REVIEW

In vitro and *in silico* evaluation of phytochemicals from water lettuce (Pistia stratiotes L.) as glutathione S-transferase (GST) inhibitors in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith)

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ABSTRACT

Plant-derived natural products are widely recognized as valuable resources for pharmaceutical and crop-protection development. This study evaluated the inhibitory effects of phytochemicals from water lettuce (*Pistia stratiotes* L.) on glutathione S-transferase (GST). GST is a detoxification enzyme associated with insecticide resistance in pests such as the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (SfGST). Methanol extracts of *P. stratiotes* were evaluated using a GST inhibition assay, followed by literature-based selection of known compounds from *P. stratiotes*. Homology modeling, structural validation, and molecular docking of 11 compounds were conducted to evaluate binding interactions with SfGST. The in vitro assay showed that the crude methanol extract exhibited notable GST inhibitory activity, with an IC_{50} of 93.30 µg/mL, suggesting potential as a natural inhibitor. *In silico* results indicated that all 11 compounds exhibited more negative binding affinities than glutathione, with vicenin-2 showing the highest affinity (–9.0 kcal/mol). Molecular dynamics simulations indicated that vicenin-2 acts as a non-covalent inhibitor, inducing moderate structural perturbations and localized flexibility in SfGST. These changes were evident in altered root-mean-square-deviation (RMSD), solvent-accessible surface area (SASA), radius of gyration (Rg), and root-mean-square fluctuation (RMSF) profiles of the enzyme-ligand complex over the 50-ns trajectory. The *in silico* findings corroborated the *in vitro* GST inhibitory activity, supporting that enzyme inhibition may be attributed to these phytochemicals. These results highlight the potential of phytochemicals from *P. stratiotes* as candidates for environmentally sustainable pest management. Field-based evaluations are recommended to confirm the laboratory findings and to explore the applicability of these phytochemicals within integrated pest management (IPM) programs.

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INTRODUCTION

The detection of fall armyworm (FAW), *Spodoptera frugiperda*, in the Philippines was reported by Navasero et al. in 2019. FAW incidences were recorded in 17 municipalities across 10 provinces. Since then, it has spread to other areas and provinces, causing massive damage to corn. The authors reported a noticeable preference of FAW for the earlier developmental stages of corn, although the pest was still capable of infesting all stages. Although the preferred management option for FAW infestation is the application of integrated pest management (IPM) (Day et al. 2017), the use of insecticides remains the major practice of farmers for control. However, continued excessive use and incorrect application of insecticides may accelerate the development of resistance. Chen et al. (2023) reported a decrease in susceptibility of FAW to chlorantraniliprole from Sichuan province in China, indicating resistance development. Moreover, Thirawut et al. (2023) reported the increase in the lethal concentrations for 50% mortality (LC_{50}) of FAW to emamectin benzoate, indoxacarb, and chlorfenapyr for samples collected in 2021 and 2022 compared with those collected in 2019.

One of the major mechanisms underlying insecticide resistance is the elevated activity of detoxifying enzymes such as glutathione S-transferases (GSTs), which function in phase II metabolism by catalyzing conjugation reactions that convert xenobiotics into more water-soluble, less toxic forms (Brooke et al. 2001). GSTs are

KEYWORDS

Pistia stratiotes, fall armyworm, glutathione S-transferase, molecular docking, vicenin-2

involved in the development of resistance to a wide range of exogenous xenobiotics, including chemotherapeutic medicines, chemical carcinogens, herbicides, and insecticides (Gawande and Khambalkar 2014). Diamondback moth (*Plutella xylostella*) and housefly (*Musca domestica*) were both reported to have an increase in GST activity that influences their resistance to pesticides (Huang et al. 1998; Enayati et al. 2005). The GST activity of the mulberry pest (*Glyphodes pyloalis* Walker) significantly increased after treatment with the insecticides phoxim and chlorfenapyr; however, larval mortality rates exposed to chlorfenapyr increased when GST was silenced (Liu Z. et al. 2022).

The use of synergists is considered one of the strategies to combat resistance development in insect pests. Synergists are chemicals that inhibit detoxifying enzymes, including GST and cytochrome P450 monooxygenases, preventing the rapid degradation of insecticides and leading to prolonged exposure and enhanced efficacy. Among the well-known insecticide synergists is piperonyl butoxide (PBO), a known inhibitor of P450 monooxygenase in several pyrethroid formulations (Romero et al. 2009). Other synergists include S,S,S-tributyl phosphorotrithioate (DEF), an inhibitor of esterase (López-Soler et al. 2011), and diethyl maleate (DEM), a known inhibitor of GST (Wang J. et al. 2013). However, the negative effects on human health and the environment raised concerns. Thus, alternative enzyme inhibitors from plant sources with low toxicities have been explored.

