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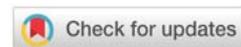
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Research Article

Antioxidant and detoxifying enzymes response of stored product insect pests to bioactive fractions of botanical extracts used as stored grains protectant

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Abstract

Recurrent exposure of stored product insects to synthetic insecticides resulted in the development of resistance which occurs due to changes in insect metabolic enzymes. The inhibitory effect of ethyl acetate active fraction of *Mitracarpus villosus*, *Bridelia micrantha*, and *Clerodendrum capitatum* on antioxidant and detoxifying enzymes of stored product insects was investigated in this study. *Sitophilus oryzae*, *Tribolium castaneum*, and *Rhizopertha dominica* were exposed to 5 and 20 μ l concentrations of ethyl acetate active fraction VI 50:50 derived from glass column chromatography in a fumigation chamber and were homogenized separately. The results showed that SOD, CAT, GPx, GSH, and GST activities were dosage-time-dependent. Adult insects exposed to different dosages of *C. capitatum* active fractions significantly inhibited GST and GPx activities, and SOD, CAT, and GSH activities were induced in comparison to insects in control. While, *M. villosus* and *B. micrantha* extracts exhibited a significant increase ($p < 0.05$) in SOD, CAT, and GSH in contrast to the inhibitory effects on GST and GPx. These results clearly show that stored beetles differ in their response to different enzymatic activities and that the evaluated plant materials may be used as an eco-friendly biopesticide in the IPM strategy for safeguarding stored food grains against stored produced insect pests.

Introduction

Food availability in unindustrialized nations seriously centers on the ability of subsistence farmers to preserve the post-harvest quality of their produce. These resource-poor farmers customarily preserve their food grains in their own small storage facilities with little or no high-tech ideas; thus, influencing great quantifiable and qualitative losses due to insect infestation and damage.

Keeping stored grains and milled grain products from insect infestations is a major challenge that causes disquiets in all nations to ensure food security. *Tribolium castaneum*,

Sitophilus oryzae, and *Rhizopertha dominica* are amongst agriculturally important insect pests attacking a lot of stored agricultural products. Consequently, it is imperative to explore good protective materials for the stored products against beetle infestation.

Synthetic insecticides and fumigants such as methyl bromide or phosphine play a vital role in controlling this problem, but they have been known to cause serious toxicological and environmental problems. Consequently, there is an urgent need to develop affordable, safe, sound insect pest control agents and techniques [1,2]. Botanical materials have played an important role in traditional methods of stored

product protection since time immemorial when mixed with stored grains. Natural products from locally available plants with insecticide activity represent a low-cost and sustainable alternative to protect agricultural produce.

Several research articles reported that organophosphates induced oxidative stress in non-mammalian systems mainly insects [3]. To protect against the effects of oxidative stress, insects have a variety of detoxifying enzymes at their disposal, such as catalase, glutathione reductase, glutathione peroxidase, and glutathione-S-transferases play an important role in eliminating harmful substances [4,5]. Hence, this study conceptualized the effect of the bioactive fraction of *Mitracarpus villosus*, *Bridelia micrantha*, and *Clerodendrum capitatum* extracts on antioxidant and detoxifying enzyme activities of stored product insects.

Materials and methods

Experimental setting

The study was set up at the Institute of Bioresources and Sustainable Development (IBSD), Insect Chemical Ecology Laboratory, in Imphal, Manipur, India (Latitude 24° 49' N and Longitude 93° 57' E), under ambient environments of 27 °C, 70° R.H, and 10:14 photoperiod (L:D).

Raising insects

The fully-grown rice weevil *Sitophilus oryzae* L. (Coleoptera: Curculionidae), lesser grain borer *Rhizopertha dominica* (Coleoptera: Bostrichidae), and rust red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) were sourced from pest-ridden rice and wheat grains at the storeroom of Food Corporation of India (FCI), Imphal, Manipur, India. *T. castaneum*, *S. oryzae*, the sub-cultures were raised in the laboratory at 27.2 °C, 70.5% relative humidity, and a photoperiod of 10:14 (L:D) until the appearance of adults on wheat flour having 2% yeast powder and whole wheat grains, correspondingly.

