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Full Length Research Paper

Inhibition of glutathione S-transferases (GSTs) activity from cowpea storage bruchid, *Callosobrochus maculatus* Frabiricius by some plant extracts

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Crude ethanolic extracts of *Tithonia diversifolia, Cyperus rotundus* and *Hyptis suavolens* have insecticidal activity against *Callosobrochus maculatus* Frabiricius. The ethanol extracts of the plants have positive results for alkaloids, saponin, tannins and flavonoids. Antioxidant and reducing properties were also determined in the crude ethanol extracts. Cowpea storage bruchid, *C. maculatus* glutathione S-transferases was a potential target for the plants extracts. Gluathione S-transferase from cowpea storage bruchid was purified by affinity gel chromatography of glutathione sepharose. Inhibition effect of the plant extracts on the GST was studied by spectrophotometric method. The binding of the extract was competitive by the Dixon plot with Ki of 84, 132 and 180 ug/mL for *T. diversifolia , C. rotundus* and *H. suavolens,* respectively. We suggest that reported efficacy of the extract is due to the antioxidant properties and competitve binding inhibition on GST may contribute to the pharmacological basis of the efficacy against cowpea storage bruchid, *C. maculatus* Frabiricius and its attendant managements.

Key words: Glutathione S-transferases, cowpea storage bruchids, plant extracts, inhibition.

INTRODUCTION

Cowpea, *Vigna unguiculata* L. (Walp) is an important crop in tropical countries especially in West Africa where it is a cheap source of dietary protein (Labeyire et al., 1981). Cowpea storage bruchid (*Callosobruchus maculatus*) depredates stored cowpea (Jackai and Adalla, 1986). The huge post harvest losses and quality detoriation caused by this insect pest are major problems of assuring food security in developing countries like Nigeria (Lale, 1992). Effective and efficient control of storage insect pests are centred mainly on synthetic insecticides. The use of these synthetic chemical is hampered by many attendant problems: development of resistant insect strains, toxic residues in foods and humans; workers' safety and high cost of procurements (Adedire, 2003). These have necessisated research on the use of alternative eco-friendly insect pest control methods amongst which are the use of plant product. Lale (1992) reported that plant materials and local traditonal methods are much safer than insecticides and suggested that their use needed exploitation.The effects of some plants extracts on the biological parameters of the herbivourous insects (e.g. oviposition, mortality rate, adult emergence, developmental rate,mortality, fecudity and egg viablity) has been reported. Boeke et al. (2001) has undertaken a comprehensive review on the subject. Studies by Adedire and Lajde (1999) and Adedire and Akineye (2004) showed that powder and ethanolic extract of *Tithonia diversifolia, Cyperus rotundus, Hyptis suavolens* and *Aframomum melegueta* have insecticidal activity against cowpea storage bruchid, *C. maculatus* F.

Two enzymatic detoxification systems were found to be involved in the adaptation of some insects to their allelopathic host plants: Myrosinase (β-thioglucosidase, EC 3.2.3.1) and glutathione S-transferases. However glutathione S-transferases play more important role in detoxification (Francis et al, 2005). Glutathione S-transferases (GSTs; EC 2.5.1.18) have attracted attention in insects because of their involvement in the

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defense towards insecticides mainly organophosphates, organochlorines and cyclodienes (Clark et al., 1986; Grant and Matsumura, 1989; Reidy et al., 1990; Fournier et al., 1992). Fakae et al. (2000) has tested *Piliostigma tholnningii, Ocimum gratissium*, *Nauclea latifolia* and *Alstonia boonei* against gastrointestinal helminthes of animals and man. The nematodes glutathione S-transferases are potential drug target and inhibitory properties of these plant extracts against the GST may contributed to the pharmacological basis of their efficacy.

Currently, comprehensive studies on glutathione S-transferases from cowpea storage bruchid (*C. maculatus*) are lacking. This work is aimed at investigating the pharmacological basis of the efficacy of ethanolic extacts of *T. diversifolia* , *C. rotundus*, *H. suavolens* and *A. melegueta* against Cowpea storage bruchid (*C. maculatus*) by spectrophotometric method.

MATERIALS AND METHODS

Glutathione and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were from Sigma Company St. Louis, USA; 1-chloro-2,4- dinitrobenzene was purchased from Aldrich Chemicals, USA. Glutathione-Sepharose was from Armersham Pharmacia, Upsalla, Sweden. Other reagents used were of analytical grade and water used was Milli Q.