Plant-derived natural products have long been recognized as valuable resources for drug and pesticide development. Taxifolin and (+)-lariciresinol 9'-p-coumarate, compounds found in conifers, exhibited inhibitory activities against the GST of the Colorado potato beetle, with the former demonstrating greater activity than DEM (Wang et al. 2014). Although often seen as unwanted in agriculture, numerous weed species also hold great potential for use in pharmaceuticals, pest management, and medicine. Crude ethanolic extracts of weeds such as *Tithonia diversifolia*, *Cyperus rotundus*, and *Hyptis suaveolens* displayed insecticidal activity against cowpea weevil (Kolawole et al. 2009). Root extracts of *Cyperus rotundus* were found to be effective against *Aphis craccivora* Koch and *Planococcus lilacinus* (Cockerell), with cyperotundone, one of the identified chemicals in the extract, showing significant inhibition of acetylcholinesterase and GST in the target insects (Singh et al. 2024). In this context, the aquatic plant *P. stratiotes* holds substantial potential as a source of bioactive compounds. Research has shown that the *P. stratiotes* contains various secondary metabolites, including flavonoids and polyphenols, which are known for their wide range of biological activities (Sudirman et al. 2017). Previous studies indicate that *P. stratiotes* extracts possess diverse bioactivities, including insecticidal effects against *Anopheles* larvae (Ma et al., 2019) and dose-dependent anthelmintic activity in earthworms (Karim et al., 2015). In addition, these extracts exhibit allelopathic effects on seedling germination (Bich & Kato-Noguchi, 2012), anti-algal activity through the inhibition of *Microcystis aeruginosa* growth (Wu et al., 2013), as well as anti-inflammatory and analgesic effects in Swiss albino mice (Hussain et al., 2018). Although very few studies focused on the use of *P. stratiotes* in pest management, it has potential use in crop protection and sustainable agriculture.

In this study, the crude methanol extract of *P. stratiotes* was evaluated for its potential inhibitory effects against the GST of the fall armyworm using an *in vitro* enzyme assay. After confirming inhibitory activity, phytochemicals previously reported from *P. stratiotes* were computationally assessed through docking and molecular dynamics analyses. The findings will serve as preliminary results, providing a foundation for future laboratory and field validation. The results could guide the development of eco-friendly plant protection products and inform future research on insecticide and synergist formulations.

MATERIALS AND METHODS

Crude Plant Extract Preparation and SfGST Extraction

The leaves of *P. stratiotes* were collected at Masaya, Bay, Laguna, Philippines (14°08'56.7"N 121°16'45.1"E). The plant specimen was authenticated at the Plant Health Clinic of the National Crop Protection Center, where voucher samples are maintained for reference. The leaves were oven-dried at 40 °C for 72 h and subsequently pulverized using a heavy-duty laboratory grinder. The powdered leaves were soaked in AR-grade methanol (1:5 w/v) for 48–72 hours, and the resulting extract was concentrated using a rotary evaporator to obtain the crude extract. A stock solution of the crude extract (1000 µg/mL) was prepared in methanol and subsequently diluted to obtain working concentrations of 10, 20, 50, 70, 100, and 120 µg/mL.

Meanwhile, mature adult fall armyworm (FAW) were collected from corn fields near the UPLB Central Experiment Station (14°09′58.8″N, 121°15′13.7″E) and reared under laboratory conditions at room temperature, where males and females were allowed to mate, and the resulting eggs were collected and transferred to separate containers. The resulting larvae were fed an artificial diet (Southland Products, Arkansas, USA) until maturity. Eggs produced from laboratory-reared adults were incubated, and the resulting larvae were reared under controlled conditions. Emerging larvae were fed until the third instar stage. Once larvae reached the fourth instar, larvae were collected for GST enzyme extraction.

Around 100 fourth-instar larvae were gathered and preserved at –20 °C. Larvae were thawed on ice and dissected using a sterile scalpel under a stereomicroscope. The abdominal segments were opened to isolate the midgut and abdomen, while discarding the cuticle and head capsule. The dissected abdomens and midguts were pooled and homogenized (1:5 w/v) in ice-cold 0.1 M sodium phosphate buffer (pH 6.5) and placed in conical tubes. The homogenate was then centrifuged at 10,000 rpm for 15 minutes at 4 °C. The supernatant, containing the crude enzyme extract, was carefully collected and stored at –20 °C for later use.

In vitro GST inhibition bioassay

To determine the potential inhibition of the crude extract of water lettuce, *P. stratiotes*, against the crude SfGST, an assay was conducted using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich) following the method of Wang et al. (2014) with slight modifications. Each well of a 96-microplate contained 220 µL of reaction mixture, and four wells were used per extract concentration. The reaction mixture consisted of 194 µL of 0.1M sodium phosphate buffer (pH 6.5), 6 µL CDNB, 6 µL crude plant extract (inhibitor), 6 µL crude SfGST incubated for 30 mins at 30 °C, and 8 µL of 100 mM lyophilized GSH (Sigma-Aldrich) to start the reaction. For the negative control, wells contained the complete reaction mixture with crude SfGST but without the plant crude extract. The reaction was monitored at 340 nm. The percent inhibition of crude extracts of *P. stratiotes* against the crude SfGST (% GST Inhibition) was calculated using Equation 1:

$$\%\ \text{GST Inhibition} = \frac{A_{control} - A_{extract}}{A_{control}} \times 100\% \quad (1)$$

where $A_{control}$ represents the absorbance of wells containing the complete reaction mixture with active enzyme and extract diluent (negative control), and $A_{extract}$ represents the absorbance of wells containing the crude enzyme and the test plant extract. Wells lacking CDNB were used as blanks to correct background absorbance. The IC_{50} value was determined by linear interpolation of % GST inhibition versus crude extract concentration.

Computational Simulations
Computational simulations were performed on a dual-boot laptop running Windows 10 Home and Ubuntu 20.04, equipped with an AMD Ryzen 7 processor and 16 GB RAM.