Extract preparation

In a Soxhlet kit, 200 grams (g) of pulverized *Mitracarpus villosus*, *Bridelia micrantha*, and *Clerodendrum capitatum* leaves powder were sequentially extracted with a succession of solvents with increasing polarization, petroleum ether, hexane, ethyl acetate, acetone, chloroform, and methanol. To eliminate the solvent from the extract, it was vaporized in a spinning evaporator under low pressure and below 60 °C. Employing activated charcoal, the dark-green residue's chlorophyll was removed. Following concentration, the solvent completely evaporated, leaving behind the chlorophyll-free filtrate.

Insecticidal activity of the active fractions of plant extracts

The insecticidal action of the active portions of *M. villosus*, *C. capitatum*, and *B. micrantha* was assessed by fumigation. For all types, ten insects were utilized per treatment. The adult insects were subjected to active fraction dosages ranging from 0 to 100

g/L for 24 hours. Five (5) replications of each dose were used for each species, and there were an equivalent number of untreated control replicates. Injecting the fumigant required the use of gas-tight micro syringes. For each dosage, five replicates were employed and mortality rates for adult insects were calculated 24 hours after exposure. Extract with promising activity was selected for column chromatography.

Column chromatography using glass

The crude extract with encouraging insecticidal action was run through column chromatography by means of a glass column with a diameter of 3 cm and a length of 100 cm, packed with silica gel (60 - 120 mesh) and eluted with hexane (100:0, I). Subsequently, a stepwise gradient of ethyl acetate, acetone, chloroform, and methanol was run through the column in the following order: I 100:0, II 80:20, III 70:30. The insecticide action of each of the five 300 mL fractions was evaluated after collection and condensing under reduced pressure. Components that are insecticide-active were combined and concentrated even more and subjected to a second round of column chromatography using a glass column (length 50 cm; diameter 3 cm) before being eluted with ethyl acetate, acetone, chloroform, and methanol [6]. The component with insecticidal potential was combined and concentrated before being used in enzymatic bioassay.

Preparation of homogenates for enzymatic activity

Twenty (20) adult insect species were exposed to five (5) replicates with ethyl acetate active fraction of the designated plant extracts at varied concentrations (5 and 20 µl) in a desiccator that functions as a fumigant chamber. Injecting the fumigant required the use of gas-tight micro syringes. The insects were removed after 3, 6, and 12 hours post-exposure. Additionally, untreated insects were used as a control. To remove insect tissue debris, the insects were normalized in 2 ml of 0.1 M phosphate buffer (7.4 pH) in a glass tissue grinder set in an icebox. The homogenate was then centrifuged at 1000 rpm for 5 min at 4 °C in a chilled centrifuge. To stop the enzyme from becoming inactive, the supernatant was divided into fresh tubes and stored in an ice box until use. However, due to the dissimilarity in the homogenization buffer, insects used to measure glutathione S-transferase activities were normalized with 2 ml Tris-HCl [7].

Superoxide dismutase activity (SOD): According to Marklund and Marklund [8], pyrogallol (2 mM) auto-oxidation was used to assess Superoxide Dismutase (SOD) activity. The enzyme was combined with pyrogallol in 0.1 M tris buffer (pH 8.2) in the reaction mixture (whole insect homogenate). Results were expressed as units/mg protein for the reaction, which was started by adding the substrate, and were read at 420 nm for 3 minutes at intervals of 1 minute. One unit of enzyme activity is the quantity of the enzyme that reduces auto-oxidation by 50%.

Reduced glutathione (GSH): Using the Ellman (1959) method, reduced glutathione (GSH) content was calculated.

Centrifuging at 2000 rpm for 10 minutes after preparing 10% whole insect homogenates in 5% w/v TCA, the supernatant (GSH) was combined with 10 mM DTNB in 0.1 M phosphate buffer (pH 8.0). The combination was allowed to stand at room temperature for 10 minutes while the color was measured at 412 nm. A standard curve was used to determine the glutathione content, which was then expressed as g/mg protein.

Catalase activity (CAT): CAT activity was measured using the Aebi [9] technique. The reaction mixture contains 0.05 M of phosphate buffer and 3% H₂O₂ (pH 7.0). About 100µl of the enzyme (whole insect homogenates) was added to start the reaction. After three minutes, the change in absorbance at 240 nm was recorded, and the activity was reported as n mole H₂O₂/min/mg protein.

