Integrated experimental and *In Silico* evaluation of spice - based Phytonova Balls for the management of red flour beetle, *Tribolium castaneum* (Herbst)

DHANUSRI S

(24PZO001)

Thesis submitted to
Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641043

In Partial Fulfilment of the Requirements for the Degree of
MASTER OF SCIENCE IN ZOOLOGY

April 2026

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Signature of Head of the Department

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Signature of the Supervisor

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I. INTRODUCTION

Stored grains constitute a critical component of global food security, serving as the primary source of energy, protein, and essential nutrients for both human populations and livestock. Cereals, pulses, and oilseeds collectively form the backbone of food systems worldwide, with global production exceeding two billion tonnes annually to meet the nutritional demands of a rapidly growing population. Advances in agricultural practices, including improved crop varieties, mechanization, and agronomic interventions, have significantly enhanced grain production over recent decades. However, despite these achievements, ensuring food security remains a persistent global challenge due to constraints such as limited arable land, climate change, population expansion, and biotic stresses affecting crops both pre- and post-harvest (Baliota *et al.,* 2022).

Among these constraints, post-harvest losses represent one of the most critical yet often underestimated issues, particularly in developing countries located in tropical and subtropical regions. It is estimated that a substantial proportion of harvested grains is lost during storage due to inadequate infrastructure, poor handling practices, and pest infestations. These losses not only reduce food availability but also directly impact the economic stability of farmers and national food systems. Insects, in particular, are responsible for a major share of these losses, causing both quantitative and qualitative deterioration of stored commodities (Tadesse, 2020).

A wide range of insect pests infest stored grains and grain products, with major species including *Sitophilus zeamais* (maize weevil), *Sitophilus oryzae* (rice weevil), *Sitotroga cerealella* (Angoumois grain moth), *Callosobruchus* spp. (pulse beetles), and *Tribolium castaneum* (red flour beetle). These pests exhibit high reproductive potential and adaptability to storage conditions, enabling them to rapidly colonize and damage stored products. Reports indicate that infestations can lead to grain weight losses ranging from 41% to 80%, depending on storage duration and environmental conditions (Tadesse, 2020). In addition to grain weight loss, insect activity leads to contamination of grains with excreta, cast skins, and dead bodies, thereby reducing food quality, nutritional value, and safety.

Effective storage management is essential to mitigate these losses; however, in many developing regions, the lack of proper storage facilities, combined with insufficient awareness of post-harvest practices, exacerbates the problem (Abdullahi and Dandago, 2021). Environmental factors such as high temperature and relative humidity further promote pest proliferation and microbial growth. Consequently, post-harvest losses represent a multifactorial problem that requires integrated management strategies combining improved storage practices and effective pest control measures (Paul *et al.,* 2020).

Stored grain deterioration is a complex process influenced by both biotic and abiotic factors. Among the biotic factors, insect infestation is considered the most destructive. Insects not only feed on grains but also facilitate secondary infestations by fungi and bacteria, leading to spoilage, toxin production, and reduced seed viability. Most stored grain pests belong to the orders Coleoptera and Lepidoptera, with beetles generally considered more destructive due to their feeding habits and longer persistence in storage environments.

Among the coleopteran pests, *Tribolium castaneum* (Herbst), commonly known as the red flour beetle, is one of the most widespread and economically significant species. It is a cosmopolitan pest with a broad host range, feeding on cereals, flour, starch-based materials, and processed grain products. The insect is commonly found in flour mills, warehouses, and food processing units, where it thrives on broken grains and grain debris. Infestation by *T. castaneum* results in the production of fine powder and dust, leading to substantial losses in both quantity and market value of stored products (Chaubey, 2023).

The biology of *T. castaneum* contributes significantly to its pest status. Its life cycle comprises four stages, egg, larva, pupa, and adult. Females exhibit high fecundity, laying approximately 400–450 eggs during their lifetime. The eggs hatch within a few days under favourable conditions, giving rise to highly active larvae that feed extensively on grain products. The larval stage is particularly destructive, followed by pupation and emergence of adults capable of further reproduction. Both larvae and adults contribute to grain damage, and their ability to survive on minimal food resources enhances their persistence in storage environments. These biological traits, combined with rapid reproduction and adaptability, make *T. castaneum* a major challenge in stored grain protection, even though it is a secondary pest.

Traditionally, the management of stored grain pests has relied heavily on chemical insecticides and fumigants. Organophosphorus compounds such as malathion and fumigants like phosphine and methyl bromide have been widely used due to their effectiveness in controlling insects at various life stages. Fumigation is particularly advantageous because of its ability to penetrate bulk grain and eliminate hidden infestations. However, the extensive and indiscriminate use of synthetic insecticides has led to several adverse consequences.

One of the most significant issues is the development of insecticide resistance, where insect populations evolve mechanisms to tolerate previously effective compounds. Resistance in *T. castaneum* has been widely reported, reducing the efficacy of commonly used insecticides. In addition, chemical control methods pose serious environmental and health concerns. Residual toxicity in food products, contamination of soil and water resources, and

adverse effects on non-target organisms, including beneficial insects, have raised significant ecological and public health concerns (Mubayiwa *et al.,* 2021). These limitations highlight the urgent need for alternative pest management strategies that are both effective and environmentally sustainable.

In this context, botanical insecticides have emerged as promising alternatives. Derived from plant sources, these compounds are biodegradable, eco-friendly, and generally less toxic to non-target organisms. Plants produce a wide array of secondary metabolites that exhibit insecticidal, repellent, antifeedant, and growth-regulating properties. Compounds such as cinnamaldehyde, eugenol, limonene, pinene, and curcumin have been reported to interfere with insect physiological processes, including feeding behaviour, reproduction, and development (Suleiman, 2021).

Botanical insecticides can be formulated in various forms, including powders, extracts, essential oils, and pellets. Spices and medicinal plants such as clove, cinnamon, pepper, turmeric, bay leaf, garlic, and citrus peels have demonstrated significant efficacy against stored grain pests. These materials are often readily available, cost-effective, and suitable for use in small-scale and traditional storage systems. The development of novel formulations, enhances the practicality and efficiency of botanical pest control by enabling controlled release of active compounds.

Despite the advantages of botanical insecticides, their interaction with insect detoxification systems remains a critical area of investigation. Insects possess sophisticated detoxification mechanisms that enable them to metabolize and eliminate xenobiotic compounds, including both synthetic insecticides and plant-derived chemicals. These mechanisms are broadly categorized into three phases: Phase I (functionalization reactions mediated by cytochrome P450 monooxygenases), Phase II (conjugation reactions involving enzymes such as glutathione S-transferases), and Phase III (transport and excretion mediated by ATP-binding cassette transporters) (Chafik *et al.,* 2023).

Understanding these detoxification pathways is essential for evaluating the efficacy of botanical insecticides and predicting potential resistance development. In this regard, computational and molecular approaches provide valuable tools for investigating insect–phytochemical interactions at the molecular level. Pathway analysis using databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) enables the identification of key genes and metabolic pathways involved in detoxification and resistance mechanisms.

Furthermore, advances in functional genomics, particularly CRISPR-Cas9 gene-editing technology, have revolutionized the study of gene function in insects. This technology allows

precise modification of target genes, facilitating the validation of their roles in detoxification and resistance. In *T. castaneum*, CRISPR-Cas9 has been successfully applied to study gene function, providing insights into molecular mechanisms underlying insecticide tolerance (Shamjana *et al.*, 2021).

In addition to experimental approaches, in silico techniques such as molecular docking and protein modeling have become integral components of modern pest management research. These methods enable the prediction of binding interactions between phytochemicals and target proteins, providing insights into their inhibitory potential and mode of action. Molecular docking, in particular, allows the evaluation of binding affinity and interaction stability, serving as a cost-effective preliminary screening tool prior to laboratory validation.

The integration of experimental bioassays with computational analyses offers a comprehensive approach to understanding pest management strategies. By combining the evaluation of botanical formulations with molecular insights into detoxification pathways, it is possible to develop targeted and sustainable pest control solutions.

With this background, the present study focuses on the development and evaluation of a novel botanical formulation for the management of *Tribolium castaneum*, along with an integrated *in silico* analysis of detoxification pathways and phytochemical interactions. This combined approach aims to provide both practical and mechanistic insights into eco-friendly pest management strategies for stored grain protection. The objectives of the present study were formulated as follows:

- To formulate Phytonova Balls (PNB), Diatomaceous Earth Balls (DEB) and Neem Balls (NB) to control *Tribolium castaneum*.
- To screen and select major phytochemicals in PB with Gas Chromatography and Mass Spectroscopy (GC-MS) analysis
- To evaluate the repellent, insecticidal, and progeny inhibition effects of PNB, DEB and NB against *T. castaneum*.
- To identify and analyze detoxification-related genes and metabolic pathways in *T. castaneum* using KEGG pathway analysis and CRISPR-Cas9 approaches.

To assess the interaction potential of selected phytocompounds with CYP6BQ9, CYP6BQ11 and GSTD1 through molecular docking.

II. Review of Literature

The literature pertaining to the research study entitled "**Integrated experimental and *In Silico* evaluation of spice - based Phytonova Balls for the management of red flour beetle, *Tribolium castaneum* (Herbst)"** were discussed under the following headings:

2.1. Stored grain insect pests and its economic importance

2.2. Biology and damage potential of *T. castaneum*

2.3. Limitations of synthetic insecticides in grain storage

2.4. Potential of botanicals in stored grain protection

2.5. Insecticidal properties of certain spices

2.6. Formulation approaches for botanical pest control

2.7. Mode of action of plant derived allelochemicals

2.8. Detoxification enzyme systems in stored product insects

2.9. CRISPRCAS9 as a functional genomics tool in entomology

2.10. *In silico* approaches for CRSIPR target validity

2.11. Protein modelling and docking in botanical insecticides

2.12. Research gap and rationale of the present study

2.1. Stored grain insect pests and its economic importance

Stored grains constitute a fundamental component of global food security, particularly in developing countries where they serve as a primary food source during off-season periods. However, these stored commodities are highly susceptible to infestation by a wide range of insect pests, leading to substantial post-harvest losses in both quantity and quality. Such infestations result in direct consumption of grains, contamination with insect fragments and excreta, deterioration of nutritional value, and reduction in market acceptability, thereby causing significant economic losses (Ahmad *et al.*, 2021). It is estimated that stored grain losses range from 10–30% globally, with higher magnitudes reported in tropical regions due to favorable temperature and humidity conditions that enhance pest proliferation (Phillips and Throne, 2010).

Stored grain insect pests are broadly categorized into primary and secondary pests based on their feeding behavior. Primary pests, such as *Sitophilus* spp. and *Rhyzopertha dominica*, infest intact grains, while secondary pests attack broken or processed grains. Among the latter, the red flour beetle, *Tribolium castaneum*, is one of the most economically important pests worldwide. Its ability to reproduce rapidly, adapt to diverse environmental conditions, and develop resistance to conventional insecticides makes it a persistent and challenging pest in storage ecosystems (Hagstrum *et al.*, 2012).

The economic significance of *T. castaneum* extends beyond direct grain loss. Moreover, contamination caused by this pest contributes to reduced grain quality standards, affecting both domestic utilization and international trade. Indirect losses also arise from increased storage costs, pest management expenses, and potential health risks associated with consumption of contaminated food (Boxall, 2002).

Effective management of stored grain pests, particularly *T. castaneum*, is therefore essential to minimize losses and maintain food quality. Traditional pest control strategies have relied heavily on synthetic insecticides and fumigants such as phosphine. However, the widespread and indiscriminate use of these chemicals has led to several challenges, including the development of resistance in pest populations, environmental contamination, and adverse effects on non-target organisms (Arthur *et al.*, 2018). These limitations necessitate the development of safer and more sustainable pest management approaches.

Contemporary strategies emphasize Integrated Pest Management (IPM), which combines preventive, cultural, physical, biological, and chemical methods. Preventive measures such as proper drying of grains, maintenance of sanitation, and use of airtight or hermetic storage structures play a crucial role in minimizing initial infestations. Monitoring tools, including pheromone traps and regular sampling, enable early detection and timely intervention. In recent years, eco-friendly alternatives such as botanical insecticides, essential oils, inert dusts like diatomaceous earth, and biological control agents have gained prominence due to their reduced environmental impact and effectiveness against resistant pest populations (Kumar and Kalita, 2017).

Furthermore, advances in molecular biology and biotechnology have opened new avenues for targeted pest control. Techniques such as RNA interference (RNAi) and CRISPR-based gene editing are being explored for their potential to disrupt essential genes in pests like *T. castaneum*, offering species-specific and environmentally benign control strategies. These approaches, when integrated with conventional practices, provide a promising framework for sustainable stored grain pest management.

Thus, stored grain insect pests, particularly *Tribolium castaneum*, pose a significant threat to food security and economic stability. A comprehensive understanding of their biology, ecological adaptability, and management strategies is essential for effective control. The integration of traditional storage practices with modern technological interventions offers a sustainable pathway to mitigate post-harvest losses and ensure the long-term preservation of stored grains.

2.2. Biology and damage potential of *T. castaneum*

Tribolium castaneum (Herbst), commonly known as the red flour beetle, is one of the most extensively studied stored-product insects due to its dual significance as a major pest and a model organism in biological research. It is a cosmopolitan secondary pest that primarily infests processed and damaged grains, including flour, semolina, and other cereal-based products. Its global distribution, high reproductive potential, and adaptability to diverse environmental conditions make it a persistent problem in storage systems, contributing significantly to post-harvest losses (Chaubey, 2023). The species is particularly important in tropical and subtropical regions, where favorable climatic conditions facilitate rapid population buildup and continuous breeding.

Biologically, *T. castaneum* exhibits a holometabolous life cycle consisting of egg, larval, pupal, and adult stages. The developmental duration and survival of each stage are strongly influenced by environmental factors such as temperature, humidity, and food substrate. Early studies demonstrated that while spatial distribution of life stages may vary across temperature gradients, core biological parameters such as development time and survival remain relatively stable under varying conditions, indicating a high degree of physiological adaptability (Amos and Waterhouse, 1969). The species also exhibits remarkable reproductive capacity, with females laying substantial numbers of eggs depending on the food source. Variation in wheat varieties significantly influences fecundity, developmental duration, and intrinsic rate of increase, highlighting the role of host substrate in population dynamics (Din *et al.*, 2018).