Protein Preparation
Since no crystal structure of *Spodoptera frugiperda* (J.E. Smith) glutathione S-transferase (SfGST) is available, its amino acid sequence was obtained from the UniProt database (A0A5Q0TYT2, https://www.uniprot.org/) and used to generate a predicted three-dimensional structure via the AlphaFold Protein Structure Database (Jumper et al., 2021). The model quality was assessed using the structure assessment tool of the SWISS-MODEL website (https://swissmodel.expasy.org/) using a reference GST from *Bombyx mori* (PDB: 3AY8). Before performing molecular docking, polar hydrogens were incorporated into the protein structure. The pH of the system was set to 7.0 to mimic the physiological conditions in the insect's body using the ProteinPrepare application of the PlayMolecule Viewer Webserver (Harrison, 2001; Torrens-Fontanals, 2024).

Ligand mining and preparation
A keyword search on Google Scholar (https://scholar.google.com/) was conducted to gather journal articles on phytochemical studies of *P. stratiotes.* The articles were screened, and phytochemicals identified from the plant were summarized in Table 1. Only journal articles indexed in either Scopus or Web of Science (WOS) were considered. The 3D conformers of the identified phytochemicals were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and imported into PyRx 0.8 (https://openbabel.org/) using the OpenBabel package (O'Boyle et al., 2011). Each ligand was then energy-minimized using a 1000-step conjugate gradient method with the Universal Force Field (UFF) to generate PDBQT files.

Table 1: List of phytochemicals identified from *P. stratiotes*

Phytochemicals	Reference
Kiwiionoside	Greca et al. 1996
Vicenin-2 Vitexin Orientin Lucenin-2 Kuromanin	Zennie et al. 1977
Luteolin Beta sitosterol Daucosterol 7beta-hydroxysitosterol Chrysoeriol	Liu et al. 2008

Molecular Docking
Molecular docking was conducted using AutoDock Vina v1.1.2 (Trott & Olson, 2010). The docking calculations employed an iterated local search combined with gradient local optimization approach, with the protein kept rigid and the ligands treated as flexible. Prior to molecular docking, the binding site of SfGST was predicted using CavityPlus webserver (http://www.pkumdl.cn/cavityplus) (Yuan et al., 2013). The grid box was centered at 10.53, -2.69, 3.89 Å with the dimensions 17.33, 20.66, 19.32 to fully enclose the binding site of SfGST based on conserved catalytic residues mentioned in the literature. The conformation with the lowest binding energy among the nine output modes per ligand was selected as the best. The molecular docking results were analyzed and visualized using BIOVIA Discovery Studio version 21.1.0.20298 (BIOVIA Dassault Systèmes, 2021).

Molecular Dynamics Simulation
The best model in complex with the top ligand was subjected to 50-ns molecular dynamics simulation using the Desmond 2020.1 module. Molecular dynamics simulations were conducted employing the OPLS_2005 force field and the SPC water model within an orthorhombic periodic boundary box. Systems were neutralized with Na^+/Cl^- counterions and simulated under the NPT ensemble. Trajectories were recorded every 50 ps, yielding 1000 frames (Castrosanto et al., 2022; Chow et al., 2008). A similar method was done to SfGST only to serve as a control. Analysis of protein-ligand stability was done by comparing the root-mean-square deviation (RMSD) of the complex with that of the protein only. The root-mean-square-fluctuation (RMSF) of the protein in the complex was also calculated, as well as the ligand interaction throughout the simulation time. The number of hydrogen bonds, Rg, and SASA were also determined. Lastly, total energy was obtained from molecular dynamics post-processing, which includes the Coulombic, van der Waals, bond, angle, and torsional contributions of the protein–ligand system.

RESULTS AND DISCUSSION

***In vitro* GST inhibition assay**
Fall armyworm (FAW) is a globally significant insect pest that causes extensive agricultural damage across continents. Its high reproductive rate, extensive migratory behavior, remarkable dispersal capacity, and robust flight ability (enabling it to fly up to 500 km before oviposition) contribute significantly to its economic impact. Chemical control through insecticides remains the primary management strategy for FAW. However, continuous misuse and overapplication has accelerated resistance development (Whalon et al. 2012). FAW is one of the most insecticide-resistant pest species (Sparks et al. 2020), with resistance mechanisms primarily involving increased activity of detoxification enzymes and reduced target site sensitivity (Liu J. et al. 2022). Specifically, enhanced activity of glutathione S-transferases facilitates the degradation of insecticides like fluxametamide (Roy et al. 2023), while overexpression of GSTs contributes to resistance to organophosphates, carbamates, and pyrethroids (Liu J. et al. 2022). This study explored the potential of phytochemicals to inhibit *Spodoptera frugiperda* (J.E. Smith) GST. *P. stratiotes* was selected as the candidate plant material due to its abundance, potential insecticidal, allelopathic, and antimicrobial properties, and lack of economic significance.

To determine the potential inhibitory effects of the crude methanol extract of *P. stratiotes* against the crude GST from FAW, an assay was carried out with 1-chloro-2,4-dinitrobenzene (CDNB) serving as the reagent (Figure 1). Methanol was chosen as the extraction solvent because it can solubilize a broad spectrum of compounds, encompassing both polar and non-polar molecules. According to Truong et al. (2019), methanol is recommended as an ideal solvent for extracting high levels of phytochemical constituents and antioxidant compounds in *Severinia buxifolia*.