Glutathione peroxidase: The modified Rotruck, et al. [10] method was used to quantify glutathione peroxidase (GPx) activity. The reaction mixture contains 2 ml of pure water, 0.05 M sodium phosphate buffer (pH 7.4), 10 mM sodium azide, 4 mM reduced GSH, and 0.042% (w/w) H₂O₂. After 10 minutes at 37 degrees Celsius, the reaction mixture was stopped by adding 0.5 ml of 10% TCA and centrifuging it for 15 minutes at 5000 rpm. The color generated was measured at 412 nm after 200 µl of supernatant was added to 3 ml of 0.3 M disodium hydrogen phosphate and 50 l of 10 mM DTNB reagent. The amount of GSH used during the process was used to compute the enzyme activity.

Glutathione-S-transferase: The Warholm, et al. [11] method was used to measure the glutathione-S-transferase (GST) activity. In 0.1 M phosphate buffer (pH 7.4) with 1 mM EDTA, the reaction mixture contained 20 mM GSH and the 20 µl supernatant (whole insect homogenate treated with or without active fraction). 30 mM 1-chloro-2-4-dinitrobenzene (CDNB) was added to start the reaction, and the change in absorbance was recorded at 344 nm. Mole CDNB conjugate/min/mg protein was used to express the enzyme activity. Using BSA as the reference, the protein content was calculated using the Lowry, et al. method.

Experimental design and data analysis

Five replications of the enzymatic activity studies, each containing 10 unsexed insects, were set up in a completely

randomized designed manner. To assess if there were significant differences in the treatment means, a one-way analysis of variance (ANOVA) was used, followed by Tukey's multiple comparison tests. The SAS computer software was used for analysis and to express the data as the mean Standard Error of the Mean (SEM). At $p < 0.05$, the results were deemed significant.

Results

Insecticidal activity of the active fractions of plant extracts

Though not presented, the fumigant activity of the crude plant extract showed that ethyl acetate exerted significant adult mortality and was selected for column chromatography. In the second round of column chromatography fraction II 80:20 exhibited promising insecticidal activity and was selected for the antioxidant and detoxifying enzymatic activities.

Effect of bioactive fractions of botanical extracts on insect antioxidant and detoxifying enzyme system

The activities of GST and GPx in adults *S. oryzae* exposed to ethyl acetate active fraction of *C. capitatum* active fractions indicated that the extract had distinct inhibitory effects in a dosage-time-dependent manner (Figure 1). A similar trend was observed in *R. dominica* and *T. castaneum* exposed to active fractions of *M. villosus* (Figure 2) and *B. micrantha* (Figure 3) ethyl acetate extracts respectively. The GST and GPx activities were significantly inhibited ($p < 0.05$) with increased extract concentration and exposure period in the treated insects compared to the significantly lower ($p < 0.05$) inhibition rate recorded in the control sample. (Figure 1).

SOD, CAT, and GSH enzymatic activities were significantly ($p < 0.05$) induced in the insects exposed to different concentrations of the botanical's active fraction compared to insects in control (Figures 4-6). The induction of enzymatic activities was equally observed to be dosage-time dependent for all insects treated with the active fractions increased with increasing concentrations and exposure duration for all the treatments with the highest activities recorded at 20 µl and 12 h post-treatment (Figures 4-6).

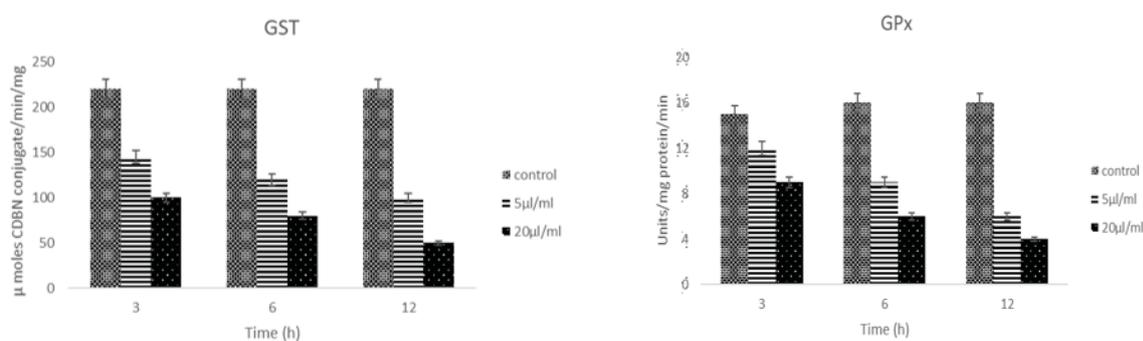


Figure 1: Effect of different concentrations of active fractions from *C. capitatum* on GST and GPx activities in *S. oryzae*.