Preparation of Plant/Sample preparation

Fresh leaves of *T. diversifolia*, *C. rotundus* and *H. suavolens* were collected from a piece of land at Federal University of Technology, Akure, Nigeria. The leaves were later taken to the Crop Production Department of the Federal University of Technology for botanical identification. The plant extracts were prepared as described by Adedire and Akinneye (2003) with slight modification. Two hundred gram (200 g) of each sample was air dried, pulverized and soaked in the 200 ml of 95% absolute ethanol for 24 h and boiled at 60˚C for 30 min on a heating mantle. The solution was then percolated through Whatman No 1 filter paper and the resulting filtrate was kept in a brown bottle and used as stock solution stored at 4˚C. Solutions of lower concentrations were derived by diluting the stock with ethanol prior to phytochemical screening. Aliquots for GST inhibition studies was centrifuged at 30 000 X g for 15 min at 4˚C. The clear supernatant was frozen dried and stored for 4˚C prior to the toxicity bioassay on GST.

Phytochemical screening

The ethanolic extract was screened for the presence of some secondary metabolites such as saponins, tannins and flavonoids. This was done according to the methods described by Sofowora (1993).

Total phenol

The concentrations of phenolic compounds in the ethanolic extracts, expressed as tannin equivalents, were measured using a modified method of Singleton et al. (1999) with some modifications. Ethanolic extract (0.1 g) was dissolved in 5 ml of acetone for 10 min on ice. To 0.5 ml of the solution, 0.5 ml of distilled water, 0.5 ml of folin's reagent (1:1) and 2.5 ml of 20% sodium carbonate were added. The reaction mixtures were kept in the dark for 40 min, after which the absorbance was read at 750 nm. Phenol contents were extrapolated from standard tannin calibration curve.

Reducing property

The ability of ethanolic extracts to reduce ferric chloride was measured according to a modified method of Pullido et al. (2000). The reducing property was expressed as tocopherol (Vitamin E) equivalent. The ethanol extracts (5 g) was dissolved in 10 ml of water and filtered. To 2.5 ml of the filtrate, 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of potassium ferrocyanide were added. The mixtures were incubated at a temperature 40℃. 10% trichloroacetic acid was added. The resulting mixtures were centrifuged for 10 min. The supernatant (5 ml) was mixed with 5 ml of distilled water and 1.0 ml of 0.1% ferric chloride. The absorbance of the standard and the sample were read at 700 nm against reagent blank made of ethanol instead of ethanolic extract.

Free radical scavenging

The scavenging activity of the Vitamin E and *T. diversifolia,C. rotundu* and*, H. suavolens* ethanolic leaf extract on DPPH radicals were determined according to the method of Chu et al. (2000). An aliquot of 0.5 ml of 0.1 mM DPPH radical in ethanol was added to test tubes containing 1 ml of different concentrations (0 – 5 mg/ml) of the ethanolic extract. The reaction mixture was mixed at room temperature and kept for 20 min. The absorbance was read at 520 nm against distilled water. Radical scavenging capacity of each extract has been calculated as the percent DPPH radical scavenging affect which is:

$$\text{DPPH Scavenging Effect (\%)} = ([A_o - A_i)] / A_o \text{ X } 100$$

Where A_o is the absorbance of the control with ethanol and A_1 is the absorbance of the sample in the presence of the extracts.

Preparation of cytosolic fraction and purification of GST

C. maculatus adults were obtained from naturally infested cowpea seeds Sokoto white cultivar of cowpea from Oba Market in Akure, Nigeria. The whole organism was homogenized in a buffer (35% w/v, 10 mM Tris-HCl, 250 mM sucrose 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol pH 7.4) and centrifuged at 14,000 rpm for 40 min to remove cell debris using Eppendorf cold centrifuge 5810 R. The supernatant was purified on GSH-Sepharose column as described by Kolawole and Ajele (2004). The glutathione removed by ultralfitration by Millipore Amicon ultralfitration and desalting column. The enzyme was stored at -70˚C until used.

Determination of protein concentration and glutathione S-transferases assay

GST activity was determined according to Habig et al. (1974) as modified by Ajele and Afolayan (1992). For a typical assay, the reaction mixture of 3 ml containing a final concentration, 100 mM potassium phosphate buffer pH 6.5 and 1 mM each of Glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB), together with an appropriate amount of enzyme. Three replicates were used for each measure. One unit of GSTs activity is defined as the amount of enzyme producing l mol thioether per min. The extinction coefficient for CDNB conjugate at 340 nm is 0.0096 μM^{-1} cm^{-1} (Habig et al., 1974). The protein concentration was determined by the method of Bradford Method (1976). Two replicates were used for each experiments using bovine serum albumin as a standard. A Shimadzu UV-Visible 1601 double beam digital spectrophotometer was used for the assay. The kinetic analysis of inhibition and inhibition interaction was done by adding known concentrations of plant extracts to the assay buffer prior to assay.