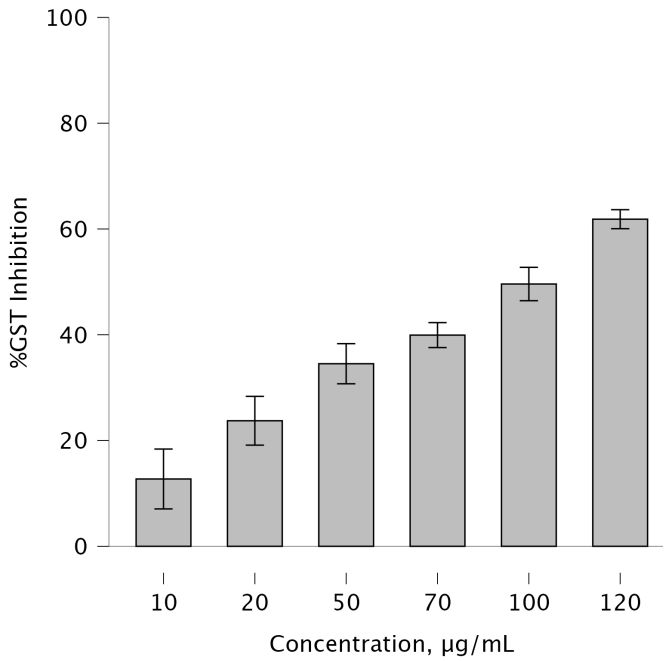

Figure 1: *In vitro* GST inhibition of crude methanol extracts of water lettuce (*P. stratiotes*). Values are mean ± SE for four determinations (n = 4).

The GST inhibitory assay demonstrated a clear dose-dependent response within the tested concentration range (10–120 µg/mL), with higher extract concentrations producing stronger enzyme inhibition. The inhibition values ranged from 12.73% to 62.66% (Figure 1), with variability indicated by the error bars, which may reflect biological or technical variation, such as differences in enzyme activity, pipetting, or extract composition. One-way ANOVA confirmed significant differences among treatments ($F(5,18) = 22.41$, $p < 0.001$). Regression analysis indicated a strong linear relationship between concentration and inhibition ($R^2 = 0.97$), supporting the dose-dependent effect. The calculated half-maximal inhibitory concentration (IC_{50}) was 93.30 µg/mL, indicating moderate inhibition compared to purified phytochemicals, but notable for a crude plant extract. It is worth noting, however, that no positive control was included in the assay, which limits direct comparison of the extract's potency with established GST inhibitors. Therefore, further confirmatory studies, ideally incorporating known GST inhibitors, are recommended to validate these findings. In the study by Wang et al. (2014), phytochemicals from conifer extracts exhibited inhibitory effects on GST isolated from the Colorado potato beetle. The calculated IC_{50} of *Pinus banksiana* cone extract was 10 µg/mL, which was considerably lower than the IC_{50} value obtained in our study. In addition, Fakae et al. (2000) reported the inhibitory potential of Nigerian medicinal plants against GSTs from nematodes, with IC_{50} values ranging from 2-28 µg/mL.

Protein Preparation

Results from the *in vitro* GST inhibition assay indicate that compounds present in the extract of *P. stratiotes* have the potential to inhibit SfGST activity. To further investigate this potential, molecular modeling and docking studies were performed on the phytochemicals of *P. stratiotes*. As no crystal structure of SfGST was available in the Protein Data Bank, its three-dimensional structure was predicted using AlphaFold Protein Structure Database with an average pLDDT of 97.44% (very high) (Jumper et al., 2021). Sequence analysis revealed that *Bombyx mori* GST shares 76.89% sequence identity with SfGST, which supported the generation and validation of a reliable homology model (Kakuta et al., 2011). While SfGST naturally exists as a homodimer, a single monomeric chain was used in the simulations to reduce computational cost. Within the monomer, ligands were docked specifically to the H-site (hydrophobic substrate-binding pocket), as this region is responsible for interactions with xenobiotics and inhibitors (Ebihara and Niwa, 2023). The resulting model demonstrated high quality, with a MolProbity score of 0.70, a clash score of 0.59, 98.58% of residues in favored Ramachandran regions, and no outliers (Waterhouse et al., 2024). Additional structure refinement was not performed since the AlphaFold DB already provided a high-quality structure. Figure 2A shows the AlphaFold-predicted tertiary structure of SfGST, highlighting the predicted binding pocket and the secondary structure distribution. The predicted binding site includes residues Ser11, Leu35, His52, Glu66, Ser67, and Leu118—residues consistent with those identified in the GST of *Ostrinia furnacalis* and *Bombyx mori* (Castrosanto et al., 2022; Kakuta et al., 2011). The Ramachandran plot and secondary structure assignment (Figure 2B), along with other validation metrics and the identification of known catalytic residues, provide strong evidence for the reliability of the model. These validations not only confirm its structural soundness but also highlight its functional relevance, making it suitable for structure-based virtual screening and docking analyses. The presence of well-defined alpha-helices and beta-sheets (Figure 2C) suggests proper protein folding, which is critical for accurate docking and interaction analysis.