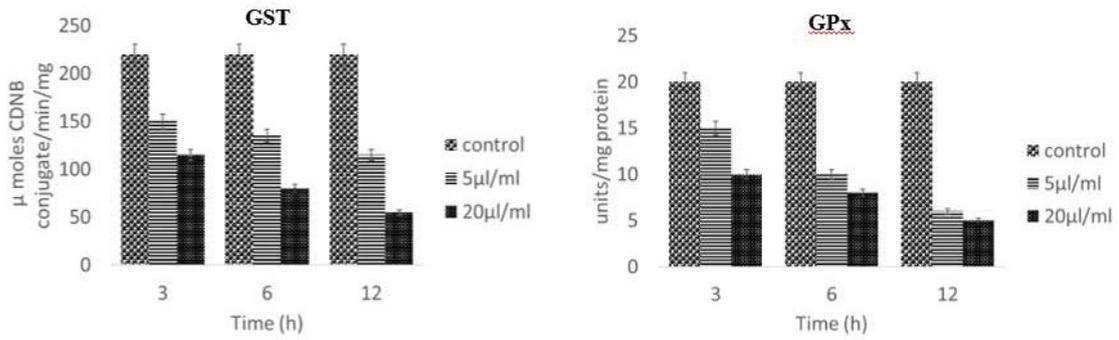


Figure 2: Effect of different concentrations of active fractions from *M. villosus* on GST and GPx activities in *T. castaneum*.

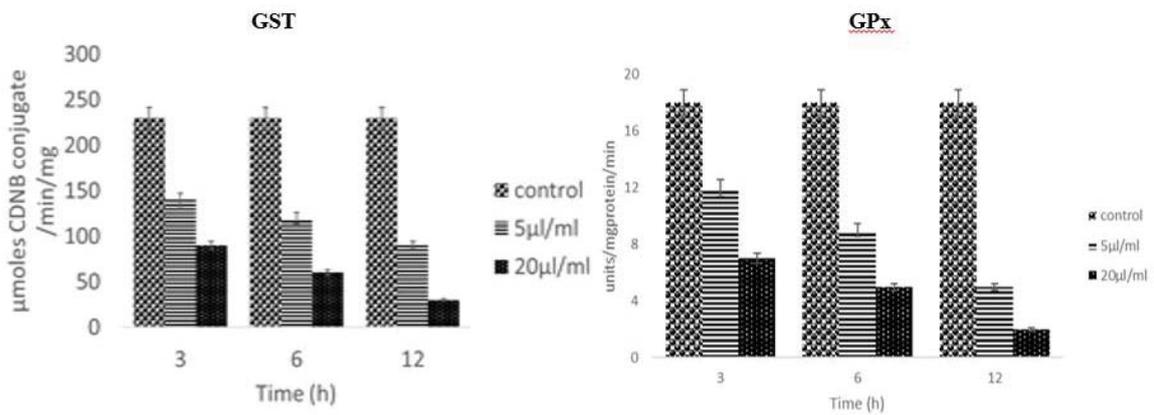


Figure 3: Effect of different concentrations of active fractions from *B. micrantha* on GST and GPx activities in *R. dominica*.

