^{-1} as the product extinction coefficient. One enzyme unit of GST activity was defined as the amount of enzyme that could catalyze the formation of 1 µmol of thioether in one minute while the specific activity of the enzyme is expressed as µmol/min/mg protein. Bradford method (Bradford, 1976) was used for the determination of protein concentration.

3.2 Purification of Crude Enzyme to apparent homogeneity

The purification process was previously described by Bamidele *et al*. (2017). The crude enzyme solution was gently applied on the equilibrated ion-exchange column (2.5 x 20 cm) packed with DEAE-Sephacel and further purified on affinity chromatography column (1 x 1 cm) pre-packed with GSH-Sepharose 4B (GSTrap 4B^{TM}). The fractions with GST activity were pooled and stored at -4 °C until required for subsequent studies. The purification process was conducted at 4 °C.

3.3 Determination of Molecular Weight

The Subunit molecular weight of the purified GST was estimated by sodium docecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide as described by Laemmli, (1970) while the native molecular weight of the enzyme was determined by gel filtration.

3.4 Inhibitory studies on Purified GST

Inhibitory studies of purified GST was investigated according to earlier described method (Tahir *et al*., 1985; Lumjuan *et al*., 2007) using the ethanolic extract of *C. annum* and some pure plant-based insecticides; Anisaldehyde and Terpeneol compounds. Preparation of stock solutions of inhibitors were carried out by adding ethanol to inhibitors and diluted with chosen buffer, to give highest ethanol concentration (1%) in the test mixtures. One hundred microliters of the purified GST and 50 µL of each inhibitor solution with appropriate concentration range of 0-100 µM were incubated at room temperature for 10 min and the incubated mixture was added to the assay medium earlier described. The control reaction

has no inhibitor. The IC_{50} which is the inhibitor concentration giving 50% enzyme activity was calculated from the logarithmic plot of fractional velocity against concentration of inhibitor using Graphpad Prism 6 software. Inhibition pattern of inhibitors was determined (Lumjuan *et al*., 2007; Dou *et al*., 2009). The assay of GST activity was carried out in the presence and absence of varied concentrations of ethanolic *C. annum* extract (0 – 1.5 mg/mL) and α-terpeneol (0-0.75 mg/mL). Inhibitor solutions were diluted to maintain 1% of organic solvent in the assay medium. Dataset obtained were analyzed by Graphpad prism 6 (Graphpad Software, San Diego, CA, USA)

4.0 Results

4.1 Purification of the Glutathione transferase from *D. maculatus*

The summary of the purification process is presented in Table 1. The ratio of initial velocity and protein concentration (specific activity) of purified GST after purification was 0.34 µmol/min/ml while the fold of purification was 107.50 with an activity recovery of 7.84%. This enzyme was adjudged homogenous from its estimated sub-unit molecular weight and native molecular weights as 28.01 kDa (Figure 1) and 53.63 kDa respectively.

4.2 Inhibition Studies

The conjugating activity of GST at constant concentration of *C. annum* extract and varied concentration of GSH, estimated K_i was 0.96 mg/ml (Figure 2) while at varied concentration of CDNB, K_i was 2.80 mg/ml (Figure 3). Also at constant concentration of α-Terpeneol and varied concentration of GSH (Figure 4), the K_i was 0.55 mg/ml while at varied concentration of CDNB as 1.85 mg/ml (Figure 5). There was no observable pattern of inhibition with anisaldehyde compound and it was not determined. All reciprocal plots of the reaction of GST with its substrates at constant concentrations of *C. annum* extract give a straight line and the convergence of the lines were below 1/GST axis. Similarly, the graph in Figure 6 is a sigmoidal curve for the three compounds (Anisaldehyde and α-Terpeneol, *C. annum* extract) tested. The IC_{50} is the inhibitor concentration giving 50% activity of GST. The estimated IC_{50} values calculated from the graph are shown on Table 2 were 0.6, 0.5 and 0.1 mg/ml for *C. annum* extract, Anisaldehyde and α-Terpeneol respectively with α-Terpeneol having the lowest IC_{50} value and most potent of the inhibitors.