Further insights into its biology reveal that *T. castaneum* can complete multiple generations per year, with rapid larval development and relatively short life cycles under optimal conditions. Studies on alternative substrates such as Brazil nut have demonstrated detailed reproductive parameters, including pre-oviposition, oviposition duration, larval instars, and survival rates, emphasizing its ability to exploit diverse food sources (Pires *et al.*, 2019). Such biological plasticity enhances its survival and persistence in storage ecosystems, making it a difficult pest to manage effectively.

The damage potential of *T. castaneum* is substantial and multifaceted. Both larvae and adults feed on broken grains and processed products, primarily targeting the germ region, leading to direct weight loss and deterioration of grain quality. In addition to physical damage, the beetle secretes quinones that impart a characteristic pungent odor and discoloration, significantly reducing the palatability and market value of infested products. Contamination with insect fragments, cast skins, and metabolic wastes further compromises food safety and

quality standards (Chaubey, 2023). Such qualitative losses often exceed quantitative losses in economic importance, particularly in commercial storage and export scenarios.

Beyond its role as a pest, *T. castaneum* has emerged as a powerful model organism bridging fundamental and applied sciences. It was the first coleopteran insect to have its genome fully sequenced, and the availability of advanced genomic resources has facilitated extensive research in genetics, developmental biology, neurobiology, and evolutionary studies. Compared to classical models like *Drosophila melanogaster*, *Tribolium* provides unique insights into developmental processes, as differences in segmentation and morphological traits allow comparative evolutionary analyses (Brown *et al.*, 2009). The species is highly amenable to functional genomics approaches, including systemic RNA interference (RNAi), CRISPR-Cas9 genome editing, and transgenic techniques, enabling precise investigation of gene function across developmental stages.

Importantly, *T. castaneum* serves as an excellent model for studying insecticide resistance mechanisms. It has developed resistance to multiple classes of insecticides, including organophosphates, carbamates, pyrethroids, and neonicotinoids, making it a valuable system for understanding detoxification pathways and resistance evolution (Rösner *et al.*, 2020). The integration of genomic tools with behavioral and ecological studies has further enhanced its utility in pest management research, particularly in understanding responses to pheromones, kairomones, and environmental cues.

In recent years, there has been increasing emphasis on sustainable management strategies targeting *T. castaneum*, including the use of plant-derived biopesticides and essential oils. These eco-friendly alternatives interfere with insect growth, development, and reproduction while minimizing environmental and health risks associated with synthetic insecticides (Gawali *et al.*, 2022). The application of such approaches, combined with advances in molecular biology, offers promising avenues for effective and sustainable control.

To summarize, *Tribolium castaneum* represents both a significant economic pest and a versatile model organism. Its well-characterized biology, high adaptability, and susceptibility to advanced genetic manipulation make it indispensable for both fundamental research and applied pest management. Understanding its life history traits and damage mechanisms is crucial for developing innovative and sustainable strategies to mitigate its impact on stored grain systems.

2.3. Limitations of synthetic insecticides in grain storage

The protection of stored grains has traditionally relied on the extensive use of synthetic insecticides and fumigants. Although these chemicals have been effective in reducing pest

populations, their long-term sustainability is increasingly questioned due to multiple ecological, biological, and socio-economic constraints. One of the foremost limitations is the development of insecticide resistance among key stored grain pests such as *Tribolium castaneum*, *Sitophilus* spp., and *Rhyzopertha dominica*. Continuous and indiscriminate application of insecticides, particularly phosphine and contact insecticides, has accelerated resistance evolution, thereby reducing efficacy and necessitating higher doses or more frequent applications (Rösner *et al.*, 2020; Arthur *et al.*, 2018).

Another critical concern associated with synthetic insecticides is the accumulation of chemical residues in stored grains. These residues pose significant health risks to consumers, including potential carcinogenic and neurotoxic effects, and often lead to regulatory restrictions in international trade. Residue persistence also affects non-target organisms and disrupts ecological balance within storage environments. In developing countries, where regulatory frameworks may be less stringent, the misuse and overuse of pesticides exacerbate these risks, further compromising food safety (Boxall, 2002).

Environmental contamination is an additional drawback of synthetic pesticide usage. Many commonly used insecticides are non-biodegradable or degrade slowly, leading to accumulation in soil, water, and food chains. This not only impacts biodiversity but also contributes to long-term ecological instability. Furthermore, the application of fumigants such as phosphine requires strict handling protocols, as improper use can result in occupational hazards, including acute toxicity and accidental poisoning among workers (Phillips and Throne, 2010).

Field-based studies have further highlighted the limitations of synthetic insecticides under practical storage conditions. Machekano *et al.*, (2019) demonstrated that while conventional pesticide treatments provide some level of protection, alternative approaches such as Diatomaceous Earth (DE) combined with low doses of bio-insecticides like spinosad or deltamethrin exhibited superior or comparable efficacy in controlling major storage pests, including *T. castaneum*. These findings suggest that reliance solely on synthetic insecticides may not be the most efficient or sustainable strategy for pest control, particularly in smallholder farming systems.

Similarly, Mubayiwa *et al.*, (2021) reported that hermetic storage technologies significantly outperformed synthetic pesticide-based methods in maintaining grain quality and minimizing pest infestation under hot and arid conditions. Weight losses in pesticide-treated grains were notably higher compared to hermetic systems, and certain pests, including *T. castaneum*, were able to survive and develop even in insecticide-treated environments. This

indicates potential limitations in the residual efficacy and penetration of chemical treatments, especially under varying environmental conditions.

In addition to these challenges, synthetic insecticides often fail to provide comprehensive control across different life stages of pests. Eggs and pupae may remain protected within grain kernels or storage structures, allowing reinfestation after treatment. Moreover, the cost of synthetic pesticides and the need for repeated applications impose economic burdens on small-scale farmers, limiting accessibility and adoption (Kumar and Kalita, 2017).

Recent research has therefore emphasized the need for safer and more sustainable alternatives. Botanicals, essential oils, inert dusts, and biological control agents have emerged as promising substitutes due to their biodegradability, low toxicity, and reduced risk of resistance development. Moreover, IPM strategies that combine physical methods such as hermetic storage with biological and botanical interventions offer a holistic approach to grain protection. While synthetic insecticides have played a significant role in stored grain protection, their limitations, including resistance development, residue accumulation, environmental hazards, and reduced field efficacy necessitate a transition toward sustainable IPM strategies. The adoption of alternative approaches, supported by advancements in research and technology, is essential for ensuring long-term food security and environmental safety.

2.4. Potential of botanicals in stored grain protection

The increasing limitations associated with synthetic insecticides have intensified research into plant-derived alternatives for stored grain protection. Botanical insecticides, derived from medicinal and aromatic plants, have been used since antiquity and are now gaining renewed scientific interest due to their eco-friendly nature, biodegradability, and reduced toxicity to non-target organisms. Stored grain infestation caused by insects at various developmental stages leads to severe quantitative and qualitative losses; hence, the adoption of safer and sustainable pest management strategies has become imperative (Trivedi *et al.*, 2018). Botanicals encompass a diverse array of bioactive compounds, including alkaloids, terpenoids, flavonoids, phenolics, and essential oils, which exhibit multiple modes of action against insect pests. These compounds function as repellents, antifeedants, fumigants, contact toxicants, and growth regulators. In many cases, they interfere with the insect nervous system, disrupt endocrine regulation, or inhibit reproductive processes, thereby reducing pest population buildup. In addition, certain plant-derived compounds act as chemosterilants and oviposition deterrents, further limiting pest proliferation (Prakash *et al.*, 2022). Such multifaceted modes

of action reduce the likelihood of resistance development compared to conventional insecticides.

A wide range of plant extracts and essential oils have demonstrated efficacy against major stored grain pests, including *T. castaneum*. Experimental studies have shown that extracts from plants such as *Azadirachta indica* (neem), *Piper nigrum* (black pepper), *Mentha spicata* (mint), and *Allium sativum* (garlic) exhibit significant insecticidal, repellent, and growth inhibitory effects. Notably, neem-based products have shown high repellency and moderate toxicity against *T. castaneum*, highlighting their potential as grain protectants (Ahmad, 2024). Similarly, various plant extracts have been reported to induce high mortality and suppress progeny development in different stored-product insect species, with efficacy varying based on concentration, exposure time, and extraction solvent (Al-Akhdar and Boraie, 2015).

Botanical insecticides also play a crucial role within IPM frameworks. They can be applied alone or in combination with other control methods such as inert dusts, pheromone traps, and biological agents to enhance overall efficacy. Their compatibility with IPM strategies and suitability for organic agriculture have contributed to their growing acceptance, particularly in developing countries where cost-effective and locally available pest control options are essential (Athanassiou *et al.,* 2014).

Recent advances have further expanded the potential of botanicals through the incorporation of modern technologies. Plant Volatile Organic Compounds (PVOCs) have been identified as potent bioactive agents with fumigant and behavioral effects on insect pests. In addition, nanotechnology-based formulations, such as essential oil nanoparticles and nano-encapsulated botanicals, have improved the stability, persistence, and delivery efficiency of plant-derived compounds. These innovations address some of the inherent limitations of botanicals, such as volatility and rapid degradation, thereby enhancing their practical applicability in stored grain systems (Kale *et al.,* 2021).

Despite their numerous advantages, the large-scale adoption of botanical insecticides faces several challenges. Variability in chemical composition, lack of standardization, limited shelf life, and inconsistencies in efficacy under field conditions hinder commercialization. Furthermore, regulatory constraints, high production costs, and limited awareness among farmers restrict their widespread use. Addressing these challenges requires coordinated efforts in research, policy support, and extension services to promote the development and adoption of botanical-based pest management solutions (Rajashekar *et al.*, 2025; Vijayan *et al.*, 2023).

Importantly, botanical insecticides are increasingly being evaluated using computational and molecular approaches to understand their mechanisms of action and safety profiles. Such studies facilitate the identification of active compounds and their target sites, enabling the design of more effective and specific pest control agents. The integration of traditional knowledge with modern scientific tools thus holds great promise for advancing botanical insecticides as sustainable alternatives in stored grain protection (Prakash *et al.,* 2022).

Thus, botanical insecticides represent a viable and environmentally sustainable alternative to synthetic pesticides for stored grain protection. Their diverse modes of action, safety profile, and compatibility with integrated pest management make them particularly suitable for addressing current challenges in pest control. However, overcoming limitations related to standardization, formulation, and large-scale implementation is essential to fully harness their potential. Continued research and technological innovation will play a critical role in promoting botanicals as mainstream solutions for sustainable grain storage management.

2.5. Insecticidal properties of spices

A substantial body of research has investigated the insecticidal potential of individual plant-derived substances, particularly spices and medicinal plants, against *Tribolium castaneum*. These botanicals exhibit diverse bioactivities including repellency, contact toxicity, fumigant action, antifeedant effects, and inhibition of growth and reproduction. Certain findings across multiple plant sources, emphasizing their efficacy and underlying limitations in stored grain protection were discussed as follows.

Clove (*Syzygium aromaticum*) has been widely reported as one of the most effective botanical insecticides against *T. castaneum*. Studies have demonstrated strong repellency and contact toxicity, with clove powders and extracts showing dose-dependent effects on insect mortality and behavior. Al-Dakhil (2025) reported that clove exhibited the highest repellency among tested plant powders, while Ho *et al.,* (1994) observed moderate mortality but complete inhibition of progeny emergence, indicating its potential as a reproductive suppressor. The insecticidal activity of clove is primarily attributed to eugenol and other phenolic compounds, which interfere with the insect nervous system (Bandara *et al.,* 2023). However, variability in efficacy depending on extraction method (polar vs non-polar) and relatively lower toxicity against *T. castaneum* compared to other pests highlight certain limitations.

Cinnamon (*Cinnamomum* spp.) is another extensively studied botanical with significant insecticidal activity. It has been shown to possess strong repellent and toxic effects, often second only to clove in efficacy (Al-Dakhil, 2025). Bioassays have demonstrated high

mortality rates (up to 90–99%) at higher concentrations and exposure durations (Ali *et al.*, 2019). Active compounds such as cinnamaldehyde and eugenol contribute to its bioactivity by disrupting physiological and neurological functions in insects. Also, cinnamon has shown synergistic effects when combined with microbial agents such as *Bacillus thuringiensis*. Despite these advantages, its effectiveness is highly concentration-dependent, and volatility may limit its persistence under storage conditions.

Turmeric (*Curcuma longa*) has been investigated for its insecticidal, antifeedant, and growth regulatory properties. Extracts of turmeric have demonstrated significant reduction in larval development, adult emergence, and grain damage caused by *T. castaneum* (Ali *et al.*, 2014). Bioactive compounds such as ar-turmerone and curcuminoids are responsible for its pesticidal activity. However, turmeric generally exhibits lower toxicity compared to other botanicals like garlic or tobacco, and its efficacy may vary across insect species (Lee *et al.*, 2001). This suggests that while turmeric is useful as a supportive component in pest management, it may not be sufficiently potent as a standalone control agent.

Ginger (*Zingiber officinale*) has shown strong toxic and repellent properties against *T. castaneum*. Studies indicate that higher concentrations of ginger extracts can achieve substantial mortality (up to ~86%) and complete repellency within short exposure periods (Atta *et al.*, 2020). Ginger also significantly reduces feeding activity and grain damage, indicating its role as an effective antifeedant. Nevertheless, its efficacy is influenced by exposure duration and concentration, and its rapid degradation may limit long-term protection.

Black pepper (*Piper nigrum*) is another potent botanical that exhibits strong repellent and insecticidal effects. Experimental studies have shown that black pepper powder and extracts can induce higher mortality and repellency compared to several other plant powders (AL-Joboory, 2019). Its bioactivity is attributed to alkaloids such as piperine, which affect insect nervous and metabolic systems. However, similar to other botanicals, its effectiveness is dose-dependent and may decline over time, necessitating repeated application.

Garlic (*Allium sativum*) has demonstrated high insecticidal efficacy against *T. castaneum*, often outperforming several other plant extracts. It significantly reduces population growth, grain damage, and weight loss, and exhibits strong toxic and repellent effects (Ahmad *et al.*, 2019). Sulfur-containing compounds such as allicin are responsible for its bioactivity. Despite its effectiveness, strong odor and potential impact on grain organoleptic properties may limit its practical application in food storage systems.

Citrus peel extracts, particularly from orange (*Citrus sinensis*), have shown promising insecticidal and reproductive inhibitory effects. Higher concentrations of citrus peel powders

and essential oils significantly increased mortality, reduced oviposition, and inhibited adult emergence in *T. castaneum* (Nta *et al.*, 2017; Zia *et al.*, 2013). The presence of limonene and other volatile compounds contributes to their fumigant and toxic effects. However, their efficacy often requires relatively high concentrations, and volatility may reduce long-term effectiveness.