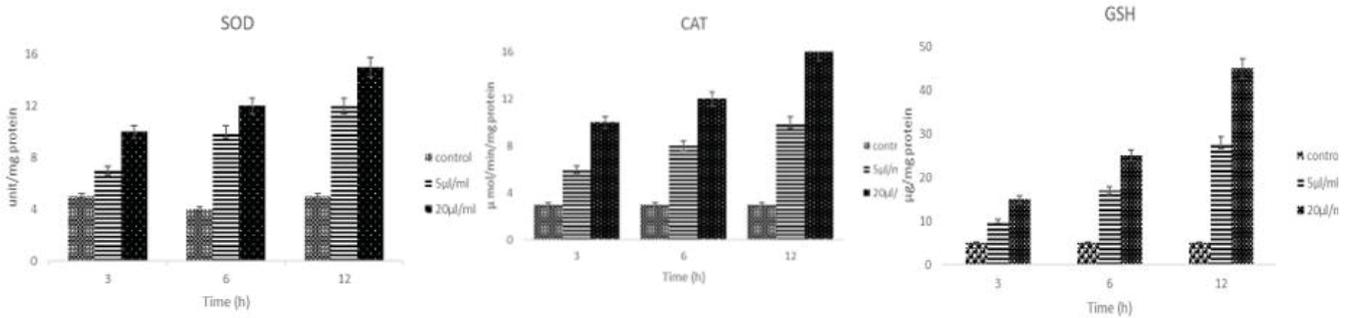


Figure 4: Effect of different concentrations of active fractions from *C. capitatum* on SOD, CAT and GSH content in *S. oryzae*.

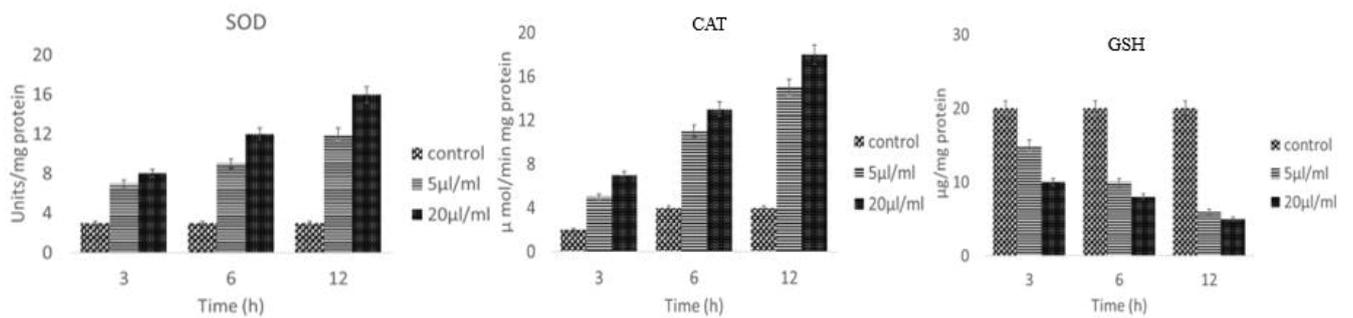


Figure 5: Effect of different concentrations of active fractions from *M. villosus* on SOD, CAT and GSH activities in *T. castaneum*.

GC-MS analysis of plant extracts active fraction

The GC-MS analysis of an active fraction of the various plant extracts revealed the presence of different phytochemical constituents showing the presence of large peaks including other minor peaks. The GC-MS profile of *B. micrantha* ethyl acetate extract shows; 13-docosen-1-ol and 4-Hydroxyphenylacetic acid as the phytochemicals with the highest peak area percentage and 2-Butenoic acid and 2 propenyl with the lowest peak (Figure 7). The highest peak in *M. villosus* petroleum ether extract contains Tetracosanoic acid and Methyl 20-methyl-heneicosanoate and Nonane, 2, 6-dimethyl Octane, 3-5-dimethyl decane recorded at the lowest peak (Figure 8). While 8-Methyl-1-undecene and Hexahydrofarnesyl acetone were observed at the highest peak and Octanoic acid Trans-2

Octenal were the lowest peak phytochemicals obtained from *C. capitatum* hexane extract (Figure 9).

Discussion

Medicinal plants are rich sources of secondary metabolites with antioxidant properties. Many secondary metabolites from medicinal plants have diverse biological effects, including the inhibition and induction of several important enzymes [12]. The toxicity of natural products particularly plant extracts has been implicated in the alterations of biochemical parameters [13].