Table 1: Summary of the purification of GST from *D. maculatus* larvae

Step	Volume (ml).	GST Activity (µmol/miin/ml)	Protein concentration (mg/ml)	Total GST Activity (µmol/miin)	Total Protein (mg)	Specific activity (µmol/miin/mg)	Yield (%)	Fold
Crude extract	70.0±5.33	0.89±0.06	277.69±12.40	62.09±8.65	19438.30±387.98	0.0032±0.00	100.00±0.00	1.00±0.00
Ion Exchange chromat.	22.2±1.88	1.44±0.01	167.11±10.20	32.01±1.64	3709.84±25.62	0.0086±0.00	19.09±1.66	2.70±0.35
Affinity chromat	15.6±1.74	33.56±3.01	97.74±8.77	523.53±15.60	1524.74±82.53	0.3434±0.00	7.84±2.44	107.50±11.4

Notes: Purification was conducted at 4°C. Values are mean±standard deviation of three repetitions

Table 2: Estimated inhibition constants and Kinetic values of GST from *D. maculatus*

Kinetic parameters	K_m (mM)	V_{max} (µmol/min/ml)
GSH	0.548±0.03	88.49±2.75
CDNB	0.442±0.10	52.63±4.31
Inhibitors	K_i (mg/ml)CDNB	K_i (mg/ml)GSH
C. annum extract	2.80±0.12	0.96±0.01
Anisaldehyde	nd	nd
Alpha-Terpeneol	1.85±0.08	0.55±0.04

Values are mean±standard deviation of three repetitions

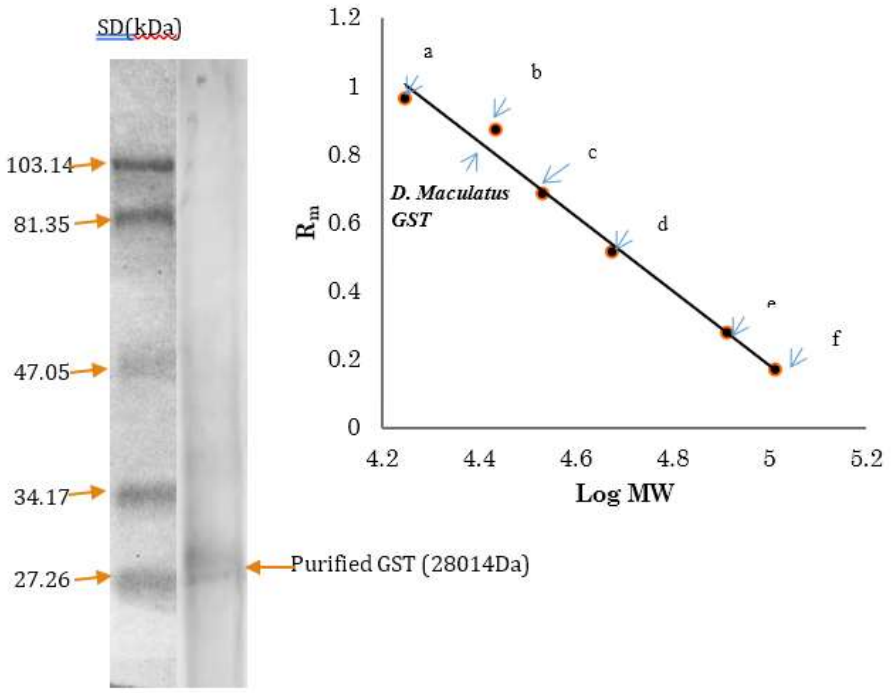

Figure 1: Electrophorectogram of purified GST from *D. maculatus* on 10% polyacylamide gel slab. Molecular weight (MW) of standard proteins used ranges from 17.27-103.14 kDa. a – Lysozyme (17670 Da) b - Soybean trypsin inhibitor (27260 Da) c - Carbonic anhydrase (34170 Da) d – Ovalbumin (47050 Da) e - BSA (81350 Da) f - Phosphorylase b (103140 Da)