Similarly, other botanicals such as bay leaf (*Laurus nobilis*) and spring onion (*Allium fistulosum*) have demonstrated moderate insecticidal and repellent activities. While these plants contain bioactive compounds capable of affecting insect survival and behavior, their efficacy is generally lower compared to more potent botanicals like clove or garlic, and they are less extensively studied against *T. castaneum*.

Thus, these studies clearly demonstrate that individual botanical sources possess significant insecticidal potential against *T. castaneum*, acting through multiple mechanisms including neurotoxicity, growth inhibition, and reproductive suppression. However, despite their promising efficacy under laboratory conditions, several limitations hinder their large-scale application. These include variability in chemical composition, lack of standardization, dependence on high concentrations, rapid degradation and volatility, short residual activity, and inconsistent performance under field conditions. Also, issues related to formulation, storage stability, and large-scale commercialization remain major challenges.

To sum up, while individual botanicals such as clove, cinnamon, garlic, and black pepper exhibit strong bioactivity against *T. castaneum*, their practical application requires further refinement through improved formulations, standardization of active compounds, and integration into comprehensive pest management strategies. These limitations highlight the need for advanced approaches, including nano-formulations and synergistic combinations, to fully exploit the potential of botanical insecticides in stored grain protection.

2.6. Formulation approaches for botanical pest control

The practical application of botanical insecticides in stored grain protection is largely determined by the efficiency of their formulation. Although plant-derived compounds exhibit promising insecticidal properties, their direct use is often constrained by physicochemical limitations such as high volatility, poor water solubility, rapid degradation under environmental conditions, and inconsistent bioavailability. These constraints significantly reduce their persistence and efficacy in real storage environments, necessitating the development of advanced formulation strategies to enhance their stability and performance (Sarmah *et al.*, 2025).

Traditional formulations of botanical pesticides include powders, crude extracts, oils, and Emulsifiable Concentrates (ECs). Among these, EC formulations have gained considerable attention due to their improved dispersion, ease of application, and enhanced bioefficacy. Purkait *et al.,* (2019) successfully developed EC formulations from plant seed oils that demonstrated significant insecticidal activity comparable to synthetic insecticides. These formulations improved emulsion stability, storage properties, and delivery efficiency, thereby enhancing the practical applicability of plant-based insecticides in pest management systems. However, conventional formulations still face challenges such as rapid degradation and limited residual activity. To overcome these limitations, recent research has focused on innovative formulation technologies, particularly nano- and micro-based delivery systems. Techniques such as nanoemulsions, microemulsions, nanoencapsulation, and microencapsulation have emerged as promising approaches for improving the stability and efficacy of botanical insecticides. These systems enable controlled and sustained release of active compounds, protect them from environmental degradation, and enhance their penetration and interaction with target pests (Sarmah *et al.,* 2025).

Nanoemulsions, characterized by their small droplet size and high surface area, improve the solubility and bioavailability of hydrophobic plant compounds. Similarly, encapsulation techniques involve entrapping active ingredients within polymeric or lipid-based carriers, which shield them from oxidation, photodegradation, and volatilization. These advanced delivery systems not only prolong the residual activity of botanicals but also reduce the required dosage and frequency of application, thereby improving cost-effectiveness and minimizing environmental impact (Saikumar *et al.,* 2025).

In addition to enhancing efficacy, formulation strategies also aim to improve target specificity and reduce non-target toxicity. Controlled-release systems allow gradual release of active compounds, maintaining effective concentrations over extended periods while minimizing exposure to beneficial organisms. Furthermore, synergistic formulations combining botanicals with other bioagents, such as entomopathogens or nanoparticles, have shown enhanced insecticidal activity by targeting multiple physiological pathways in pests (Pant *et al.,* 2016).

Despite these advancements, several challenges remain in the formulation and commercialization of botanical insecticides. Variability in the chemical composition of plant extracts due to environmental and genetic factors complicates standardization and quality control. Moreover, large-scale production of stable and effective formulations requires sophisticated technologies and infrastructure, which may increase production costs. Regulatory

constraints and lack of uniform guidelines further hinder the commercialization and widespread adoption of these products (Guleria and Tiku, 2009; Divekar, 2023).

Another critical limitation is the insufficient number of field-based studies validating the performance of advanced formulations under diverse storage and environmental conditions. While laboratory studies have demonstrated promising results, translating these findings into real-world applications remains a significant challenge. Issues such as formulation stability during storage, interaction with grain matrices, and long-term efficacy under fluctuating temperature and humidity conditions need further investigation (Sarmah *et al.*, 2025).

Formulation plays a pivotal role in determining the success of botanical insecticides in stored grain protection. Advances in nano- and micro-formulation technologies have significantly improved the stability, efficacy, and delivery of plant-based insecticides, offering a viable alternative to conventional synthetic pesticides. However, challenges related to standardization, scalability, cost, and field validation must be addressed to facilitate their widespread adoption. Continued research integrating formulation science with molecular and ecological studies will be essential for developing efficient, sustainable, and commercially viable botanical pest control strategies.

2.7. **Mode of action of plant derived allelochemicals**

Plant-derived allelochemicals represent a diverse group of bioactive secondary metabolites that exert multifaceted effects on insect physiology, biochemistry, and behavior. In *T. castaneum*, these compounds act through multiple target sites, distinguishing them from conventional synthetic insecticides that typically operate via single-site toxicity. This multi-target mode of action is one of the key reasons for considering botanicals as promising alternatives for sustainable pest management. However, a detailed understanding of their mechanisms is essential for optimizing their efficacy and ensuring long-term applicability.

A primary mechanism of allelochemical action involves disruption of the insect nervous system. Many plant-derived compounds, particularly phenylpropenes (eugenol, cinnamaldehyde) and monoterpenes (terpinen-4-ol, α-terpinene), act as neurotoxins by inhibiting acetylcholinesterase (AChE), a critical enzyme responsible for terminating nerve impulses. Inhibition of AChE leads to accumulation of acetylcholine at synaptic junctions, resulting in continuous nerve stimulation, paralysis, and eventual death of the insect. Saad *et al.*, (2019) demonstrated significant AChE inhibition in *T. castaneum* exposed to these compounds, along with suppression of ATPase activity, indicating disruption of ion transport and neuronal signaling. In addition, interference with ATPases suggests impairment of

membrane potential regulation and cellular energy homeostasis, further contributing to toxicity.

Beyond neurotoxicity, allelochemicals significantly affect metabolic and digestive pathways. Enzymes such as α-amylase, Acid Phosphatase (ACP), and Alkaline Phosphatase (ALP) are essential for nutrient digestion and assimilation in insects. Botanical compounds have been shown to modulate these enzyme activities, leading to impaired digestion and reduced energy availability. Oyeniyi and Akinnuoye (2025) reported that exposure to botanical extracts altered the activity of these enzymes in *T. castaneum*, with significant variation depending on diet, dosage, and exposure duration. Furthermore, detoxification enzymes such as Glutathione S-Transferase (GST) are also affected, limiting the insect's ability to neutralize toxic compounds. This dual impact on digestion and detoxification creates metabolic stress, ultimately reducing survival and reproductive fitness.

Allelochemicals also interfere with insect growth and developmental processes by acting as Insect Growth Regulators (IGRs). Certain compounds mimic or disrupt hormonal pathways, particularly those involving ecdysteroids and juvenile hormones, which regulate molting and metamorphosis. García *et al.,* (2003) demonstrated that sesquiterpenes such as eudesmane and eremophilane derivatives caused prolonged pupal duration, developmental delays, and morphological abnormalities in *T. castaneum*. These abnormalities included incomplete sclerotization, deformities in appendages, and failure to emerge as viable adults. Such chronic effects are particularly advantageous in pest management, as they suppress population growth over successive generations.

Behavioral modifications constitute another important mode of action. Many allelochemicals act as repellents, antifeedants, or oviposition deterrents by interacting with the insect's chemosensory system. These compounds interfere with olfactory and gustatory receptors, preventing host recognition and feeding behavior. Sesquiterpenes like 3-oxo-γ-costic acid exhibit strong repellency, while other compounds may act as attractants depending on concentration and chemical structure (García *et al.*, 2003). Monoterpenes and essential oils have also been shown to disrupt orientation, aggregation, and mating behaviors, thereby reducing infestation levels in stored grains.

At the cellular level, allelochemicals induce oxidative stress and cellular damage. Many plant compounds generate Reactive Oxygen Species (ROS), leading to lipid peroxidation, protein denaturation, and DNA damage. This oxidative stress disrupts cellular integrity and function, particularly in metabolically active tissues such as the midgut and nervous system. Additionally, interference with mitochondrial respiration and ATP production further

compromises cellular energy balance, contributing to insect mortality. The inhibition of ATPases observed in *T. castaneum* larvae supports the hypothesis that energy metabolism is a critical target of botanical insecticides (Saad *et al.,* 2019).

Another significant aspect of allelochemical action is the interaction between multiple compounds. Botanical extracts often contain complex mixtures of bioactive constituents that may act synergistically or antagonistically. Synergistic interactions enhance toxicity by targeting multiple physiological pathways simultaneously, while antagonistic interactions may reduce overall efficacy. Oyeniyi and Akinnuoye (2025) reported that combinations of botanical extracts exhibited both synergistic and antagonistic effects depending on the host diet and experimental conditions. This highlights the importance of understanding compound interactions for developing effective formulations.

Despite these diverse and potent mechanisms, the effectiveness of allelochemicals is influenced by several extrinsic and intrinsic factors. Environmental conditions such as temperature and humidity, the physicochemical properties of the compounds, and the physiological state of the insect (e.g., developmental stage, nutritional status) all play a role in determining efficacy. Moreover, variability in plant composition due to genetic and environmental factors can lead to inconsistent results, posing challenges for standardization and large-scale application.

2.8. Detoxification enzyme systems in stored product insects

Plant-derived allelochemicals represent a diverse class of bioactive compounds that exert toxic, repellent, antifeedant, and growth-regulatory effects on pests. Unlike synthetic insecticides that typically act on a single molecular target, allelochemicals influence multiple physiological and biochemical pathways, thereby offering a complex mode of action. However, despite this multi-target nature, *T. castaneum* exhibits remarkable adaptive plasticity, necessitating detailed investigation into both mechanisms of action and resistance development.

Despite the diverse modes of action, *T. castaneum* can develop resistance through multiple adaptive mechanisms. The most prominent among these is **metabolic detoxification**, mediated by enzyme systems such as cytochrome P450 monooxygenases, glutathione S-transferases, and carboxylesterases.

Cytochrome P450 Monooxygenases (CYPs): CYP enzymes play a central role in the oxidative metabolism of xenobiotics. Genome-wide analysis has revealed a large and diverse CYP gene family in *T. castaneum*, reflecting its capacity to detoxify a wide range of compounds (Zhu *et al.,* 2013). Specific CYP genes such as CYP6BQ7 and CYP346B

subfamily members are significantly induced upon exposure to plant toxins and fumigants. Functional studies using RNA interference have demonstrated that silencing these genes increases susceptibility to plant-derived toxicants, confirming their role in resistance (Zhang *et al.*, 2021; Wang *et al.*, 2020). Moreover, CYP gene expression can be rapidly upregulated even at low insecticide concentrations, indicating a highly sensitive adaptive response (Liang *et al.*, 2015).

Glutathione S-Transferases (GSTs): GSTs are phase II detoxification enzymes that conjugate toxic compounds with glutathione, facilitating their excretion. Increased GST activity has been consistently associated with insecticide resistance in *T. castaneum*. Resistant strains exhibit elevated GST levels and enhanced detoxification capacity (Reidy *et al.*, 1990). Furthermore, specific GST genes such as TcGSTu1 are upregulated in response to plant compounds like eucalyptol, and gene silencing significantly increases insect susceptibility (Gao *et al.*, 2025). Genome-wide analyses have identified extensive expansion of GST gene families in *T. castaneum*, suggesting evolutionary adaptation to chemical stress (Shi *et al.*, 2012).

Carboxylesterases: Carboxylesterases contribute to resistance by hydrolyzing ester-containing compounds. Enhanced esterase activity has been linked to resistance against organophosphates and other insecticides. For example, malathion-resistant strains exhibit significantly higher esterase activity due to qualitative changes in enzyme properties (Haubruge *et al.*, 2002). Recent studies also indicate that esterase genes are transcriptionally regulated and play roles in both detoxification and reproductive fitness (Wei *et al.*, 2019).

ABC Transporters: ABC transporters are membrane proteins that actively efflux toxic compounds out of cells. These transporters are highly expanded in *T. castaneum* and contribute to insecticide resistance by reducing intracellular toxin concentrations (Broehan *et al.*, 2013). Upregulation of ABC transporter genes has been observed following exposure to insecticides, and inhibition of these transporters increases insect susceptibility (Rösner and Merzendorfer, 2020). Their role in transporting both synthetic and plant-derived compounds highlights their importance in resistance mechanisms.

Gene Expression Plasticity and Adaptation: Transcriptomic studies have revealed that *T. castaneum* exhibits extensive gene expression plasticity in response to chemical stress. Exposure to toxic compounds such as benzoquinones results in large-scale changes in gene expression, particularly in pathways related to oxidation–reduction processes and metabolic detoxification (Guo *et al.*, 2023). This plasticity enables rapid adaptation to environmental toxins, including allelochemicals.

Enzyme Induction and Species-Specific Responses: Comparative studies between *T. castaneum* and related species demonstrate that differences in detoxification enzyme activity can influence susceptibility to botanical compounds. Variations in GST and oxidase activity determine differential tolerance levels between species. Such findings emphasize the role of enzymatic regulation in resistance development.

2.9. CRISPRCAS9 as a functional genomics tool in entomology

The advent of CRISPR/Cas9 technology has revolutionized functional genomics by providing a precise, efficient, and programmable system for genome editing across diverse organisms, including insects. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system, coupled with CRISPR-associated (Cas) proteins, particularly Cas9, enables targeted modification of DNA sequences through site-specific double-strand breaks. These breaks are subsequently repaired via endogenous cellular mechanisms such as Non-Homologous End Joining (NHEJ) or Homology-Directed Repair (HDR), resulting in gene disruption or precise genetic modification (Banerjee *et al.*, 2023).