Determining the inhibitory abilities of exogenous compounds on the activities of enzymes in the insect body is an important method for evaluating insecticidal activities

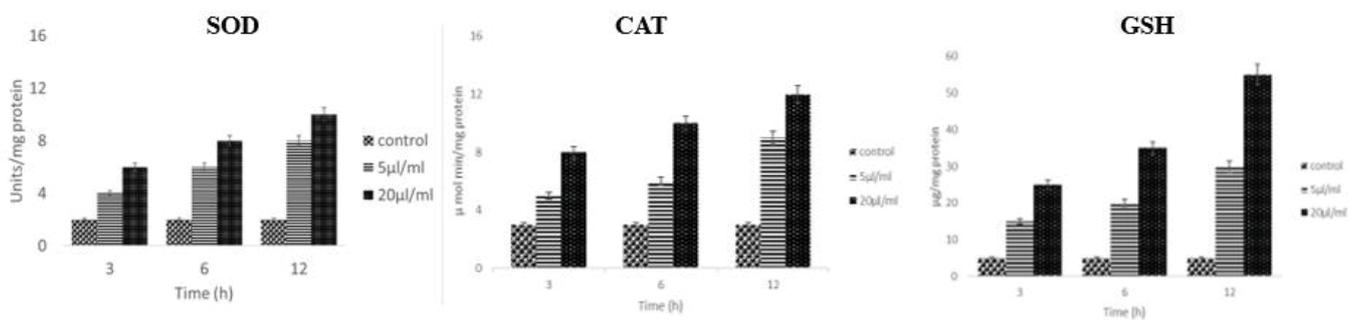


Figure 6: Effect of different concentrations of active fractions from *B. micrantha* on SPD, CAT and GSH activities in *R. dominica*.

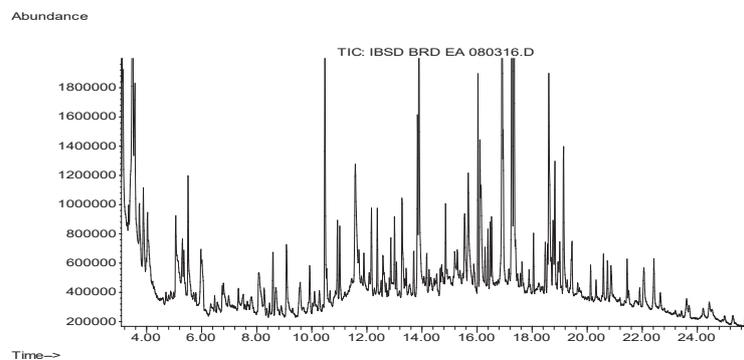


Figure 7: Chromatographic profile GC-MS of ethyl acetate active fraction of *Bridelia micrantha*.

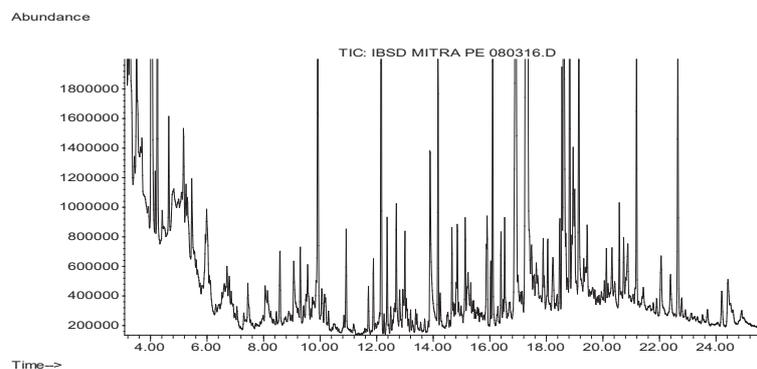


Figure 8: Chromatographic profile GC-MS of ethyl acetate active fraction of *Mitracarpus villosus*.