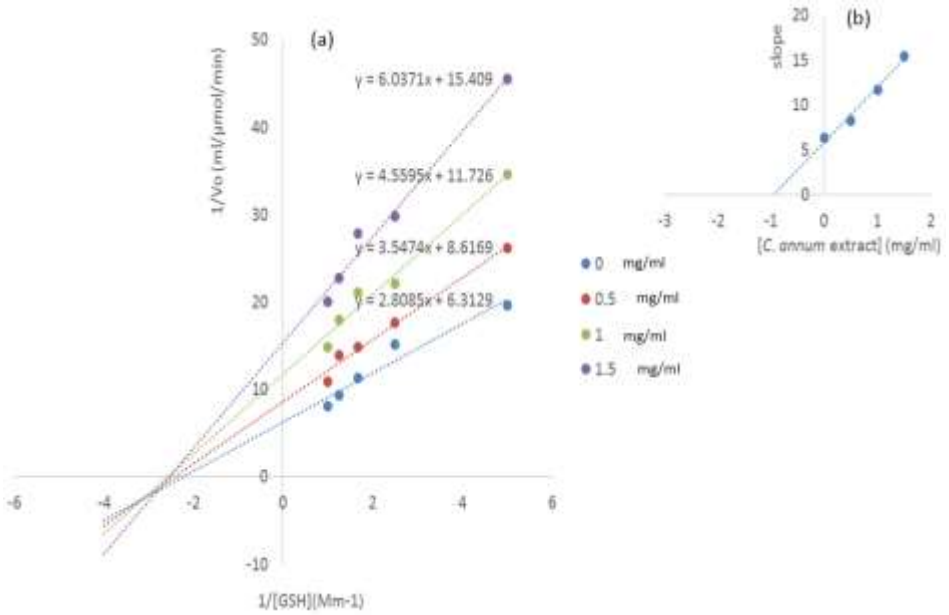

Figure 2: (a) Lineweaver-Burk Plot showing reaction of GST with *C. annum* extract at different concentrations (0 mg/ml 0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml) and varied concentration of GSH (b)Inset: Secondary replot of slopes in (a) against concentration of *C. annum* extract

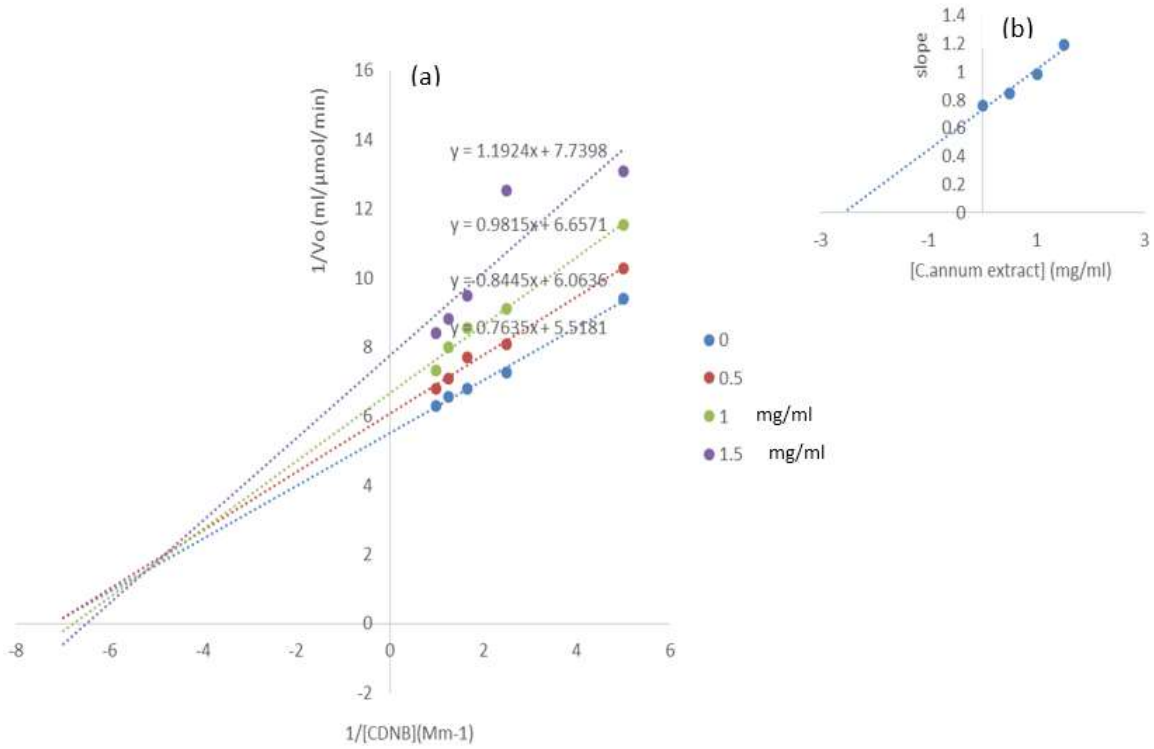

Figure 3: (a) Lineweaver-Burk Plot showing reaction of GST with *C. annum* extract at different concentrations (0 mg/ml 0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml) and varied concentration of CDNB (b)Inset: Secondary replot of slopes in (a) against concentration of *C. annum* extract