Mechanistically, CRISPR/Cas9 relies on a guide RNA (gRNA) that directs the Cas9 nuclease to a complementary DNA sequence, enabling highly specific genome targeting. This programmable specificity distinguishes CRISPR/Cas9 from earlier genome-editing tools such as Zinc Finger Nucleases (ZFNs) and TALENs, making it more accessible and versatile for entomological research. In insects, this system has facilitated not only gene knockout studies but also gene knock-in, transcriptional regulation, and functional annotation of genes involved in development, physiology, and pest behavior (Lei *et al.*, 2016).

In the context of *Tribolium castaneum*, a well-established model organism in coleopteran genetics, CRISPR/Cas9 has significantly enhanced functional genomic studies. Early demonstrations of CRISPR efficiency in *T. castaneum* revealed high mutation rates, with 55–80% of injected individuals carrying targeted mutations and up to 100% germline transmission (Gilles *et al.*, 2015). These findings underscore the robustness of CRISPR-mediated genome editing in this species, enabling stable heritable modifications across generations. Such high efficiency is particularly advantageous for studying gene function in developmental biology, insect physiology, and pest management.

Further advancements include the development of **transgenic Cas9-expressing lines,** which improve editing efficiency and reduce the need for repeated microinjections. Transgenic lines expressing Cas9 under constitutive or inducible promoters have demonstrated effective genome editing even without external induction, thereby streamlining experimental workflows and enabling large-scale genetic studies (Rylee *et al.*, 2022). These innovations have expanded

the CRISPR toolkit in *T. castaneum*, making it comparable to other model organisms such as *Drosophila melanogaster*.

CRISPR/Cas9 has also been instrumental in elucidating **metabolic and developmental pathways** in insects. Targeted knockout of the vermilion gene, encoding tryptophan 2,3-dioxygenase, resulted in disruption of the ommochrome pathway and produced a white-eyed phenotype in *T. castaneum*. This study demonstrated the ability of CRISPR to generate precise frame-shift mutations leading to loss of gene function, thereby validating gene function through phenotypic analysis (Adrianos *et al.,* 2018). Such targeted gene disruptions provide valuable insights into biochemical pathways and their physiological relevance.

Beyond basic research, CRISPR/Cas9 holds immense potential in **applied entomology and pest management.** Functional genomic studies enabled by CRISPR can identify key genes involved in insecticide resistance, detoxification pathways, reproduction, and behavior. This knowledge can be exploited to design targeted pest control strategies, including gene knockouts that reduce fitness or disrupt reproductive capacity. Additionally, CRISPR-based gene drives have been proposed as a tool for controlling pest populations, although ethical and ecological considerations remain critical.

In stored grain pests like *T. castaneum*, CRISPR/Cas9 can be particularly valuable in understanding **molecular mechanisms of resistance**, including genes encoding detoxification enzymes such as cytochrome P450s, glutathione S-transferases, and esterases. Functional validation of these genes through knockout or gene-editing approaches can establish causal relationships between gene expression and resistance phenotypes. This is especially important in the context of increasing resistance to both synthetic insecticides and botanical compounds. Despite its transformative potential, several challenges limit the widespread application of CRISPR in entomology. Efficient delivery of CRISPR components into insect embryos, off-target effects, and variability in editing efficiency across species remain significant constraints. Moreover, regulatory frameworks and biosafety concerns surrounding genetically modified organisms must be addressed before field-level applications can be realized.

CRISPR/Cas9 has emerged as a powerful functional genomic tool in entomology, enabling precise manipulation of insect genomes and advancing both fundamental and applied research. In *Tribolium castaneum*, the system has demonstrated high efficiency in gene editing, facilitating studies on development, metabolism, and resistance mechanisms. Continued advancements in CRISPR technology, including improved delivery systems and reduced off-target effects, are expected to further enhance its utility in sustainable pest management strategies.

2.10. *In silico* approaches for CRSIPR target validity

The successful application of CRISPR/Cas9 genome editing relies heavily on the accurate design and validation of guide RNA (gRNA), which determines the specificity and efficiency of gene targeting. In recent years, *in silico* approaches have emerged as indispensable tools for predicting gRNA performance, minimizing off-target effects, and optimizing genome editing outcomes prior to experimental validation. These computational strategies integrate sequence analysis, predictive modeling, and genomic databases to streamline CRISPR-based research, particularly in non-model organisms such as *T. castaneum.*

A fundamental step in CRISPR/Cas9 workflows is the selection of efficient gRNA sequences. Although multiple candidate gRNAs can be designed based on the presence of Protospacer Adjacent Motif (PAM) sites, their editing efficiencies vary significantly. Computational tools evaluate parameters such as GC content, nucleotide composition, thermodynamic stability, and sequence context to predict on-target activity. However, studies have demonstrated that high in silico scores do not always correlate with actual editing efficiency. The variation in indel formation rates among gRNAs with similar predicted scores highlights the necessity for validation beyond computational prediction (Kim *et al.,* 2021). This underscores that *in silico* design serves as a preliminary filtering step rather than a definitive predictor of CRISPR efficiency.

Beyond efficiency prediction, a critical component of *in silico* analysis is the identification of potential off-target sites. Off-target effects occur when gRNAs bind to genomic regions with partial sequence similarity, leading to unintended mutations. Computational algorithms scan entire genomes to identify sequences with allowable mismatches, thereby predicting potential off-target loci. Advanced workflows integrate these predictions with experimental validation techniques such as GUIDE-Seq and SITE-Seq, enabling comprehensive off-target profiling. Notably, *in silico* predictions alone often underestimate off-target events, emphasizing the importance of combining computational and empirical approaches for accurate validation (Patel *et al.,* 2020).

Recent advancements in machine learning and predictive modeling have further improved the accuracy of gRNA selection. Models such as gradient-boosted regression trees analyze large datasets of experimentally validated gRNAs to identify key determinants of editing efficiency. Features such as nucleotide identity, positional sequence preferences, and microhomology patterns have been identified as significant contributors to CRISPR activity (Fusi *et al.,* 2015). These models enable researchers to prioritize highly efficient and specific gRNAs, thereby reducing experimental trial-and-error.

In the context of insect genomics, the availability of high-quality genome assemblies and annotation datasets significantly enhances *in silico* CRISPR design. For *T. castaneum,* improved genome assemblies and gene annotations have facilitated precise identification of target genes, transcription start sites, and regulatory regions. The integration of RNA-Seq data and gene models allows for accurate targeting of functional regions, including coding sequences, splice variants, and Untranslated Regions (UTRs), thereby increasing the success rate of gene editing experiments (Herndon *et al.,* 2020). These genomic resources are particularly valuable for functional studies involving detoxification genes, metabolic pathways, and resistance-associated loci.

Another important aspect of *in silico* CRISPR validation is the integration of functional genomics and pathway analysis. Computational tools can predict the impact of gene knockout on metabolic pathways, enabling researchers to select targets with high biological relevance. The targeting genes involved in detoxification pathways or insecticide resistance can be strategically planned using pathway databases and gene interaction networks. Such integrative approaches enhance the translational potential of CRISPR technology in pest management.

Furthermore, *in silico* methods play a crucial role in designing multiplex CRISPR strategies, where multiple genes are targeted simultaneously. Computational tools assist in selecting compatible gRNAs that minimize interference and maximize editing efficiency. This is particularly relevant in complex traits such as insecticide resistance, where multiple genes and pathways are involved.

Despite these advancements, several limitations persist in *in silico* CRISPR validation. Predictive models may not fully account for chromatin accessibility, epigenetic modifications, and cellular context, which significantly influence editing efficiency *in vivo*. Also, variability in genome quality and annotation accuracy can affect target prediction, especially in non-model organisms. Therefore, in silico predictions must be complemented with experimental validation to ensure reliability.

In silico approaches form a critical foundation for CRISPR/Cas9 target validation by enabling efficient gRNA design, off-target prediction, and functional target prioritization. The integration of computational modeling, genomic resources, and experimental validation provides a robust framework for precise genome editing. In *T. castaneum*, these approaches significantly enhance the feasibility of functional genomics studies and pave the way for advanced applications in insect pest management and resistance analysis.

2.11. Protein modelling and docking in botanical insecticides

Protein modelling and molecular docking have emerged as powerful in silico tools for understanding the interaction between bioactive phytochemicals and target proteins in insect pests. These approaches provide mechanistic insights into how plant-derived compounds exert insecticidal, repellent, or antifeedant effects at the molecular level. In recent years, the integration of computational biology with phytochemical research has significantly advanced the discovery and optimization of botanical insecticides.

Protein modelling involves the prediction of three-dimensional (3D) structures of target proteins, particularly when experimentally resolved structures are unavailable. In insect pest research, key molecular targets include enzymes such as acetylcholinesterase (AChE), glutathione S-transferases (GSTs), cytochrome P450 monooxygenases, and digestive proteases. Accurate structural modelling of these proteins enables the identification of active sites and binding pockets, which are essential for subsequent docking analysis. Advances in structural bioinformatics, including homology modelling and AI-based prediction tools, have greatly improved the reliability of protein models used in entomological studies.

Molecular docking, on the other hand, simulates the interaction between ligands (phytochemicals) and target proteins to predict binding affinity, stability, and interaction patterns. Docking studies help identify potential lead compounds with strong inhibitory activity against essential insect enzymes. Phytochemicals such as kaempferol and diosmetin derived from *Artemisia absinthium* demonstrated higher binding affinity towards acetylcholinesterase than the standard insecticide malaoxon, suggesting their potential role in neurotoxicity-mediated insect control (Yaseen *et al.*, 2025). Such findings validate the role of plant secondary metabolites as effective enzyme inhibitors.

In addition to neurotoxic targets, docking studies have also explored interactions with **olfactory and behavioral proteins**, such as Odorant-Binding Proteins (OBPs), which play a crucial role in host recognition and feeding behavior. Compounds like camphor, carvacrol, and oleic acid have shown strong binding interactions with OBPs, indicating their potential as repellents by disrupting insect olfactory signaling pathways (Gopal and Kannabiran, 2013). This highlights the broader applicability of molecular docking beyond toxicity, extending to behavioral modification strategies in pest management.

Another important application of docking is in evaluating **enzyme inhibition and metabolic disruption.** Botanical extracts such as those from neem (*Azadirachta indica*) and rosemary (*Rosmarinus officinalis*) have been shown to interact with key enzymes like DNA gyrase and metabolic proteins, with compounds such as limonene exhibiting strong binding affinity and stability in molecular dynamics simulations (Alhaithloul *et al.*, 2023). Similarly,

secondary metabolites from *Ocimum basilicum* demonstrated inhibitory effects on proteolytic enzymes, including trypsin-like serine proteases and metalloproteases, supported by docking and ADMET analysis (Darrag *et al.*, 2022). These interactions indicate that botanical compounds can impair digestion and metabolism, leading to reduced insect growth and survival.

Docking studies are also increasingly integrated with **formulation and nanotechnology-based approaches.** Nanoemulsions of insecticides have been shown to enhance delivery and efficacy, with docking analysis confirming strong interactions between active compounds and target enzymes such as AChE, ATPase, and GST (Badawy *et al.*, 2022). This combination of formulation science and molecular docking provides a comprehensive understanding of both efficacy and mechanism of action.

One of the major advantages of molecular docking is its ability **to screen multiple compounds rapidly**, thereby reducing the time and cost associated with experimental assays. By prioritizing compounds with high binding affinity and favorable interaction profiles, researchers can focus on the most promising candidates for in vitro and in vivo validation. Additionally, docking studies provide insights into binding modes, hydrogen bonding, hydrophobic interactions, and conformational stability, which are critical for rational pesticide design.

However, despite its advantages, molecular docking has certain limitations. The accuracy of docking results depends on the quality of protein structures and the algorithms used. Moreover, docking does not fully account for dynamic biological conditions, such as protein flexibility, cellular environment, and metabolic transformations. Therefore, docking results must be complemented with experimental validation, including bioassays and enzyme inhibition studies.

Thus, protein modelling and molecular docking serve as essential tools in botanical insecticide research by elucidating the molecular mechanisms underlying insecticidal activity. These approaches enable the identification of target-specific interactions, facilitate the discovery of novel bioactive compounds, and support the development of eco-friendly pest management strategies. When integrated with experimental studies and advanced formulations, docking-based research significantly enhances the efficiency and precision of botanical pesticide development.

2.12. Research gap and rationale of the present study

Despite extensive research on stored grain pests and their management, several critical gaps persist in the current understanding and application of control strategies against *T. castaneum*. Although earlier studies have comprehensively documented the biology, ecology, and economic significance of this pest, there remains a lack of integration between fundamental biological insights and applied pest management approaches. Most investigations treat insect biology, insecticide resistance, and control strategies as independent domains, thereby limiting the development of holistic and effective management systems. Furthermore, while the limitations of synthetic insecticides including resistance development, environmental persistence, and toxic residues have been widely reported, the transition toward sustainable alternatives has not been fully realized in practical storage systems.

Botanical insecticides have emerged as promising eco-friendly alternatives; however, existing studies are largely confined to the evaluation of individual plant extracts or isolated phytochemicals under laboratory conditions. There is a notable lack of research on synergistic combinations of botanicals, particularly spice-based formulations, which could potentially enhance efficacy through multi-component interactions. Also, investigations on the insecticidal properties of individual spices such as clove, cinnamon, turmeric, garlic, and black pepper have demonstrated significant repellency, toxicity, and growth inhibition effects against *T. castaneum*. Nevertheless, these studies remain fragmented and lack standardization in terms of formulation, dosage optimization, and long-term efficacy assessment. Importantly, the translation of such findings into practical, user-friendly delivery systems suitable for grain storage environments remains largely unexplored.

Although recent advancements in formulation technologies, including nanoemulsions, microencapsulation, and controlled-release systems, have improved the stability and efficacy of botanical insecticides, their application in low-cost and farmer-adoptable storage solutions is still limited. This highlights a clear disconnect between advanced formulation research and real-world applicability. Moreover, while studies on the mode of action of allelochemicals have identified multiple biochemical targets such as acetylcholinesterase inhibition, disruption of metabolic enzymes, and interference with insect growth and development, there is insufficient understanding of the combined effects of multiple phytochemicals present in botanical mixtures. The complexity of interactions between these compounds and insect physiological systems remains inadequately addressed.

In addition, the advent of molecular and computational tools such as CRISPR/Cas9 genome editing, molecular docking, and in silico pathway analysis has opened new avenues

for understanding insecticide targets and resistance mechanisms. However, their application in stored grain pest management is still in its infancy. There is a lack of studies integrating gene-level insights, protein–ligand interactions, and biochemical responses with practical pest control strategies. Similarly, molecular docking studies evaluating phytochemical interactions with insect target proteins are often conducted in isolation without validation through experimental bioassays. Another major gap lies in the limited attention given to resistance development against botanical insecticides. Although botanicals are often considered less prone to resistance, emerging evidence suggests that insects may still develop adaptive responses through detoxification enzymes and metabolic pathways, necessitating further investigation.