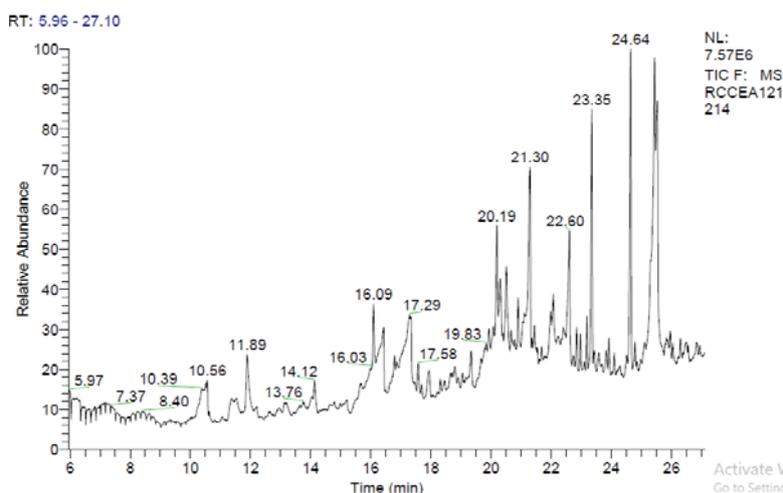


Figure 9: Chromatographic profile GC-MS of ethyl acetate active fraction of *C. capitatum*.

[14,15]. Detoxification enzymes are largely proven as the enzymatic defense against alien compounds, and various plant xenobiotics inclusive and play significant roles in maintaining their normal physiological functions. The inhibition of these enzymes after exposure to insecticide and fumigants indicated that they are not responsible for the detoxification of insecticide and possibly increase the susceptibility of insect pests to these insecticides [16].

Therefore, the present findings reveal that the effect of active fractions of *C. capitatum*, *M. villosus*, and *B. micrantha* on the antioxidant system of stored grain pests was studied in different concentrations and exposure periods. The investigation clearly shows that active fraction caused significant impairment in the antioxidant enzyme system (SOD, CAT, GSH, GPx, and GST) of treated insects. The enzymes, SOD, CAT, and GPx are the major cellular antioxidant defenses. The results have shown increased antioxidant enzyme (SOD, CAT, and GSH) activities in the insect homogenate by active fractions, whereas in the case of GPx and GST activities, it significantly decreased in the treated insects. This corroborates the findings of Adesina, et al. [17] The active fraction also significantly alters the enzyme activities when treated to insects, indicating induction of the enzymes that would contribute to the enhanced antioxidant defenses of the insects. Overall, the study shows that a high level of oxidative stress was induced by active fractions from the plant active fractions.

Superoxide Dismutase (SOD) is the major attractive metalloprotein in the antioxidant family and the first line of defense against oxygen-free radicals. This enzyme removes superoxide radicals (O_2^-) through the process of dismutation to oxygen and hydrogen peroxide [18,19]. The ability of SOD to scavenge O_2^- is temporary and limited [20]. SOD is an antioxidant enzyme that can protect normal cells from ROS. The ability of this enzyme to overcome the toxic effects of ROS in insects has been documented [21]. It is also plausible to suggest that at higher concentration of the herbal insecticides, more O_2^- is generated, which may accumulate to an extent that may overwhelm the scavenging ability of the SOD enzyme,

SOD activity for all the herbal insecticide formulation-treated insects increases with increased concentration and exposure period. The increase in SOD might be connected to multivarious chemical components of the plant's active fractions. The increased active fractions concentration in all the treated samples stimulated the synthesis of SOD, resulting in higher dismutation of superoxide anion (O_2^-). Thus, preventing the production of hydroxyl radical (OH^-) - a highly reactive species [22].

GSH (glutathione) is a thiol group-containing major non-enzymatic antioxidants that play a very important role in the defense against ROS. GSH participates in the protection against oxidative stress by its involvement in the ascorbate-GSH cycle, regulation of protein thiol-disulfide redox status, and reduction of H_2O_2 to water [23]. It is a tripeptide (γ -glutamyl-cysteinyl glycine), ubiquitously found in cells. The antioxidant activity of glutathione protects the cells against oxidative damage by free radicals and represents an important cellular defense mechanism. GSH pool is maintained in the cells by the restoration of the oxidized form of glutathione (GSSH) [24]. This finding shows that GSH increased in treated insects with both doses indicating higher oxidative stress brought about by the active fraction.

The exposure of the storage beetles to the active fractions might have brought about the induction of the GSH synthesis and the effect of herbal insecticides formulation could be a result of the medley of allelochemicals that characterized the active fractions [25]. The response of the beetle GSH to the insecticides could demonstrate the insect sensitivity to xenobiotics and might be connected to enhance the utilization of GSH as co-substrate for GSH transferase or cofactor for GSH POX [26]. Glutathione and glutathione-dependent enzymes have been known to play a central role in the protection and detoxification of peroxides and hydroperoxides. Marked depletion of GSH and its dependent enzymes in the insects by active fractions indicates a major deleterious effect on the insects. This finding revealed that the oxidative imbalance may be involved in the toxic effects of active fractions from *C. capitatum*, *M. villosus*, and *B. micrantha*.