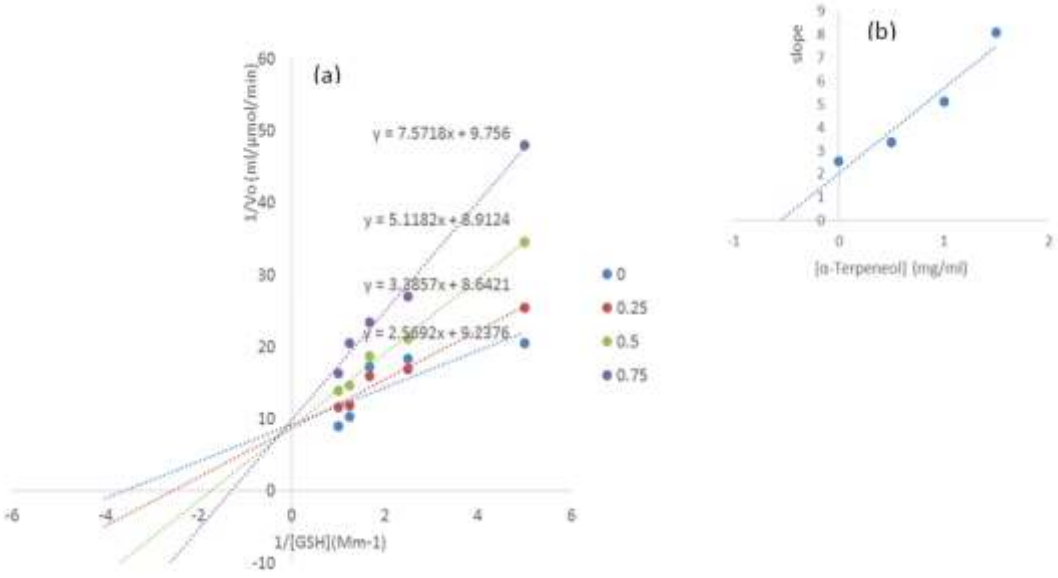

Figure 4: (a) Lineweaver-Burk Plot showing reaction of GST with α-Terpeneol at different concentrations (0 mg/ml 0.25 mg/ml, 0.5 mg/ml and 0.75 mg/ml) and varied concentration of GSH (b)Inset: Secondary replot of slopes in (a) against concentration of α-Terpeneol

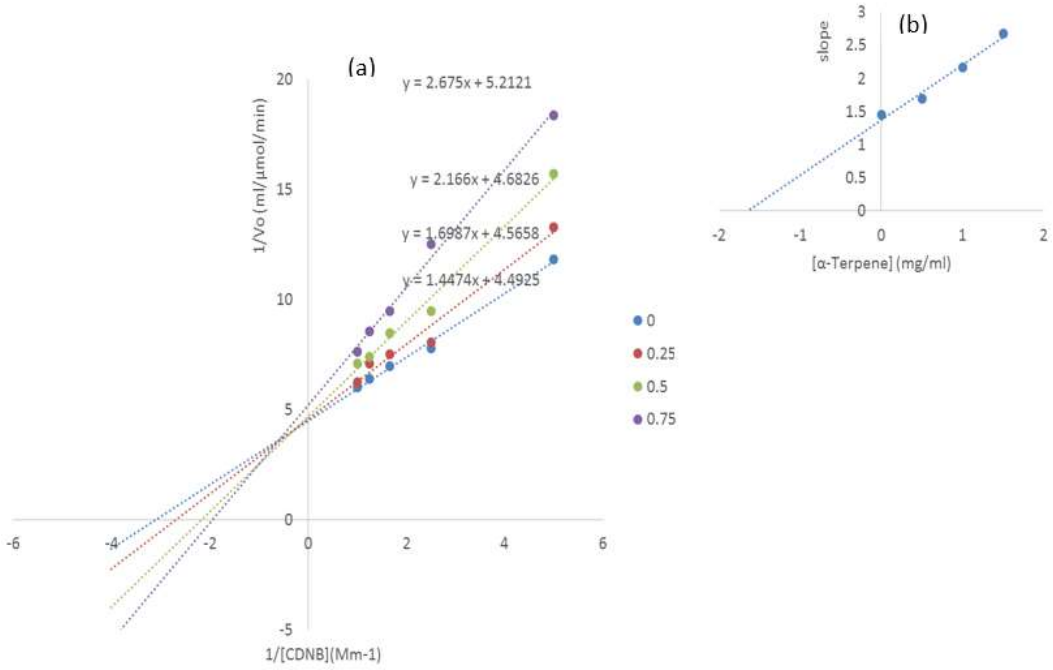

Figure 5: (a) Lineweaver-Burk Plot showing reaction of GST with α-Terpeneol at different concentrations (0 mg/ml 0.25 mg/ml, 0.5 mg/ml and 0.75 mg/ml) and varied concentration of CDNB (b)Inset: Secondary replot of slopes in (a) against concentration of α-Terpeneol