Thus, the present study entitled **"Integrated experimental and in silico evaluation of spice - based Phytonova Balls for the management of red flour beetle, *Tribolium castaneum* (Herbst)"** is conceptualized to address the major gaps identified in the existing literature. While previous studies have established the insecticidal potential of individual botanicals, there remains a lack of research on integrated, multi-component formulations that combine efficacy, stability, and practical applicability. The development of spice-based Phytonova Balls represents an innovative approach aimed at creating a sustained-release, eco-friendly pest control system suitable for stored grain environments.

This study adopts a multidisciplinary framework by integrating experimental bioassays with *in silico* analyses to provide a comprehensive understanding of both efficacy and mechanism of action. Experimental evaluations will generate data on repellency, toxicity, and biological effects on *T. castaneum*, while computational approaches such as molecular docking and pathway analysis will elucidate protein–ligand interactions and target-specific mechanisms. Such integration enables a deeper understanding of how phytochemicals interact with insect physiological systems at the molecular level.

Furthermore, the use of multi-component botanical formulations is expected to reduce the likelihood of rapid resistance development due to their complex modes of action. By targeting multiple biochemical pathways simultaneously, these formulations may offer a more sustainable alternative to conventional synthetic insecticides. The present study also aims to bridge the gap between laboratory findings and practical application by developing a formulation that is not only effective but also economical, biodegradable, and user-friendly.

III. Materials and Methods

Storage of grains and seeds is essential for food security and future crop production. During storage, their quality is mainly affected by insect pests, which cause major losses. Currently, pest control depends largely on synthetic insecticides, However, plant-based products can be used as safer alternatives to reduce grain losses. Botanicals, derived from plants, contain active compounds that helps to control storage pests. Spices have also been traditionally used to protect stored grains from insects. Thus, the methodology adopted for the present study entitled "**Integrated experimental and *In Silico* evaluation of spice - based Phytonova Balls for the management of red flour beetle, *Tribolium castaneum* (Herbst)"** were discussed under as follows:

3.1. Rearing of *T. castaneum*

3.2. Collection and processing of ingredients

3.3. Experimental designs and treatment details

3.4. Selection of detoxification genes in *T. castaneum*

3.5. Retrieval of full-length coding sequences

3.6. In- silico CRISPR Cas 9 sgRNA Design using CHOPCHOP

3.7. Protein structure Modelling of target gene

3.8. Molecular docking of spice phytochemical

3.9. Network and pathway analysis

3.10. Integration of computational analyses

3.1. Rearing of test insects *Tribolium castaneum*

Adult red flour beetles (*Tribolium castaneum* Herbst,1797) was collected from the local traders and identified with standard identification keys of Hinton (1948). The wheat flour was purchased from the local grocery. The *T. castaneum* was introduced into the flour in a glass jar. From the mother culture, 20 adults were introduced into two separate glass jars, each containing 250 grams of broken wheat. The setup was maintained under the optimal conditions of temperature (30±2°C) and relative humidity (75%). The emerging adult (F_1 generation) after 60 days was utilized for further study (Fig.1)

Fig .1. Test Insects: *Tribolium castaneum*

3.2. Collection and processing of ingredients

With the objective of developing an effective, safe, and eco-friendly alternative to synthetic pesticides, selected botanicals were evaluated for its insecticidal property. The botanicals include spices such as pepper (Ashouri and Shayesteh 2010), cinnamon (Baker and Grant, 2018), clove, turmeric (Ashouri and Shayesteh 2010), leaves such as bay leaf (Batool *et al.*,2020), spring onion (Block,1992), peels like orange peel (Romelle *et al.*, 2016), ginger peel (Ivane and Sun 2023) and garlic peel (Wei *et al.*, 2009) (Table- 1). The whole raw spices were procured from Uyir Organics Private Limited, Sai Baba colony, Coimbatore. The leaves and peels were shade dried and powdered as illustrated in Fig 2.

Table – 1 List of selected botanicals with common and scientific names

Botanicals	Common Name	Scientific Name
Spices	Pepper	*Piper nigrum*
	Cinnamon	*Cinnamomum verum*
	Clove	*Syzygium aromaticum*
	Turmeric	*Curcuma longa*
Leaves	Bay	*Laurus nobilis*
	Spring onion	*Allium fistulosum*
Peels	Orange	*Citrus sinensis.*
	Ginger	*Zingiber officinale Roscoe*
	Garlic	*Allium sativum*

Fig. 2. Selected botanicals for formulating treatment ball

3.2.1. Formulation of Phytonova Balls

Phytonova Balls (PNB) were prepared using one gram of each botanical at concentrations of 0.5 g, 1.0 g, and 1.5 g (Fig. 3.). It is a technique in which powders were converted into spherical balls by adding appropriate amount of distilled water and continuous rolling (Deb and Ahmed, 2013). The mixture was compressed under pressure to produce balls of defined shape and uniform size.

3.2.1.1.Characterization of Phytonova Balls

The ethnobotanical information was used to discern the possible bioactive molecules of the formulations using spectral analysis. The structural clarification of the bioactive molecules was determined using Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography – Mass Spectroscopy (GCMS).

3.2.2. Formulations of standards – Diatomaceous Earth balls and Neem balls

Diatomaceous Earth (DE), was procured from Unitedlys, Telangana (Amazon - United sales). The neem leaves were collected from the Campus I of Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamil Nadu. Diatomaceous Earth Balls (DEB) were prepared at concentrations of 0.5 g, 1.0 g, and 1.5 g (Fig. 4.). The Neem Balls (NB) were prepared at 1g. It is a technique in which powders were converted into spherical balls by adding appropriate amount of distilled water and continuous rolling (Deb and Ahmed, 2013). The balls were formulated in the ratio 1:1 (Fig. 5.). It was compressed under pressure to produce balls of defined shape and uniform size.

Fig. 3. Phytonova Balls formulated at different concentrations at 0.5g, 1.0g, and 1.5 g

Fig. 4. Diatomaceous Earth Balls formulated at different concentrations at 0.5g, 1.0g, and 1.5 g

Fig. 5. Neem Balls formulated at 1 g

3.2.3. Standardization of unit weight and size

The thickness and diameter of the pellets were determined using a digital Vernier caliper. Readings were obtained for PNB, DEB, and NB, and the average values were expressed at means. The pellets were shade dried at room temperature, and stored separately in Zip lock cover for future use.

3.2.4. Cost analysis

The cost analysis was done based on the market prices, available on the local stores.

Orange peel, Ginger peel, Garlic peel was collected from household waste. The market Cost of 100 grams, Spring onion ₹ 20, Clove ₹ 200, Cinnamon ₹150, Bay leaf ₹30, Pepper ₹120, Turmeric ₹60 (Table - 2) The results were expressed as cost per unit of treatment and cost per kilogram of grain protected. Only the costs of ingredients were considered, while labour, preprocessing, packaging, and transportation were excluded from the analysis

Table - 2 Cost evaluation of selected botanicals for Phytonova Balls

Botanicals	Cost in ₹ per g
Spring onion	20
Clove	200
Cinnamon	150
Bay leaf	30
Pepper	120
Turmeric	120

3.3 Experimental designs and treatment details

3.3.1 Bioassay studies

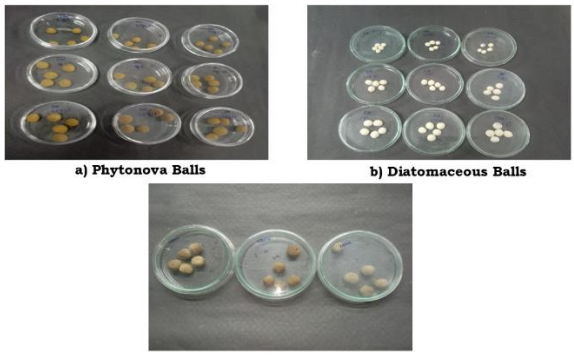
The experiment was laid on Completely Randomized Design (CRD). PNB, DEB and NB tested. The different concentrations of the PNB and DEB were 0.5 g, 1.0g, and 1.5g while NB at 1g concentrations. DEB and NB were considered as standard treatments. Twenty grams (20g) of broken wheat was taken in Ziplock cover and treated with different concentrations of PNB, DEB and NB. Ten freshly emerged insects of *T. castaneum* were introduced into each cover and the treatments and standards were replicated thrice. The following parameters were considered to assess the efficacy of PNB against *T. castaneum*.

3.3.2. Repellent activity of Phytonova Balls, Diatomaceous Balls and Neem Balls

The repellence test was conducted by the standard method described by McDonald *et al.,* (1970) with some modifications. In the Petri dish, 5 numbers of PNB, DEB (0.5g, 1g, 1.5g) and NB(1g) with different concentrations were kept individually. In the center of the dish, ten adults of *T. castaneum* (7-14 days) were introduced and closed (Fig. 6). The data on the no of insects repelled to the treatments were recorded at ½ an hour, 12 ,24 ,72 hour. The percentage repellency was calculated using the following formula.

$$R = 2(C- 50)$$

Where C is the percentage of insects in the untreated half. Positive values expressed repellence and negative values attractancy.



a) Phytonova Balls b) Diatomaceous Balls

c) Neem Balls

Fig.6. Experimental setup for repellent activity

3.3.3 Adult mortality

Mortality of the insects were observed from the control, treatments, and standard after exposure to 12, 24, 48, 72 hours and recorded (Fig.7, 8). Percent adult mortality was calculated by using Abbott's percent corrected mortality formula (1925).

$$\text{Adult mortality (\%)} = \frac{\text{\% mortality in control seeds} - \text{\% mortality in treated seeds}}{\text{\% mortality in control seeds}} X\ 100$$

Fig. 7 . Experimental setup against *Tribolium castaneum*

Fig. 8. Mortality of formulated Phtonova Balls, Diatomaceous Earth Balls, Neem Balls against *Tribolium Castaneum*

3.3.4 Adult emergence

The number of adults emerged from the seven treatments were recorded 40 days after treatments.

3.3.5. Weight loss of grains

The seeds from the treatments were weighed 40 days after storage period by removing the dust and insects from the grains. Percent seed weight loss was determined by the following formula given by Harris and Linblad (1978)

$$\text{Percentage weight loss} = \frac{\text{Original weight of seeds} - \text{Current weight of seeds}}{\text{Original weight of seeds}} \text{ X } 100$$

3.4 Data Analysis

Probit analysis was used to estimate LC_{50} values. One-way or two-way ANOVA was performed to compare treatment effects, and Duncan's Multiple Range Test (DMRT) for comparison of means.

3.5 Selection of detoxification genes in *T. castaneum*

Based on this evidence, five genes were selected for computational investigation: CYP6BQ9, CYP6BQ11 and GSTd1. The gene CYP6BQ9 has been functionally validated as a major determinant of deltamethrin resistance in *T. castaneum*, where RNAi knockdown significantly increased insect susceptibility (Zhu *et al.,* 2010). Regulation of the CYP6BQ gene cluster by transcription factors further indicates its central role in metabolic resistance (Kalsi and Palli, 2015). CYP6BQ9 and CYP6BQ11 were selected because they represent inducible detoxification enzymes capable of metabolising structurally diverse xenobiotics, including plant-derived compounds (Zhu *et al.,* 2010; Kalsi and Palli, 2015; Xiong *et al.,* 2019) Glutathione-S-transferases (GSTs) conjugate electrophilic insecticide metabolites with glutathione, increasing their solubility and facilitating excretion. GST activity has been reported to be elevated in resistant strains of *T. castaneum*, indicating a role in detoxification of both synthetic and natural toxicants (Zhu *et al.,* 2007). The gene GSTd1 was selected to represent Phase II conjugation pathways and to evaluate potential binding interactions with phytochemical metabolites. (Zhu *et al.,* 2007)

3.6 Retrieval of full-length coding sequences

Full-length coding sequences (CDS) and corresponding protein sequences of selected detoxification and resistance-associated genes (CYP6BQ9, CYP6BQ11 and GSTd1) of *T. castaneum* were retrieved from the NCBI RefSeq genome database using the most recent genome assembly. Gene identities were verified using annotated gene models and previously published resistance-related gene records. Only complete CDS containing both start and stop codons were retained for analysis. Protein sequences were obtained either directly from RefSeq protein entries or by in silico translation of nucleotide sequences using the standard genetic code. Accurate retrieval of full-length gene and protein sequences is essential for structural

modelling and docking studies, as truncated or partial sequences may lead to incorrect folding and misleading interaction predictions (Rösner *et al.*, 2020).

3.7 Network and pathway analysis

Protein–protein interaction networks of the selected detoxification genes were constructed using the STRING database with *T. castaneum* as the reference organism. Interaction networks were imported into Cytoscape software for visualization and topological analysis. Functional enrichment analysis was performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases to identify biological processes and metabolic pathways associated with xenobiotic detoxification and resistance. Docking results were integrated with network analysis by mapping ligand-interacting proteins onto enriched detoxification pathways, allowing identification of key nodes potentially involved in tolerance to botanical phytochemicals. Network-based analysis provides systems- level understanding of how multiple detoxification genes interact and cooperate in resistance mechanisms (Szklarczyk *et al.*, 2019). Pathway enrichment enables biological interpretation of molecular interaction data within metabolic and regulatory frameworks.

3.8 **In- silico CRISPR Cas 9 Sg RNA Design using CHOPCHOP**

Single guide RNAs (sgRNAs) targeting the selected detoxification genes were designed using the CHOPCHOP v3 web server. The *T. castaneum* genome was selected as the reference organism, and SpCas9 (NGG protospacer adjacent motif) was used as the endonuclease system. Exonic regions located near the 5′ end of each gene were prioritised to maximise the probability of generating functional knockouts. Candidate sgRNAs were filtered based on predicted on-target efficiency, GC content (40–70%), and minimal off-target matches within the genome. For each gene, the top three sgRNAs were selected and their genomic coordinates, PAM sequences and efficiency scores were recorded. Although no experimental genome editing was conducted in this study, sgRNA design was included to evaluate gene targetability and to support future functional validation strategies. Computational sgRNA design enables prediction of gene editability and provides a rational basis for linking structural docking results with potential genetic intervention points (Labun *et al.*, 2019).