Table 1. Plant used for insecticial activity and their phytochemical components.

Plant	Plant part used	Alkaloids	Saponin	Tannins	Flavonids
Tithonia diversifolia	Leaves	+	+	+	+
Cyperus rotundus	Roots	Trace	+	+	Trace
Hyptis suavolens	Leaves	+	+	+	+

Table 2. Antioxidant activities of the plants.

Plants (mg/ml)	Total phenols (mg/ml)	Reducing properties (mg/ml)
Tithonia diversifolia	0.26 ± 0.01	0.043 ± 0.01
Cyperus rotundus	0.21 ± 0.007	0.040 ± 0.01
Hyptis suavolens	0.15 ± 0.00	0.034 ± 0.02

RESULTS

Phytochemical Screening

The phytochemical screening of *T. diversifolia, H. suavolens* and *C. rotundus* and revealed the presence of alkaloids , saponin and tannins and flavonoids as shown in Table 1.

Antioxidant properties

The antioxidant properties of *T. diversifolia , C. rotundus* and *H. suavolens* as reveals by total phenol content was 0.26 ± 0.01, 0.21 ± 0.007 and 0.15 ± 0.01 mg/ml, respectively as tannin equivqlents (Table 2). *T. diversifolia, C. rotundus* and *H. suavolens* have reducing properties level of 0.043, 0.040 and 0.034 mg/ml, respectively. DPPH (1, 1–diphenyl–2-Picrylhydrazyl) percent scavenging activities of the plants were measured in different concentration ranging from 0 to 5 mg/ml. 5 mg/ml of *T. diversifolia* showed 60% inhibtion; *C. rotundus,* 53% and *H. suavolens,* 44% inhibition as shown in Figure 1.

Effect of the plant extracts on *C. maculatus* GST

The glutathione S-transferases from cowpea storage bruchids (*C.maculatus*) was rapidly purified by affinity gel of glutathione-agarose. The interested plant extracts were screened for their effect on GST activity apart from their antioxidant properties. The data were obtained in the experiment to determine the effect of *T. diversifolia, C. rotundus* and *H. suavolens* ethanolic extracts on glutathione S-transferases from cowpea storage bruchids (*C. maculatus*) and to establish the type of inhibition mechanism. The straight line graph result of a series of reciprocal of GST activity against inhibition concentration of all ethanolic plant extracts was observed. The series of line converged at a point away from the origin. The Ki values are 84, 132 and 180 µg/mL, respectively for *T. diversifolia , C. rotundus* and *H. suavolens* (Figures 2, 3 and 4)

DISCUSSION

The use of plant products to protect stored cowpea, *V. unguiculata* L. (Walp) against cowpea storage bruchids (*C. maculatus*) predation is an age long practice in Nigeria (Lale, 1992). The ethanolic extract of *T. diversifolia , C. rotundus* and *H. suavolens* has been reported to have potent insecticidal activity against cowpea storage bruchid, *C. maculatus* F. These plants have different degree of insecticidal potentials on the insect (Adedire and Akinneye, 2003; Adedire and Lajide, 1999)

In this present investigation our aim was to collect information on the possible pharmacological basis for the efficacy of these plant extracts on the effective management of cowpea storage bruchids (*C. maculatus*). The plant extracts were screened for the bioactive components that could be responsible for the efficacy. Our result shows that alkaloids, saponin and tannins and flavonoids are some of the major contituents of the plants extracts. The presence of these secondary metabolites might not be unconnected to their insecticidal activities. These plant extracts also have high content of polyphenols and antioxidant activity. Phenolic constituents have been studied extensively as important contributors to the antioxidant activity in plants (Aruoma, 2003; Skerget et al., 2005).There are reports in the literature which correlate the total phenolics content of a plant extract with its antioxidant activity (Coruh et al*.,* 2007a; Skerget et al*.,* 2005) and this was also the case in the present study, as shown in Table 1. The extracts with high phenolics content, also has high DPPH radical scavenging.