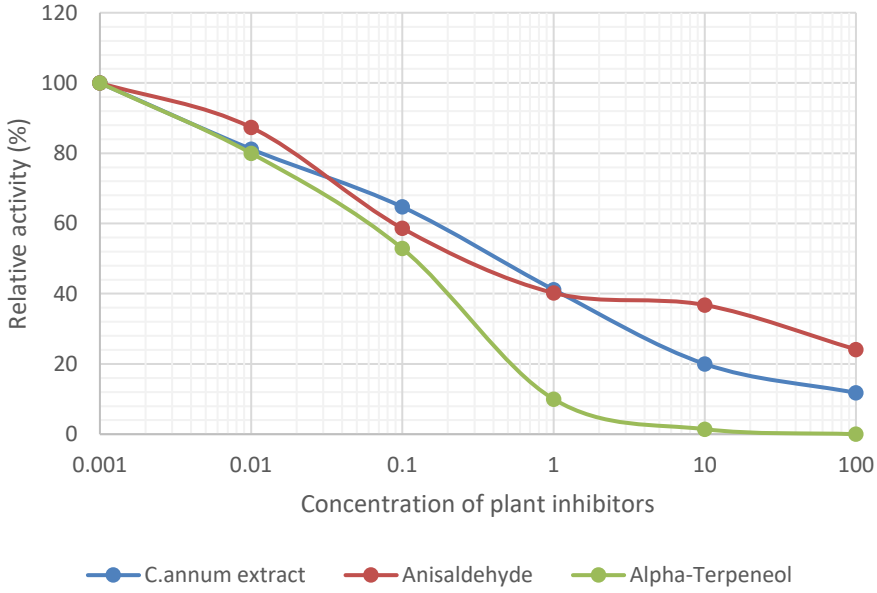

Figure 6: Logarithmic plot of relative activity against concentration of *C. annum* extract, Anisaldehyde and α-Terpeneol

5.0 Discussion

The result of glutathione transferase activity assay shows that the enzyme is present in *D. maculatus* larvae. Previous investigation suggested the conjugation efficiency of GST in the gut of the *D. maculatus* larvae to eliminate chemical insecticide makes them more water soluble for excretion (Bamidele *et al.*, 2017). The specific activity of the enzyme increase through the purification processes. This indicates that all other non-GST proteins must have been removed. The purification fold and yield obtained were

calculated and these were increased during the progress of purification. Similar purification steps have been reported previously by Konishi et al., (2005) and Shukor et al., (2014). A single protein band is shown on SDS-PAGE plate which indicates that purified *D. maculatus* GST is purified to apparent homogeneity and has an estimated subunit molecular weight of 28.014 kDa. This subunit molecular weight when compared with the native molecular weight indicates that *D. maculatus* GST appears homodimeric. Homodimeric nature of GST from *D. maculates* is in agreement with reports of (Dou et al., 2009; Wu et al., 2009).

Inhibition studies show that α-terpeneol and anisaldehyde exhibited good inhibitory effects on GST, and α-terpeneol was the most effective inhibitor. This is seeing when α-terpeneol was used as inhibitor, the inhibition constant with respect to CDNB and GSH were lower than that of the *C. annum* extract. Meanwhile, when anisaldehyde was used as inhibitor, the inhibition constant with respect to CDNB and GSH was not determined. The pattern of inhibition of α-terpeneol and *C. annum* extract on *D. maculates* larvae GST was mixed inhibition. This pattern refers to the inhibitor binding with the enzyme and enzyme-substrate complex during conjugation reaction. The patterns of inhibition supported by the lower k_i values of α-terpeneol also suggest stronger binding of α-terpeneol to the enzyme than the plant extract. Though the substrates (GSH and CDNB) have stronger attractions to *D. maculatus* GST, the inhibitors are able to cause inhibition of the enzyme activity by binding to the enzyme and its derivatives during the catalysis.

Conclusively, the presence of GST in the gut of Hide beetle (*Dermetes maculatus)* larvae has been established. The binding affinity of α-terpeneol and anisaldehyde as well as ethanolic extract of *C. annum* on *Dermetes maculatus* GST are known. These inhibitors proceed via mixed inhibition to inactivate the enzyme. Similar to α-terpeneol, the ethanolic extract of *C. annum* shows promising results in the inhibition of *Dermetes maculatus* GST. This biochemical investigation of inhibitory property of ethanolic extract of *C. annum* may be the first step in the designing of plant-based inhibitors as bio-pesticide for the control of stored product pest and enhance stored product quality and quantity. This work is the preliminary study in the process of developing effective inhibitors for the control of stored product pests. In-depth studies are required to gain insight into the interactions between plant-based inhibitors and the enzyme by exploring chemical and molecular biology techniques.

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