3.8 Protein structure Modelling of target gene

Three-dimensional protein structures of the selected detoxification enzymes were obtained using structure prediction approaches. Protein sequences were submitted to the AlphaFold Protein Structure Database to retrieve predicted structures. For proteins lacking suitable AlphaFold models, homology modelling was performed using the SWISS-MODEL server based on the closest homologous templates from the Protein Data Bank (PDB). The quality of

predicted protein structures was evaluated using Ramachandran plot analysis and model quality assessment metrics. Structures with acceptable stereochemical quality were selected as receptors for molecular docking studies. High-resolution protein structure prediction enables identification of active sites and ligand-binding cavities and has been shown to achieve near-experimental accuracy for many proteins (Jumper *et al.*, 2021). Homology modelling provides reliable structural approximations when experimental structures are unavailable (Waterhouse *et al.*, 2018).

3.9 Molecular docking of Spice Phytochemical

Major phytochemical constituents of the Phytonova Balls were retrieved from public chemical databases in SMILES format. Ligand structures were converted to three-dimensional conformations using Open Babel and energy minimised using the MMFF94 force field. Protein structures were prepared by removing water molecules and adding polar hydrogens and partial charges. Molecular docking was performed using Auto Dock Vina. Grid boxes were defined around predicted ligand-binding regions based on known catalytic residues or pocket prediction algorithms. Docking simulations were run with an exhaustiveness value of 8, generating ten binding poses per ligand–protein interaction. Binding affinities were recorded in kcal/mol and the most stable conformations were visualised using PyMOL. Protein–ligand interactions were analysed based on hydrogen bonds, hydrophobic interactions and π–π stacking. Molecular docking predicts the binding orientation and affinity of ligands within enzyme active sites and is widely used to evaluate interactions between phytochemicals and detoxification enzymes (Trott and Olson, 2010). Docking has been successfully applied to insect detoxification enzymes to infer metabolic or inhibitory roles of plant compounds (Pavela and Benelli, 2016)

3.11 Integration of computational analyses

Results from sequence analysis, protein structure modelling, molecular docking and network/pathway analysis were integrated to identify key detoxification genes potentially involved in tolerance to botanical phytochemicals. Comparative binding affinities of phytochemicals with detoxification enzymes were correlated with their predicted roles in metabolic and efflux pathways. This integrative in silico framework was used to propose a mechanistic model of detoxification-mediated resistance in *T. castaneum*. Botanical insecticides consist of multiple active compounds that interact with several detoxification pathways simultaneously. An integrated computational approach allows prediction of multi-target interactions and resistance mechanisms without the need for wet-lab experimentation (Dermauw and Van Leeuwen, 2014).

IV. Results and Discussions

The results of the experimental analysis carried out for the research work entitled "**Integrated experimental and *In Silico* evaluation of spice - based Phytonova Balls for the management of red flour beetle, *Tribolium castaneum* (Herbst)"** were interpreted and discussed under various headings as follows:

4.1. Dimensional and Cost analysis of formulated Balls

4.2. Characterization of Phytonova Balls

4.3. Bioassay Studies

4.4. *In silico* studies

4.1. Dimensional and Cost analysis of formulated Balls

4.1.1. Characterization of Formulated Balls

The diameter of formulated treatment balls, PNB, DEB, and NB was measured using a vernier caliper, and the results were presented in Table 3.

Table 3 – Measurement of formulated balls

Weight of the balls (g)	Treatment	Size in cm
0.5	**PNB**	1.20 ± 0.18
1	**PNB**	1.46± 0.05
1.5	**PNB**	1.59±0.05
0.5	**DEB**	0.89 ± 0.05
1	**DEB**	1.22 ± 0.09
1.5	**DEB**	1.41 ± 0.04
1	**NB**	1.32± 0.05

Note: Values are expressed as Mean ± SD

PNB – Phytonova Ball, DEB – Diatomaceous Earth Ball, NB – Neem Ball

The size of formulated treatment balls Phytonova, Diatomaceous Earth, and Neem exhibited a consistent and proportional increase with increasing weight, indicating uniformity in preparation and scalability of the formulations. In the case of PNB, a clear incremental trend in diameter was observed with increasing weight. The smallest balls were recorded at 0.5 g (1.20 ± 0.18 cm), followed by a noticeable increase at 1 g (1.46 ± 0.05 cm), and reaching the maximum diameter at 1.5 g (1.59 ± 0.05 cm). This progressive increase demonstrates a direct relationship between formulation quantity and ball size.

A similar pattern was evident in DEB. The 0.5 g treatment produced the smallest balls (0.89 ± 0.05 cm), which increased to 1.22 ± 0.09 cm at 1 g and further to 1.41 ± 0.04 cm at 1.5 g. Although the trend mirrors that of PNB, the overall diameters were comparatively smaller at corresponding weights, suggesting differences in material properties such as density

or binding characteristics. The NB, at 1 g, recorded balls with a mean diameter of 1.324 ± 0.05 cm. The size was intermediate, between DEB and PNB at the same weight (1g).

The relatively higher standard deviation observed in the 0.5 g PNB treatment suggests slight variability in ball formation, likely due to challenges in achieving uniformity at lower quantities. Thus, the results confirm a systematic increase in ball diameter with increasing formulation weight across all treatments, highlighting consistency in preparation and the influence of formulation composition on final ball size.

4.1.2. Cost analysis of formulated balls

To establish the economic viability of the developed formulation, a comparative cost analysis of PNB with DEB and NB were carried out and presented in Table 4 and 5.

Table 4 – Cost analysis of Phytonova Balls at different concentrations

Botanical	Cost (₹/100 g)	Cost per g (₹)	Quantity used (g)	Cost contribution (₹)
Spring onion	20	0.20	1	0.20
Clove	200	2.00	1	2.00
Cinnamon	150	1.50	1	1.50
Bay leaf	30	0.30	1	0.30
Pepper	120	1.20	1	1.20
Turmeric	120	1.20	1	1.20
Total	—	—	**6 g**	**₹6.40**

The present cost analysis (Table 4) clearly demonstrates that the PNB, developed using a blend of six botanicals at equal proportions, incurred a cost of ₹6.40 per unit, which is comparatively higher than the other tested formulations. This elevated cost is primarily due to the incorporation of high-value spice components such as clove, cinnamon, and pepper, which significantly contribute to the total formulation cost. However, these ingredients are well documented for their potent bioactive compounds, including eugenol, cinnamaldehyde, and piperine, which possess strong insecticidal, repellent, and growth-inhibitory properties.

Table 5 – Cost analysis of Diatomaceous Earth and Neem Balls at different concentrations

Ball	Cost (₹/Kg)	Cost per g (₹)	Quantity used (g)	Cost contribution (₹)
Diatomaceous Earth	350	0.35	0.50	0.18
			1.0	0.35
			1.5	0.53
Average	-	-		0.35
Neem	120	1.2	1.0	0.12

In contrast, the cost of the DEB balls, with costing ₹0.35, and the NB costing ₹0.12 per unit, represent simpler and more economical formulations. Their lower cost is attributed to the use of single active ingredients with relatively inexpensive market prices. While DE acts primarily through physical desiccation of insects and neem exerts biochemical effects mainly due to azadirachtin, and their modes of action are comparatively limited when considered individually.

Importantly, the PNB formulation offers a multi-component, synergistic mode of action, combining several botanicals that may act on different physiological and behavioral pathways of storage pests. This integrative approach enhances the likelihood of improved efficacy, prolonged protection, and reduced chances of resistance development. Moreover, the use of plant-derived materials aligns with eco-friendly pest management strategies and supports sustainable storage practices.

Therefore, although the initial cost of PNB is higher, the formulation can be considered cost-effective in a functional sense, as the enhanced bio efficacy and broader spectrum activity may reduce the frequency of application and overall losses during storage. Therefore, the PNB were considered as a value-added, sustainable, and scientifically robust alternative to conventional single-component treatments, particularly in the context of IPM for stored grains

4.2. Characterization of Phytonova Balls

4.2.1. Fourier Transform Infrared Spectroscopy (FTIR) analysis of Phytonova

Fourier Transform Infrared Spectroscopy (FTIR) is a widely used analytical technique for the identification and characterization of functional groups present in complex biological and botanical formulations. The technique is particularly useful for confirming the presence of key phytochemical constituents through their characteristic vibrational frequencies within the infrared region of the electromagnetic spectrum. In any plant-based formulations, FTIR analysis provides valuable information regarding the presence of functional groups such as phenols, alcohols, aldehydes, ketones, ethers, sulfur-containing compounds, and aromatic hydrocarbons, which are commonly associated with biologically active secondary metabolites.

In the present study, the obtained FTIR spectrum revealed a complex profile consisting of aromatic structures, oxygenated functional groups, and minor nitrogen-containing compounds, which are consistent with the phytochemical constituents derived from pepper, cinnamon, clove, bay leaf, turmeric, spring onion, orange peel, ginger peel, and garlic peel. The overall spectral pattern therefore reflects the chemical complexity of the formulation (Fig.9.)

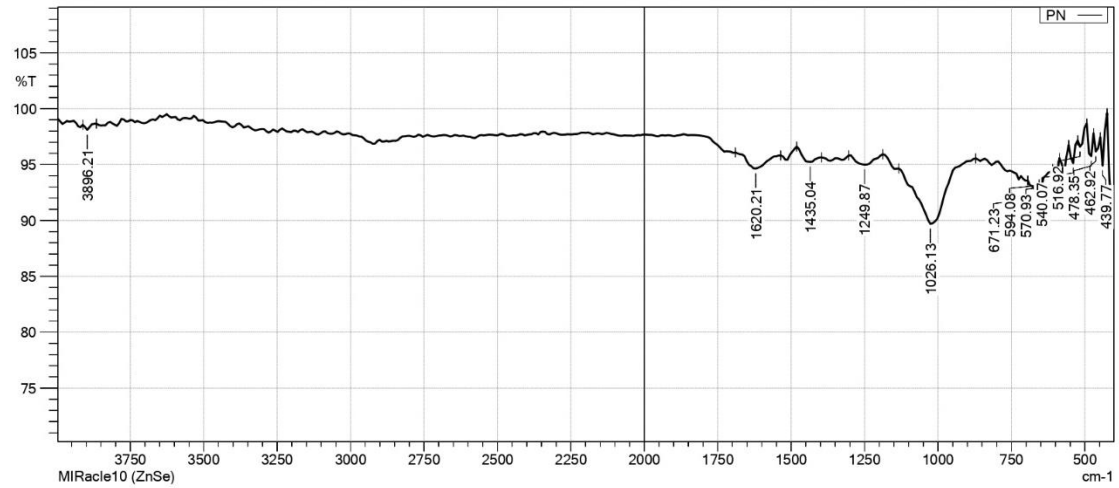


Fig. 9. FTIR analysis of Phytonova

The peak at 1620 cm-^{1} is a strong aromatic C=C stretching indicating the presence of α, β, unsaturated ketone groups. The band at 1435 with medium intensity confirms the presence of hydroxy group. The peak at 1249 corresponds to C-N stretching of amine functional group. The peak at 1026 corresponds to the fluro compounds. The strong bands between the range 516-671could be attributed to the C-Br stretching vibrations, indicating the presence of bromo compounds. The peak obtained at 1620 in the present study could be linked to the findings of Chowdhury *et al.,* (2026) who investigated the peak range from 1600-1630 might be due to the presence of eugenol, proteinaceous and polyphenolic chemicals.

The fingerprint spectrum range obtained from 1450- 400 indicates the unique structural identity of functional groups from the pepper, cinnamon, clove, bay leaf, turmeric, spring onion, orange peel, ginger peel, garlic peel. The peak obtained at 1435 in the present study could be attributed to the findings of Chen *et al., (*2017*),* who investigated that the range from 1400 –1300 might be due to the O-H bending or C-H deformation vibrations, confirming the presence of carboxylic acids and phenols in cloves. These secondary metabolites are responsible for disrupting biological processes in pests. Also, peak obtained at 1435 in the present study could be linked to findings of Wong *et al.,* (2023) who investigated that vibrations of the peak could be attributed to C=C, confirming the presence of aromatic ring-structured chemicals from zingiberene curcumene, and bisabolene in ginger essential oil.

A distinct peak obtained at 1026 in the present study could be ascribed to the findings of Alvarino *et al.,* (2023) who reported that the range from 1019-590 might be due to C- S stretch representing sulfur-containing compounds like allicin and diallyl disulfide, present in garlic peel. It also include alcohol or phenolic chemicals such as gingerols, zingerone, and shogaols that have the capacity act as antioxidants. Also, peak at 1026 in the present study

could be aligned to the works of Stan *et al.,* 2015 and Taranath *et al.,* 2015, whose findings revealed that the peak might be due to the presence of compounds in turmeric. The peak at 1249 in the present study could be associated to the findings of Hasan *et al.,* 2025 who investigated that the peaks range from 1200- 1000 might be due to C–O–C and C–O stretching vibrations, indicating the presence of ethers, alcohols, and glycosidic linkages. The peak at 671in present study could be associated with the findings of Chowdhury *et al.,* 2026 who determined that the peaks from 700–600 might be due to C-Br vibrations, indicating the presence of aliphatic bromo compounds in bay leaf. The peak at 522 in the present study aligns with similarly, Nair and mukne, 2017 who revealed that the peak from 439-594 might be due to S-S bonds in garlic.

Therefore, the FTIR spectral analysis of the Phytonova formulation revealed the presence of multiple functional groups corresponding to a diverse range of phytochemical constituents. The detected absorption bands confirmed the presence of aromatic compounds, phenolic groups, amine functionalities, sulfur-containing molecules, and ether or glycosidic linkages. These functional groups are commonly associated with plant-derived secondary metabolites such as phenylpropanoids, terpenoids, sulfur compounds, and polyphenols that are known to exhibit significant biological activities. The fingerprint region of the spectrum further demonstrated the complex chemical nature of the formulation, prepared from pepper, cinnamon, clove, bay leaf, turmeric, ginger, garlic, and citrus peel materials. The presence of these functional groups supports the hypothesis that the formulation contains multiple bioactive compounds capable of exerting biological effects.

Thus, the FTIR analysis provides preliminary confirmation of the chemical diversity and structural characteristics of the Phytonova formulation. These findings support the potential role of the formulation as a bioactive botanical preparation and provide a basis for further analytical and biological studies aimed at evaluating its pesticidal efficacy.