Our preliminary investigation shows that all the plant extract used in this study bring about the inhibition of crude GST extract from the cowpea storage bruchids (*C. maculatus*). We therefore investigated the nature of the inhibition on the purified sample of the GST. From the

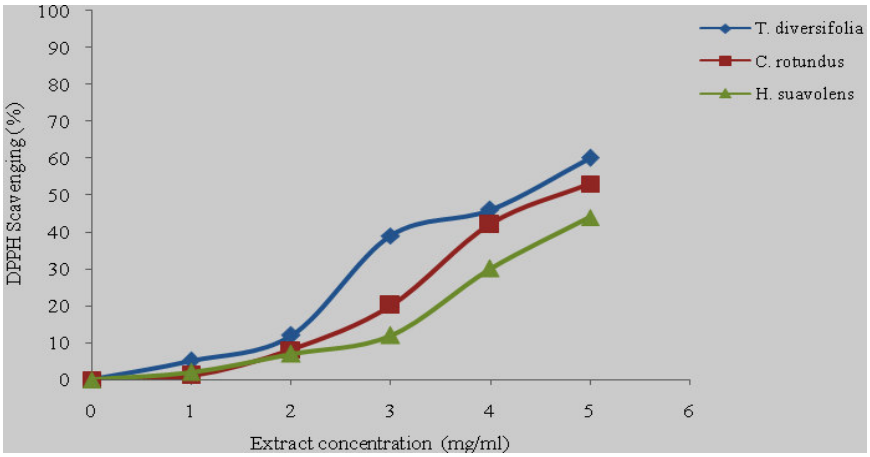

Figure 1. DPPH scavenging activities abilities of the plant extracts.

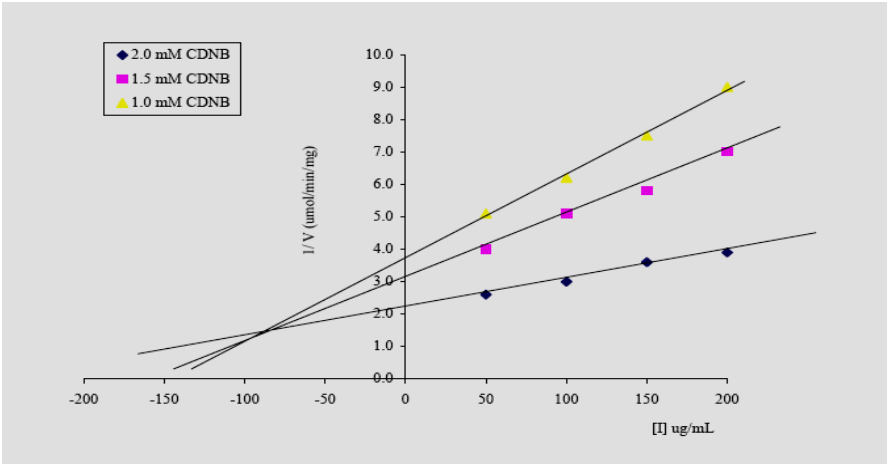

Figure 2. Dixon Plot of the reciprocal of GST activity (1/V) versus *Tithonia diversifolia* concentration utilizing 1.0 mM, 1.5 mM and 2.0 mM CDNB comcentrations. Lines of best fit were determined by least square linear regression analysis.

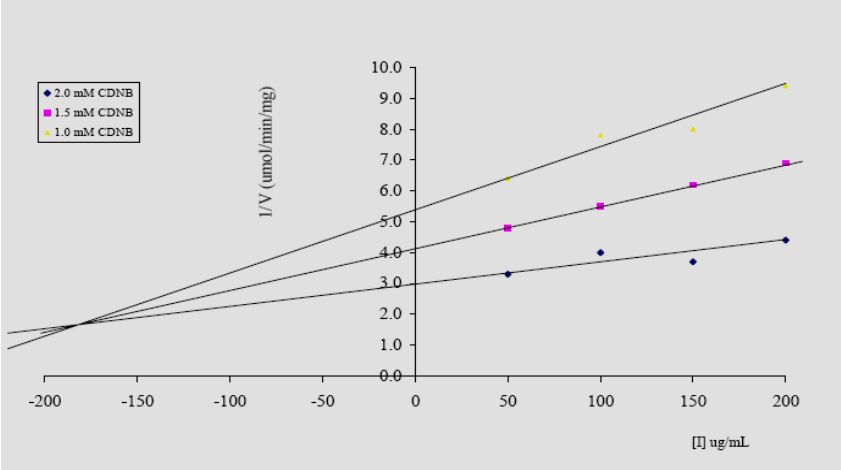

Figure 3. Dixon Plot of the reciprocal of GST activity (1/V) versus *Hyptis suavolens* concentration utilizing 1.0 mM, 1.5 mM and 2.0 mM CDNB comcentrations. Lines of best fit were determined by least square linear regression analysis.