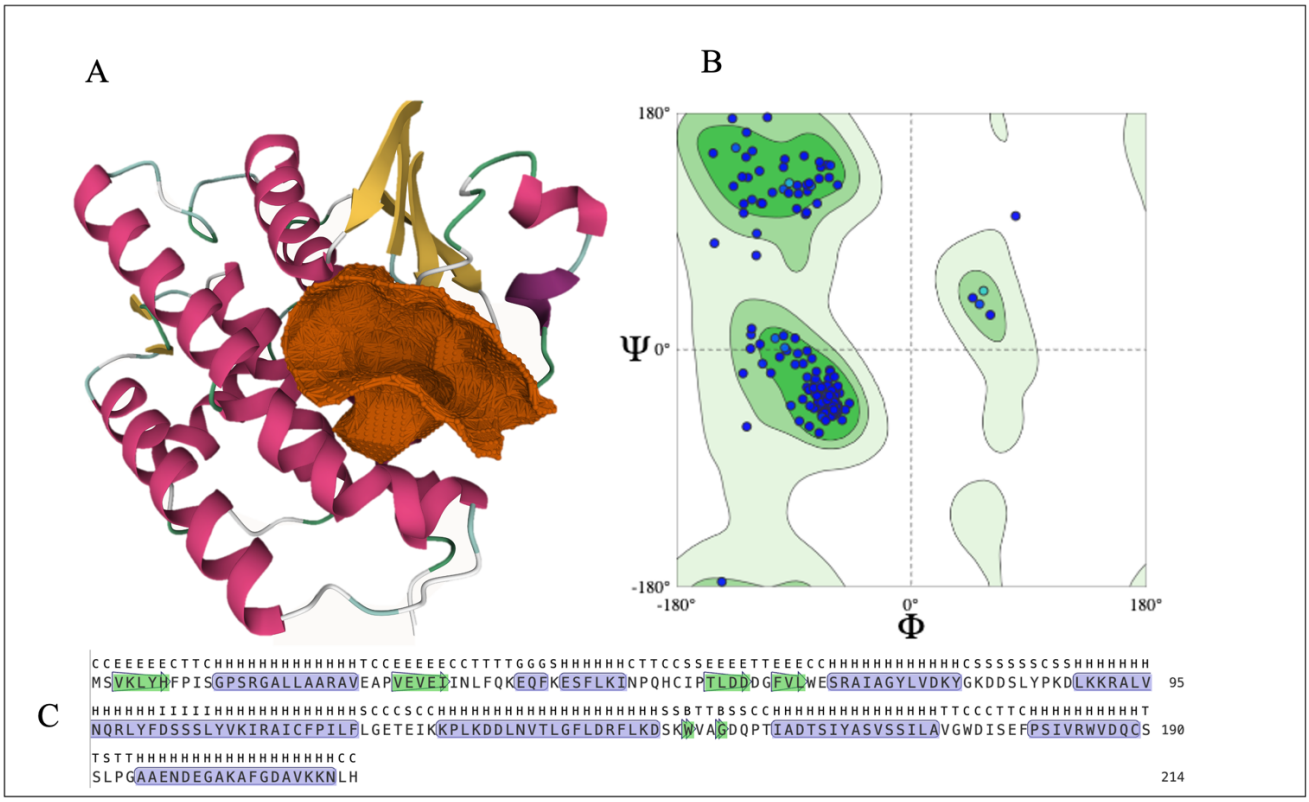

Figure 2: (**A**) Alphafold-predicted tertiary structure of *Spodoptera frugiperda* (J.E. Smith) glutathione S-transferase (SfGST) along with the predicted binding site. (**B**) Ramachandran plot of the predicted tertiary structure of SfGST and (**C**) the amino acid sequence of SfGST showing specific secondary structure – green for beta sheets and purple for alpha helixes

Molecular Docking

This validated model was subsequently used to dock phytochemicals identified from *P. stratiotes*, leading to the prediction of binding poses within the active site. Table 2 presents the binding affinities of the ligands, with glutathione, the natural substrate of GST, as the control. All docked phytochemicals exhibited stronger binding, as indicated by more negative binding affinities, suggesting their potential to interact favorably with the active site. This is consistent with possible inhibitory activity and warrants further biochemical validation. Vicenin-2 ranked first with a binding affinity of -9.0 kcal/mol, followed closely by lucenin-2 (-8.9 kcal/mol) and several sterol compounds such as 7β-hydroxysitosterol, β-sitosterol, and daucosterol (all -8.7 kcal/mol).

Table 2: Binding affinity of compounds from *P. stratiotes* on SfGST with glutathione serving as the control*.

Ligand	Binding affinity (kcal/mol)
Vicenin-2	-9.0
Lucenin-2	-8.9
7β-hydroxysitosterol	-8.7
β-sitosterol	-8.7
Daucosterol	-8.7
Vitexin	-8.2
kuromanin	-8.1
Orientin	-8.1
Chrysoeriol	-7.9
Luteolin	-7.7
Kiwiionoside	-7.2
Glutathione*	-5.4

Vicenin-2 is a naturally occurring C-glycosylated flavone. It is also referred to as apigenin-6,8-di-C-glucoside due to the two glucose units attached via carbon-carbon bonds to the apigenin backbone (Peter et al., 2015). It is relatively stable and resistant to enzymatic hydrolysis, mainly due to C-glycosylation compared to O-glycosylated flavones. Based on the literature, vicenin-2 shows promising potential as a GST inhibitor, as it was reported to modulate the mercapturic acid pathway, a GST-dependent detoxification route (Singhal et al., 2017). The authors reported that vicenin-2 not only inhibited cancer cell growth but also enhanced the efficacy of docetaxel, a standard chemotherapy drug. The synergistic effect was attributed to vicenin-2's ability to interfere with detoxification mechanisms, including the mercapturic acid pathway, which relies on GST activity for conjugation and removal of genotoxic compounds. These findings are based on mammalian models, where vicenin-2 modulates GST activity. However, this mechanism may be relevant across taxa, including insects, due to conserved catalytic residues. This finding aligns well with the objective of this study—to propose a potential insecticide synergist that specifically inhibits the insect's GST, thereby impairing its ability to metabolize the target insecticide. Lucenin-2, on the other hand, is also classified as a C-glycosyl flavone, but with a luteolin backbone instead of apigenin. Although direct studies on its GST inhibitory activity are limited, its structural similarity to vicenin-2 and other flavonoids known to inhibit GST suggests potential relevance (Boušová & Skálová, 2012).