4.2.2. GC–MS characterization of Phytonova Balls

GC–MS analysis of Phytonova Balls was performed, and the resulting Total Ion Chromatogram (TIC) were presented in Table 6 and Fig. 10. A total of fifteen compounds were identified based on their distinct retention times and corresponding mass spectral data. The compounds were separated by gas chromatography and subsequently identified by comparing their mass spectra, including characteristic mass-to-charge (m/z) fragment ions, with standard library databases. The chromatographic profile illustrates the elution pattern of the detected constituents, while the mass spectra of major compounds, showing diagnostic m/z values, which were used to confirm their identities. The relative abundance of each compound was

estimated from peak areas in the GC chromatogram, enabling semi-quantitative evaluation of the sample composition.

The peak observed at 7.85 min corresponds to cinnamaldehyde (1.23%), characterized by prominent fragment ions at m/z 131, 103, and 77, confirming its aromatic aldehyde structure and its role in fumigant toxicity against insects. The peak at 8.17 min represents 2-methoxy-4-vinylphenol (1.99%), exhibiting diagnostic fragments at m/z 150, 135, and 77, indicative of a methoxy-substituted phenolic compound with repellent and larvicidal activity. A major peak at 8.54 min was identified as eugenol (25.05%), showing characteristic ions at m/z 164, 149, 131, and 77, confirming its phenolic structure and strong insect repellent properties. The peak at 9.25 min corresponds to caryophyllene (1.04%), with key fragments at m/z 93, 133, and 41, typical of sesquiterpene hydrocarbons, followed by a peak at 10.03 min identified as naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl) (1.05%), exhibiting fragments at m/z 189, 161, and 133, supporting its sesquiterpene nature. These compounds are associated with fumigant activity, repellency, and insect growth regulation.

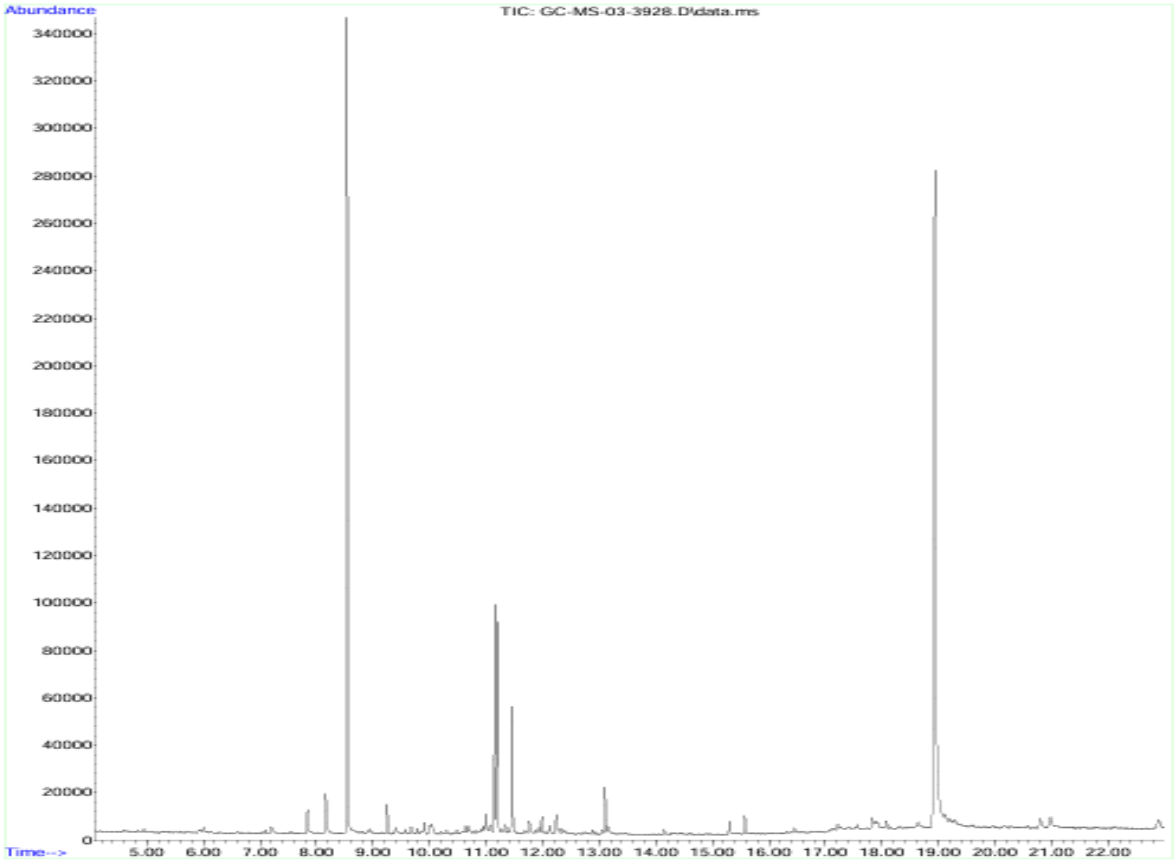


Fig. 10. GCMS spectrum of Phytonova

Table – 6 GCMS analysis of Phytonova balls with bioactive peaks

Peak	Retention time	Area%	Key m/z (Major fragments)	Compound name	Chemical group	Source	Insecticidal activity
1	7.853	1.23	131,103, 77	Cinnamaldehyde,	Aldehyde	Cinnamon	Fumigant toxicity
2	8.175	1.99	150,135,77	2-Methoxy-4-vinylphenol	Phenols	pepper	Repellent and Larvicidial
3	8.542	25.05	164,149,131,77	Eugenol	Phenols	Clove	Repellent
4	9.253	1.04	93,133,41	Caryophyllene	bicyclicsesquiterpene	Clove	Fumigant,
5	9.786	0.16	173, 145, 105, 91, 77	Benzoic acid,	Carboxylic acid	Clove	Fumigants activity
6	9.908	0.55	218, 145,91	7,11-dimethyl-3-Methylene-	Sesquiterpene hydrocarbon	Ginger	Larvicidial and Repellent activity
7	10.031	1.05	189,161,133	Naphthalene derivative	Sesquiterpenes	Cinnamon	Repellency and Insect growth regulation
8	10.986	0.56	43,58,77	Butan-2-one, 4-(3-hydroxy-2-meth.	Phenolic compound	Zinger	Antifeedant and Repellency activity
9	11.153	6.70	216, 137, 91	Ar-tumerone	Sesquiterpene ketone	Turmeric	Repellency and Insect groeth regulation
10	11.197	6.25	218, 137, 91	Tumerone	Sesquiterpene	Turmeric	Repellency and Insect groeth regulation
11	11.341	0.33	210, 105, 77	2',3',4' Trimethoxyacetophenon	Ketone	Clove	Fumigant activity

12	11.464	4.81	218, 137, 91	Curlone	Sesquiterpenoids	Turmeric	Growth regulation, Inhibition of nervous enzymes
13	11.764	0.76	60,73,129	Tetradecanoic acid	Fatty acid	Pepper	Disruptionn of cell membrane
14	15.585	0.66	131,103,77,68	trans-1-Cinnamoylimidazole	Acylimidazoles	Cinnamon	Insect growth regulators
15	18.941	41.80	285,201,135,77	Piperine	Alkaloid	Pepper	Neurotoxic effect

Further peaks at 11.15 and 11.19 min were identified as ar-turmerone (6.70%) and turmerone (6.25%), with characteristic fragment ions at m/z 216/218, 137, and 91, confirming sesquiterpene ketone structures linked to repellent and insect growth regulatory activity. Another notable compound, curlone (4.81%) at 11.46 min, also showed similar fragment ions (m/z 218, 137, 91), supporting its classification as a sesquiterpenoid with enzyme inhibition and growth regulatory effects. The most abundant compound appeared at 18.94 min and was identified as piperine (41.80%), exhibiting prominent ions at m/z 285, 201, 135, and 77, confirming its alkaloid structure and neurotoxic action in insects. In addition, minor constituents such as benzoic acid (m/z 173, 145, 105, 91, 77), 7,11-dimethyl-3-methylene (m/z 218, 145, 91), butan-2-one derivative (m/z 43, 58, 77), 2',3',4'-trimethoxyacetophenone (m/z 210, 105, 77), tetradecanoic acid (m/z 60, 73, 129), and trans-1-cinnamoylimidazole (m/z 131, 103, 77, 68) were also detected. These compounds, although present in lower concentrations, contribute collectively to fumigant, larvicidal, repellent, antifeedant activities, membrane disruption, and insect growth regulation, thereby enhancing the overall bioefficacy of the formulation.

A comparative evaluation with existing literature highlights the influence of species variation and geographical factors on the phytochemical composition of spice-derived formulations. In the present study, the most abundant compound, piperine, is consistent with the findings of Mohammed et al., (2016), who reported its significant fumigant and repellent activity. Similarly, eugenol (25.05%) identified in this study aligns with the observations of Abdelmuhsin et al., (2025), who reported a higher proportion of eugenol (58.86%), and Vargas-Méndez et al., (2019), who demonstrated that clove-derived eugenol possesses strong larvicidal and deterrent potential against stored grain insect pests. Furthermore, the presence of ar-turmerone (6.70%) and turmerone (6.25%) corresponds with the work of Lee et al., (2001), who identified ar-turmerone as a dominant sesquiterpene in Curcuma rhizomes with species-specific insecticidal and repellent activity, confirming its potential as an effective botanical insect-control agent. Also, the detection of cinnamaldehyde (1.23%) in the present study is in agreement with Silva et al., (2020), who reported its potential as an eco-friendly alternative insecticide with strong larvicidal activity, particularly within integrated pest management strategies. Thus, these findings suggest that key bioactive compounds such as piperine, eugenol, turmerones, and cinnamaldehyde serve as marker compounds across various botanical species, although their relative concentrations vary significantly depending on species identity, ecological conditions, and genetic variability. Such variations also influence the overall

aromatic profile and bioefficacy of the formulation, thereby emphasizing the importance of phytochemical diversity in determining insecticidal performance.

4.3. Bioassay Studies

4.3.1. Repellent activity of formulated treatment balls against *T. castaneum*

The repellent activity of formulated Phytonova (PNB), Diatomaceous Earth (DEB), and Neem Balls (NB) against *T. castaneum* was evaluated at different time intervals, and the results were tabulated in Table-7.

Table 7 – Percentage Repellent activity of formulated balls

Treatment	30 min%	1h%	3 h%	5 h%	7 h%	24h%	48h%	Average
0.5g PNB	81.33±1.15	85.33±6.11	90±8.71	80±0	80.6±1.15	88.67±7.02	86 ±5.29	**84.56**
1 g PNB	84±4	86.67±3.06	86.67±6.11	83.33±4.16	81.33±1.16	89.33±2.31	87.33±3.06	**85.52**
1.5g PNB	82.67±3.06	84.67±1.16	85.33±5.77	87.33±3.06	86±5.29	88.67±1.16	86±4	**85.81**
0.5g DEB	82±3.46	83.33±1.16	84 ±2	84±2	85.33±2.31	86.67±2.31	82.67±3.06	**84.00**
1 g DEB	83.33±3.06	86±2	82.67±3.06	82.67±1.16	85.33±4.16	90±2	86.67±1.16	**85.23**
1.5g DEB	82.67±3.06	82.67±1.16	83.33±2.31	83.33±2.31	82±3.46	83.33±3.06	84±0	**83.04**
1g NB	81.33±1.16	81.33±2.31	83.33±4.16	84±3.46	82.67±3.06	87.33±3.06	86±4	**83.71**
Average	**82.76**	**84.29**	**85.90**	**83.52**	**83.32**	**88.00**	**85.81**	-

Note: PB – Phytonova Balls, DEB – Diatomaceous Earth Balls, NB – Neem Balls

Table – Repellency (%) of Treatment Balls at Different Time Intervals

The percent repellent effect of formulated Phytonova (PNB), Diatomaceous Earth (DEB), and Neem Balls (NB) ranging from 80% to 90% for all treatments. From the table it was evident that all the tested balls exhibited repellent activity against *T. castaneum*. The repellent activity of the formulations were recorded the highest at 24 hrs of observations (88.00%) followed by 3 hrs (85.90) and 48 hrs (85.81%) among the treatments. Meanwhile the PNB at 1.5 g and 1g was recorded maximum with the average values of repellency of (85.81%) and (85.52 %) respectively. The treatment with 1g DEB also produced more repellent activity against *T. castaneum* (85.23%). In individual observation of repellent activity of the treatment showed the highest percentage of (90±8.71%) at 3 hours of observations. In 0.5 g PNB followed by 1g DEB (90±2%) at 24 hrs of observation.

The observed repellency range (80–90%) is in close agreement with the findings of Chahal, who reported that eugenol, a constituent of bay leaf and clove, exhibited high repellency (80–100%) against several beetle species. In addition to its repellent action, eugenol was also found to inhibit egg development and immature stages within grain kernels, suggesting a dual mode of action. The comparable repellency levels in the present study may therefore be linked to the presence of eugenol-rich components in the PNB.

Furthermore, the peak repellency observed in this study (90 ± 8.71%) for 0.5 g at 3 hours of observations aligns well with the results reported by Mutalib *et al.,* (2017), who demonstrated that *Piper nigrum* extract at 50% concentration exhibited extremely strong repellency (99.00 ± 0.000%). The authors also highlighted that piperine and related compounds possess insecticidal activity, effectively controlling forest insect pests such as *Lymantria dispar* and *Malacosoma disstria*. The inclusion of pepper in the PNB likely contributed to the enhanced repellency observed.

Similarly, the repellency range recorded in the present study for PNB corresponds with the findings of Sanga *et al.,* (2023), who reported significant repellency effects of clove and cinnamon essential oils. At concentrations of 45%, 55%, and 65%, clove oil exhibited repellency of 84%, 83.3%, and 92.7%, respectively, while cinnamon oil showed 88.0%, 81.3%, and 73.6%. These findings support the current results, as both clove and cinnamon are key constituents of the PNB and are known for their strong insect-repellent activity.

The lower bound repellency values observed in the present study (e.g., 80%) are also consistent with the work of Suthisut *et al.,* (2011), who reported that the ginger-derived compound terpinene-4-ol exhibited repellency of 77.5 ± 6.5% against *T. castaneum*. This suggests that even moderate levels of repellency can be achieved through individual phytochemicals, while the combined effect of multiple compounds in PNB may enhance overall efficacy.

Thus, the repellency observed in the present study can be attributed to the synergistic action of multiple bioactive compounds such as eugenol, piperine, and terpenoids present in the formulated PNB. The results were strongly supported by previous studies, indicating that plant-derived compounds offer effective, eco-friendly alternatives for the management of *T. castaneum*

4.3.2. Effect of formulated balls on the percent adult mortality of *T. castaneum*

The adult mortality of the *T. castaneum* exhibited a clear dose-dependent and time-dependent pattern across all treatments (Table 8). Mortality increased progressively from Day 1 onwards, with all treated groups recording significantly higher mortality than the untreated control ($P \leq 0.05$).