GST belongs to the phase II detoxification system involved in conjugation reactions and may also detoxify a number of toxic ligands by acting as a non-catalytic intracellular binding protein (Kostaropoulos, et al. 2004). GST plays an important role in protecting cells against ROS-mediated injury by detoxification of lipid hydroperoxides formed due to oxidative damage [27,28]. It is believed that these enzymes play an essential role in the survival of insects exposed to endogenous or exogenous xenobiotics [29] as they are involved in the detoxification of various plant xenobiotics [30]. They usually catalyze the conjugation of the thiol group of reduced glutathione to electrophilic toxic xenobiotics and endogenously activated compounds and molecules, thereby increasing their solubility and promoting rapid excretion or facilitating degradation [14,31,32]. GSTs are potential drug targets and plant extracts enter tissues and organs of target insects and affect the activity of various detoxifying enzymes. Several secondary plant metabolites may inhibit GSTs activity, whereas others can activate GSTs activity [30,33,34].

Results from this finding showed a drastic decrease in the activity of GST in all treated insects with *C. capitatum* and *M. villosus* active fractions, which could compromise the biochemical antioxidant defenses of the insects. However, an increase in GST activity was observed in insects exposed to different concentrations of active fractions of *B. micrantha* herbal formulations, and a more significant effect than the control treatments. This might be due to the fact that the herbal insecticide formulation toxicity induces stress causing an increase in the GST enzyme to resist oxidative damage. This result correlates with the findings of War, et al. [35] and Vinseton, et al. [36] who observed greater levels of GST in *S. litura* and *Plutella xylostella* treated with a combination of neem oil and sesame oil [36].

The dose-dependent antioxidant activities of active fractions as observed in this study are in line with Tsado, et al. [37]. This indicates that the antioxidant potentials of the various plant extracts change as the concentrations rise.

Significant alteration in the insect vital enzymes is a sign of very high insecticidal activity of the *C. capitatum*, *B. micrantha*, and *M. villosus* active fractions against the economically important storage beetles. The activities of the extract against AChE, detoxifying, and antioxidant enzymes could, therefore, be attributed to the presence of phytochemicals/bioactive molecules in the plant materials. It is demonstrated by the results that these ingredients cause high lethality in storage beetles at a very low dose and cause significant inhibition of metabolic enzymes.

Bhattacharya and Chandra [38] stated that botanicals are an important source of chemical compounds and proved to be efficient biopesticides for the control of insect pests. The efficacy of these plant products (extracts) could be accredited to the manifestation of phytochemical secondary metabolites, thus accountable for the different actions comprising insecticidal, enzyme detoxification, and antioxidant properties [39].

This study has established that extraction of active biochemical from plants is directly proportional to the polarity of the solvents used. The ethyl acetate effectiveness as observed in these findings aligned with the observation of Adesina [40] who stated that ethyl acetate is a reasonably polar solvent (polarity index of 4.4) that largely extracts steroids, alkaloids, etc., and was seen to give good results. He went further to claim that lethal secondary metabolites present in botanicals have the tendency to block ion channels, inhibit enzymes, or interfere with neurotransmission. This might have accounted for the varying degree of enzyme detoxification exhibited in this study. The observed activities of the plant volatiles in the study may not be restricted only to its major bioactive chemical components; it could also be due to some minor constituents or a synergistic effect of several constituents. The isolated compounds from the three evaluated plants have been previously implicated for antifeedant, larvicidal, oviposition deterrent, and ovicidal activities in various classes of insects [41-43].

Conclusion

The inhibitory effect of insecticides on enzymatic activities indicated that these enzymes are not responsible for the decontamination of the insecticide and might be accountable for the increase in insect susceptibility to these insecticides. The results from these findings evidently show that stored beetles differ in their response to different enzymatic activities and that the evaluated plant materials may be used as an eco-friendly biopesticide in the IPM strategy for safeguarding stored food grains against stored produced insect pests' infestation.

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