Figure 3 illustrates the binding interactions of vicenin-2 and lucenin-2 within the predicted active site of SfGST. Notably, both ligands formed hydrogen bonds with Glu66, a residue documented to be directly involved in the catalytic activity of GST (Kakuta et al., 2011). Glu66 in insect GSTs is highly conserved as it helps stabilize and orient glutathione properly. The interaction of ligands with Glu66 supports the inhibitory potential of *P. stratiotes* extract, indicating that the phytochemicals may disrupt enzymatic function. In addition, vicenin-2 exhibited unique non-covalent interactions not observed with lucenin-2, including a π–π stacking/T-shaped interaction with Phe119 and a π–cation interaction with Leu35. These interactions likely contribute to the slightly higher binding affinity of vicenin-2 and may enhance its stability within the active site, further supporting its potential as a more effective GST inhibitor.

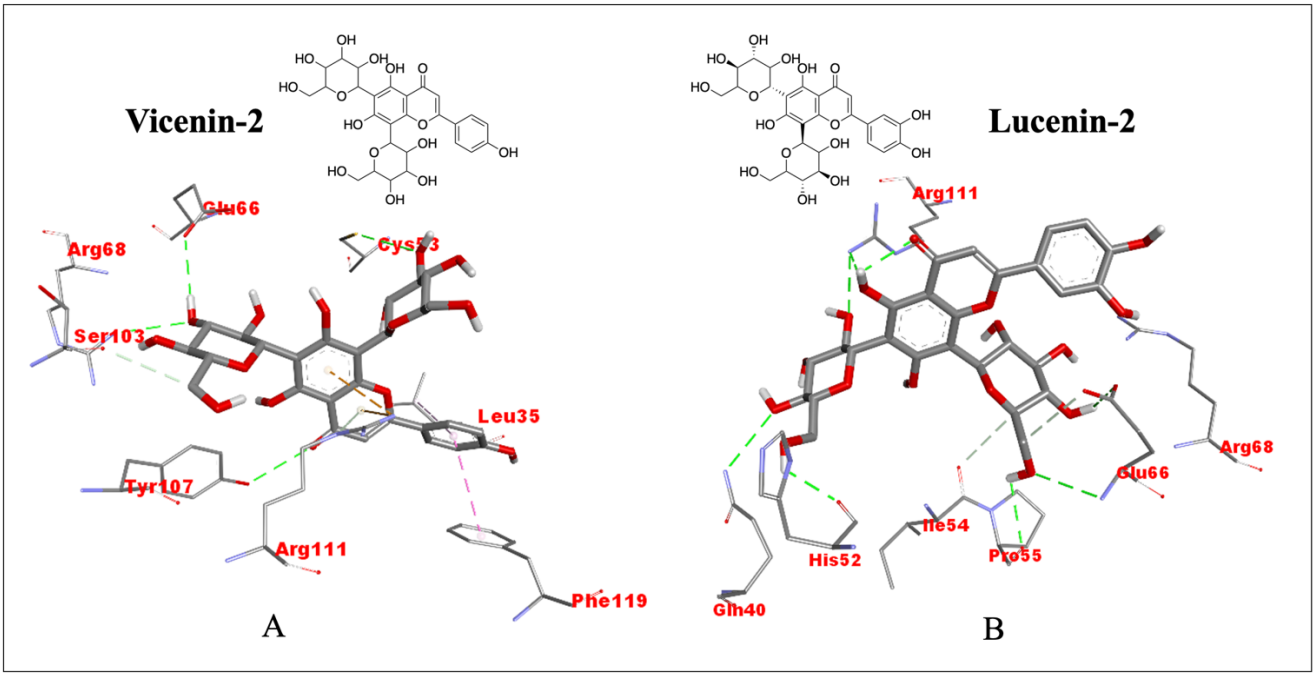

Figure 3: Molecular docking-predicted interactions between SfGST and the ligands (**A**) vicenin-2 and **(B**) lucenin-2. Hydrogen bonds are depicted by green dashed lines, π–alkyl or π–π stacking/T-shaped interactions by pink dashed lines, and π–cation interactions by yellow dashed lines.

Numerous studies highlight the GST inhibitory and insecticidal properties of phytochemicals, particularly phytosterols and flavonoids. Several sterols have been identified as potent insect GST inhibitors; for instance, compounds from *Anamirta cocculus* (stigmasterol, lupeol, gamma-sitosterol) have shown high binding affinities to GST in the Asian corn borer, highlighting their potential as GST inhibitors (Castrosanto et al. 2022). Mhalla et al. (2018) reported that the extracts of *Rumex tingitanus* were rich in phytosterols such as beta and gamma sitosterol, which they found to have a significant synergistic effect with *Bacillus thuringiensis* δ-endotoxin against *Spodoptera littoralis*.

For flavonoids, quercetin was shown to inhibit GST activity in two moth species, *Micromelalopha troglodyta* (Graeser) and *Clostera anachoreta* (Fabricius) (Tang et al. 2014), while daidzein and desmethylglycitein were identified as GST inhibitors in *Aedes aegypti* (Inaba et al. 2022). More recently, catechin was reported to effectively inhibit GST activity in *Spodoptera litura* (Ruttanaphan et al. 2023). Flavonoids, with their planar aromatic backbones and multiple hydroxyl groups, are likely to establish hydrogen bonds and π–π stacking interactions (Katalinić et al. 2010).