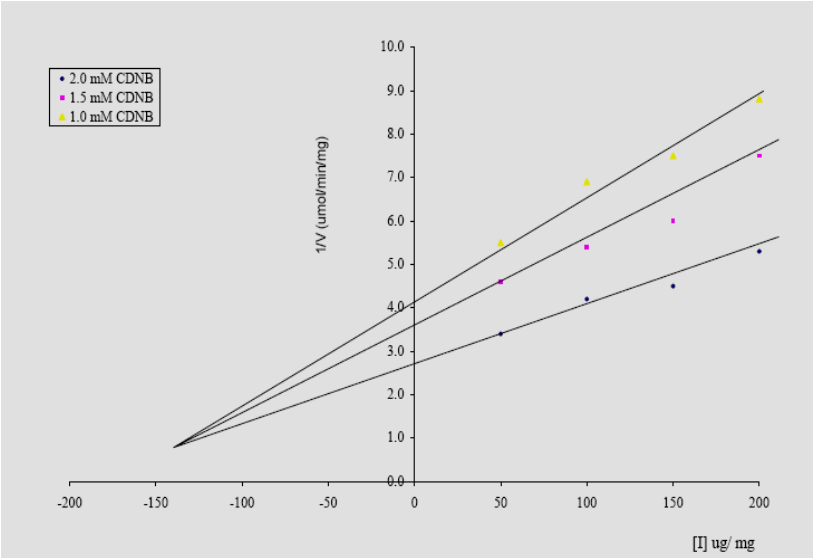

Figure 4. Dixon Plot of the reciprocal of GST activity (1/V) versus *Cyperus rotundus* concentration utilizing 1.0 mM, 1.5 mM and 2.0 mM CDNB comcentrations. Lines of best fit were determined by least square linear regression analysis.

results, the inhibition was found to be competitive as showed by the Dixon plot. This suggests that the extract binds to the active site of the enzyme and therefore prevent detoxification role of the enzyme. The enzyme has been reported to be involved in the adaptation of insects to allelochemicals and insecticides (Francis et al., 2005). Sigma class of GST is implicated to as protectors against oxidative stress in insects (Enayati et al., 2005). The observation of the *in vitro* inhibition of cowpea storage bruchids (*C. maculatus*) glutathione S-transferases by the extracts of *T. diversifolia, C. rotundus* and *H. suavolens* is in good agreement with the earlier report of Fakae et al. (2000) that the GST of parastic nematodes was inhibited by some Nigerian medicinal plants. This is also supported by the reports of Coruh et al. (2007a,b) *whose* work on *Gundelia tournefortii*, *Prangos ferulacea* (L.) Lindl.*, Chaerophyllum macropodum* Boiss. and *Heracleum persicum* Desf. extracts showed great inhibition on glutathione-S-transferase activity. The inhibitory effects of naturally occurring plant polyphenols such as tannic acid, ellagic acid, ferulic acid, caffeic acid, stilbene, quercetin, curcumin and chlorogenic acid against GST have long been reported by many researchers (Kawabata et al., 2000; Gyamfi *et a*l., 2004). Quinines are also well-known examples of covalent inhibitors of GST enzymes (Zanden et al., 2004).

From our results it appears that the extracts with high polyphenols and reducing properties have effective inhibition on the GST from the cowpea storage bruchids (*C. maculatus*). This suggests that the phytochemicals can exert insecticidal role by antioxidant activity. Francis et al. (2005) reported that glucosinolates and isothiocynates from Brassicaceae plants are easily detoxified by GST from *Myzus persicae* aphid and this is responsible for its adaptation. However, Fakae et al. (2000) suggest the combinatorial approach of some active allelochemicals in the plant extracts on the effective inhibition of GST. If this posture holds, the combinatorial approach prevents either modification of the target site or to amplified production of this detoxification enzyme (Haubruge and Amichot, 1995).

Spectrophotometric methods have succeeded in answering the question of the pharmacological basis of the insecticidal activity and the type of inhibition on the GST, a dimeric enzyme. The question still remains if the binding of this extracts brings about the cooperative binding of the extracts on the other dimmers. This merits our further investigation.

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