Molecular Dynamics Simulation

The 50-ns molecular dynamics simulations and binding interaction analyses support the hypothesis that vicenin-2 acts as a non-covalent inhibitor of SfGST. As illustrated in Figure 4, the RMSD, Rg, SASA, and RMSF profiles indicate that vicenin-2 binding induces structural perturbations and localized flexibility, particularly in regions associated with catalysis and substrate binding. The post-MD simulation analyses suggest that vicenin-2 maintains a stable interaction with SfGST, with minimal perturbation to the protein's overall structure. The average RMSD of the ligand-bound complex (1.68 ± 0.16 Å) was slightly higher than that of the unbound protein (1.54 ± 0.31 Å), indicating modest conformational adjustments likely associated with ligand accommodation. Importantly, the radius of gyration (Rg) remained comparable between the complex (17.61 ± 0.12 Å) and the apo form (17.41 ± 0.08 Å), suggesting that the compactness of the protein structure was preserved upon binding. Similarly, the solvent-accessible surface area (SASA) values for the complex (1.10 ± 0.02 μm²) and the unbound protein (1.12 ± 0.02 μm²) showed a negligible difference, implying that the protein's surface exposure to solvent remained largely unchanged. These results collectively indicate that vicenin-2 binds in a stable manner without inducing significant structural destabilization. Such behavior supports the potential of vicenin-2 as a candidate inhibitor, warranting further investigation into its functional impact on SfGST activity. The interaction energy remained stable throughout the simulation, primarily driven by van der Waals interactions and maintained by a consistent hydrogen bonding network, with an average of over 15 hydrogen bonds. Figure 5 further supports these findings by showing persistent contacts with catalytically or structurally relevant residues, including Arg68 (89%), Arg111 (86%), and Glu66 (45%). These interactions suggest strong anchoring through electrostatic and π-cation contacts. Engagement with polar and charged residues, such as His52, Gln40, and Glu66, indicates potential interference with the glutathione-binding site. Water-mediated interactions and hydrogen bonding from sugar hydroxyl groups further contribute to the stability of the complex with moderate occupancy. Water-mediated interactions between vicenin-2 and SfGST were predominantly transient, with occupancy values ranging from 30% to 35% across the simulation trajectory. These moderate occupancy values suggest that while water molecules contribute to interfacial stabilization, they are not persistent enough to form stable hydration bridges essential for long-term binding. Consequently, the overall stability of the complex appears to be maintained primarily through direct interactions, particularly with residues such as Arg111, Arg68, and Glu66. Comparable GST-ligand simulations have shown that stable binding and increased protein flexibility are consistent with inhibitory activity. The total energy (~–130 kcal/mol) is also more favorable than typical reported values (–60 to –90 kcal/mol) (Valli & Achilonu, 2023), further supporting vicenin-2's potential as a promising GST inhibitor.

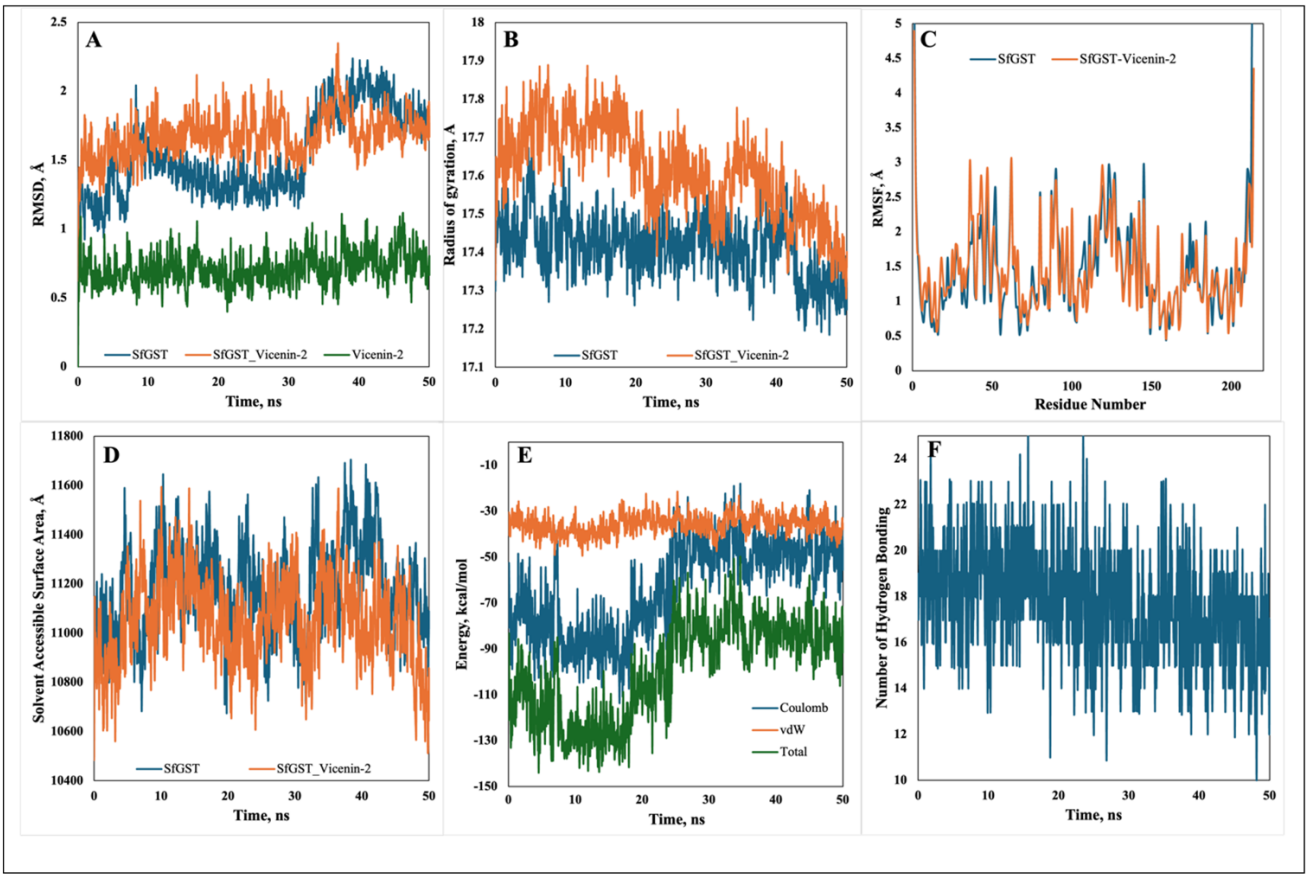

Figure 4: Molecular dynamics simulation profiles of SfGST with and without vicenin-2: (**A**) root-mean-square deviation (RMSD), (**B**) radius of gyration (Rg), (**C**) root-mean-square fluctuation (RMSF), (**D**) solvent-accessible surface area (SASA), (**E**) ligand interaction energy, and (**F**) number of hydrogen bonds formed between vicenin-2 and SfGST throughout the simulation.

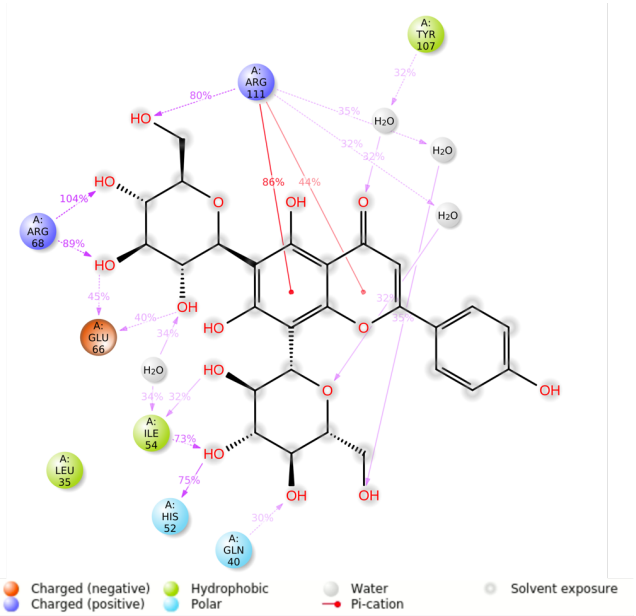

Figure 5: 2D interaction diagram illustrating the binding interactions between vicenin-2 and SfGST over a 50-ns molecular dynamics simulation. Interacting residues are categorized by physicochemical properties. Red arrows represent π–cation interactions; percentages denote the occupancy, i.e., the fraction of simulation time the interaction was present.

Early molecular dynamics studies on human GST P1-1 showed that when a ligand binds, the protein undergoes noticeable shape changes, especially around helix and nearby loops. These movements help shield the active site from the surrounding solvent, potentially affecting how the enzyme functions (Stella et al., 1999). Enzyme loop flexibility contributes to enzyme inhibition via conformational shifts (Lu et al. 2022) and increased rigidity (Assadieskandar et al. 2019). Similarly, our RMSF data highlight enhanced fluctuations in analogous loop regions, suggesting vicenin-2 may facilitate structural reorganization needed for inhibition. In simulations of insect GSTs, like those from *Anopheles gambiae*, researchers found that strong π–π interactions and hydrogen bonds played a key role in keeping ligands tightly bound (Wang Y. et al., 2013). A similar pattern was observed in our study, where stable van der Waals forces and a consistent network of hydrogen bonds (ranging from about 10 to 24) helped maintain the ligand's attachment throughout the simulation. Studies on *Schistosoma* GST using MD-based pharmacophore models have shown that selective inhibitors like bromosulfophthalein and quercetin analogs can bind stably, usually with total energies around –50 to –60 kcal/mol (Valli & Achilonu, 2023). In contrast, our energy calculations suggest that vicenin-2 binds much more strongly, with an estimated total energy of about –130 kcal/mol. While MD simulations offer valuable insights into the potential inhibitory mechanisms, biochemical validation using purified vicenin-2 is essential to confirm its precise mode of action. Such validation would support its role as a natural GST inhibitor and justify further in vivo testing, including its potential application in the development of insecticide synergists.

CONCLUSION

In conclusion, this study demonstrated that the crude methanol extract of *P. stratiotes* inhibited SfGST activity *in vitro*. Docking and simulation analyses revealed that vicenin-2, a major flavonoid component, shows strong binding affinity and structural compatibility with the SfGST active site. These findings highlight the potential of *P. stratiotes* as a source of bioactive compounds for sustainable pest management. To strengthen this potential, future research should include the isolation and structural confirmation of

vicenin-2, detailed enzyme kinetics analyses (Km, Vmax, and mode of inhibition), and insect bioassays complemented with field efficacy evaluations. Further studies on the methanolic extract of *P. stratiotes* are recommended, including comprehensive phytochemical profiling through LC–MS/MS dereplication. Subsequent isolation studies should be conducted to identify and confirm the compounds responsible for its GST inhibitory activity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

JJP Manuben and MA Castrosanto contributed to the conception of the project, study design, and data interpretation. All authors contributed to data acquisition and manuscript preparation, review, and revision.

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