Among the treatments, DB-1.5 g exhibited the most rapid action, achieving 100% mortality by Day 6, followed by Neem 1 g (Day 7) and PNB-1.5 g (Day 8). Lower concentrations such as PNB-1 g and PNB-0.5 g required up to Day 9 and Day 10, respectively, to attain complete mortality. A similar trend was observed in DB formulations, where increasing dosage significantly reduced the time required to achieve cent percent mortality. The

progressive increase in mortality over time indicates a cumulative toxic effect of the formulations, likely mediated through ingestion and subsequent physiological disruption. Although initial differences among treatments were significant, all treatments eventually reached complete mortality, indicating that even lower doses are effective with prolonged exposure.

The present study recorded 100% mortality of *T. castaneum* with treatments, which aligns well with several earlier reports on the efficacy of botanical insecticides, *T. castaneum.* Similar findings were reported by Hussein *et al.,* (2017), who observed complete mortality of adult beetles following exposure to black pepper (*Piper nigrum*) extracts, with mortality increasing progressively with concentration and exposure duration. Likewise, Ahmad *et al.,* (2023) demonstrated that botanical extracts such as *Piper nigrum*, *Allium sativum*, and *Syzygium aromaticum* produced up to 100% mortality, confirming a strong dose- and time-dependent toxicological response, which corroborates the pattern observed in the present investigation.

The present results are also consistent with Hasan *et al.,* (2025), who reported complete mortality using *Citrus sinensis* peel extract, and Ali *et al.,* (2023), documented high mortality rates (>90%) with *Cinnamomum verum* extracts, both emphasizing enhanced efficacy at higher concentrations and longer exposure periods. Similarly, Ibrahim *et al.,* (2015) reported rapid insecticidal action of clove oil, achieving 100% mortality within a short exposure duration.

Further support is provided by Dzięgelewska *et al.,* (2025), who recorded complete mortality of *T. castaneum* using spice-based botanicals such as neem, turmeric, ajwain, and cinnamon. The insecticidal action of these botanicals has been attributed to bioactive compounds such as cinnamaldehyde, which disrupt detoxification pathways, particularly glutathione metabolism (Lu *et al.,* 2020), thereby enhancing insect susceptibility.

In addition, Kumar *et al.,* (2024) reported that botanical pesticides induced complete mortality at higher concentrations, attributing their efficacy to physiological disruptions such as paralysis and interference with respiratory electron transport systems. These mechanisms likely contribute to the cumulative toxic effects observed in the present study.

Collectively, these findings strongly support that botanical formulations exert potent insecticidal activity, ultimately leading to complete mortality. This reinforces their potential as eco-friendly and sustainable alternatives to synthetic insecticides for the management of stored-product pests.

Table – 8 Effect of formulated balls on the percent adult mortality of *T.castaneum*

Treatment / Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	0.00 (0.00)^{e}	3.33 (10.54)^{e}	6.67 (14.98)^{f}	10.00 (18.43)^{f}	20.00 (26.57)^{d}	23.33 (29.01)^{d}	26.67 (31.14)^{e}	33.33 (35.26)^{a}	46.66 (43.11)^{b}	46.66 (43.11)^{b}	60	70	86.66	100
PNB-0.5 g	3.33 (10.54)^{de}	10.00 (18.43)^{d}	16.67 (24.09)^{e}	36.67 (3.30)^{e}	50.00 (45.00)^{c}	60.00 (50.77)^{c}	73.33 (58.96)^{cd}	83.33 (65.38)^{b}	93.33 (75.17)^{a}	100.00 (90.00)^{a}				
PNB-1 g	10.00 (18.43)^{bc}	16.66 (24.09)^{bc}	23.33 (29.01)^{d}	43.33 (41.20)^{d}	63.33 (52.74)^{b}	73.33 (58.96)^{b}	83.33 (65.38)^{bc}	86.66 (68.89)^{b}	100.00 (90.00)^{a}					
PNB-1.5 g	0.00 (0.00)^{e}	13.33 (21.38)^{cd}	26.66 (31.14)^{cd}	50.00 (45.00)^{c}	63.33 (52.74)^{b}	76.66 (61.16)^{b}	90.00 (71.57)^{b}	100.00 (90.00)^{a}						
DB – 0.5 g	6.66 (14.98)^{cd}	16.66 (24.09)^{bc}	33.33 (35.26)^{bc}	40.00 (39.23)^{d}	46.66 (43.11)^{cd}	63.33 (52.74)^{c}	70.00 (56.79)^{d}	83.33 (65.38)^{b}	93.33 (75.17)^{a}	100.00(90.00)^{a}				
DB -1g	10.00 (18.43)^{bc}	20.00 (26.57)^{b}	40.00 (39.23)^{b}	50.00 (45.00)^{c}	60.00 (50.77)^{bc}	73.33 (58.96)^{b}	86.66 (68.69)^{b}	100.00 (90.00)^{a}						
DB -1.5g	13.33 (21.38)^{b}	33.33 (35.26)^{a}	53.33 (46.93)^{a}	73.33 (58.96)^{a}	86.66 (68.89)^{a}	100.00 (90.00)^{a}								
Neem -1g	20.00 (26.57)^{a}	33.33 (35.26)^{a}	46.67 (43.11)^{ab}	66.66 (54.74)^{ab}	83.33 (65.38)^{a}	96.66 (79.46)^{a}	100.00 (90.00)^{a}							
Grand Mean	**13.92**	**24.44**	**33.58**	**39.77**	**48.75**	**55.97**	**66.57**	**74.81**	**78.63**	**80.39**	**95.00**	**96.25**	**98.33**	**100.00**
SED	**2.75**	**2.73**	**2.69**	**2.71**	**2.68**	**2.63**	**2.28**	**2.14**	**2.19**	**2.24**				
CD ($p \leq 0.05$)	**5.89**	**5.85**	**5.76**	**5.81**	**5.74**	**5.63**	**4.88**	**4.58**	**4.69**	**4.80**				
F ratio	**18.74***	**16.92***	**22.48***	**25.96***	**31.44***	**36.82***	**52.87***	**41.22***	**28.64***	**26.18***				

Figures in parenthesis are ARCSINE transformed before ANOVA
Means with the same letter in each column are not significantly different
SED – Standard Error Deviation, CD – Critical Difference
** - Significant at 5% level, ns – not significant

4.3.3. Adult emergence of *T. castaneum* treated with formulated balls

The observations on adult emergence clearly indicate a significant suppression of progeny development with all the treatments compared to the untreated control (Table 9)

Table – 9 Adult emergence of *T. castaneum* treated with formulated balls

Treatment	Number of adults emergence(nos)
Control	2.3^{a}
PNB-0.5 g	1.0^{b}
PNB-1 g	1.0^{b}
PNB-1.5 g	0.3^{c}
DEB – 0.5 g	0.61^{bc}
DEB -1g	1.0^{b}
DEEB -1.5g	0.3^{c}
Neem -1g	0.3^{c}
Grand Mean	0.85
SED	0.25
CD ($p \leq 0.05$)	0.54
F ratio	6.82

Means with the same letter in each column are not significantly different

SED – Standard Error Deviation, CD – Critical Difference

* - Significant at 5% level, ns – not significant

Adult emergence was highest in the control (2.3 Nos), confirming uninterrupted reproduction and successful completion of the life cycle in the absence of treatment. In contrast, all treated grains recorded markedly reduced emergence, demonstrating the inhibitory effect of the formulations on population buildup.

Among the treatments, PNB-1.5 g, DEB-1.5 g, and Neem 1 g (0.3 Nos each) were the most effective and were found to be statistically on par, forming the superior treatment group. These treatments resulted in maximum suppression of adult emergence, indicating strong interference with developmental stages such as egg hatchability and larval survival. Moderate suppression was observed in DEB-0.5 g (0.61 Nos), which was statistically comparable with both higher and lower groups (bc), suggesting an intermediate level of efficacy. Treatments such as PNB-0.5 g, PNB-1 g, and DEB-1 g (1.0 Nos each) recorded relatively higher emergence among treated groups but were still significantly lower than the control, indicating partial inhibition of development.

The statistical analysis ($F = 6.82$; $CD = 0.54$ at $P \leq 0.05$) confirms that the differences among treatments are significant, with a clear separation between high-efficacy (c group), moderate (bc), and lower-efficacy (b) treatments. Thus, the pattern demonstrates a dose-dependent reduction in adult emergence, where higher concentrations (1.5 g) resulted in greater

suppression. This suggests that the treatments exert cumulative effects on immature stages, ultimately reducing adult emergence and preventing population buildup.

The present study demonstrated a marked reduction in adult emergence, indicating effective suppression of progeny development. This finding corroborates with the reports of Amuji *et al.,* (2012), who observed reduced adult emergence in stored grain insects treated with ginger extracts, attributing the effect to bioactive compounds such as gingerols that interfere with digestive enzymes and insect growth. The reduced adult emergence recorded in the present study is also in agreement with Ali *et al.,* (2014), who reported minimal adult emergence at higher concentrations of botanical treatments, highlighting their significant inhibitory effect on insect development. Similarly, garlic-derived compounds such as methyl allyl disulfide and diallyl trisulfide have been reported to reduce egg hatchability and subsequent adult emergence in *T. castaneum*, indicating disruption of early developmental stages.

Further support is provided by Ashouri *et al.,* (2010), who demonstrated that turmeric and cinnamon powders significantly reduced adult emergence, with complete inhibition observed at higher dosages. This reduction has been attributed to interference with larval growth and developmental processes. In addition, Ashouri and Shayesteh (2010) reported that black pepper powder exhibited significant insecticidal activity, reducing both adult survival and progeny emergence, with statistically significant differences compared to the control ($P < 0.05$).

The minimum adult emergence observed at higher concentrations in the present study is in line with Plata-Rueda *et al.,* (2017), who reported that garlic essential oil affects multiple physiological systems in insects, particularly respiration, leading to paralysis and eventual mortality. Such physiological disruptions likely contribute to reduced survival of immature stages and lower adult emergence. Similarly, Nta *et al.,* (2017) demonstrated that citrus peel powders significantly reduced adult emergence, attributing their efficacy to physical and biochemical effects, including blockage of spiracles and impairment of respiration. This mode of action further supports the reduction in progeny observed in the present investigation.

The findings are also corroborated by Upadhyay *et al.,* (2017), who reported that botanical oils, particularly from Piper nigrum, significantly suppressed larval and pupal survival, resulting in a substantial decline in adult emergence. These effects were associated with insecticidal, antifeedant, and growth regulatory activities.

Thus, the present study confirms that botanical formulations effectively reduce adult emergence through cumulative effects on immature stages, including inhibition of egg hatchability, larval development, and pupation. The observed inverse relationship between

dosage and adult emergence further emphasizes the dose-dependent nature of these treatments, supporting their potential as eco-friendly and sustainable alternatives for the management of stored grain insect pests.

4.3.4 Effect of the formulated balls on weight loss of wheat grains

The data on percentage weight loss of grains after 40 days of storage reveal a significant reduction in grain damage with all the treatments compared to the control**,** indicating effective protection against *T. castaneum* infestation (Table 10).

Table 10 - Effect of the formulated balls on weight loss of wheat grains

Treatment	Percentage weight loss of grains after 30 days of storage
Control	3.76 [a]
PNB-0.5 g	2.00 [b]
PNB-1 g	1.76 [bc]
PNB-1.5 g	1.35[cd]
DEB – 0.5 g	1.25[cd]
DEB -1g	2.18[b]
DEB -1.5g	1.80[bc]
NB -1g	0.93[d]
Grand Mean	0.85
SED	0.25
CD ($p \leq 0.05$)	0.54
F ratio	6.82

Means with the same letter in each column are not significantly different

SED – Standard Error Deviation, CD – Critical Difference

* - Significant at 5% level, ns – not significant

The control recorded the highest weight loss (3.76%), reflecting unrestricted feeding and development of the insect population. In contrast, all treated samples showed markedly lower weight loss, demonstrating the efficacy of the formulations in minimizing grain damage.

Among the treatments, Neem 1 g recorded the lowest weight loss (0.93%) and was statistically superior (d group), indicating maximum protection of grains. This was followed by DEB-0.5 g (1.25%) and PNB-1.5 g (1.35%), which were statistically on par (cd group) and exhibited strong protective effects. Treatments such as PNB-1 g (1.76%) and DEB-1.5 g (1.80%) showed moderate reduction (bc group), while PNB-0.5 g (2.00%) and DEB-1 g (2.18%) recorded comparatively higher weight loss among treated groups but still significantly lower than the control (3.76%). The statistical analysis ($F = 6.82$; $CD = 0.54$ at $P \leq 0.05$) confirms that the differences among treatments are significant, with a clear separation between high-efficacy and lower-efficacy treatments.

A general dose-dependent trend is evident in PN treatments, where increasing concentration from 0.5 g to 1.5 g progressively reduced weight loss. However, in DEB treatments, slight variation in trend suggests possible differences in formulation dynamics or release pattern. Thus, treatments that recorded lower adult emergence and higher mortality correspondingly showed reduced grain weight loss, indicating a strong relationship between suppression of pest population and minimization of feeding damage.

The present study demonstrated that untreated grains recorded the maximum weight loss (3.76%), whereas all treated samples showed a substantial reduction, with the minimum weight loss (0.93%) observed in treated grains. This clearly indicates the effectiveness of botanical formulations in protecting stored grains from insect damage.

These findings are in agreement with Buba and Salisu (2023), who reported significantly higher weight loss in untreated groundnut seeds compared to those treated with ginger powder. The reduced weight loss in treated samples was attributed to the presence of bioactive phytochemicals possessing repellent, antifeedant, and toxic properties, which limit insect feeding and development. Similarly, Timothy and Esther (2009) and Amuji *et al.,* (2012) reported that higher levels of ginger residues inhibited normal growth and development of *T. castaneum*, thereby reducing feeding activity and consequent weight loss.

The present results are in accordance with Sanga *et al.* (2023), who demonstrated that citrus peel powder significantly reduced grain weight loss due to the presence of compounds such as limonene and flavonoids, which deter feeding and oviposition. Likewise, Nta *et al.* (2017) reported that citrus-based treatments reduced weight loss through both biochemical and physical mechanisms, including spiracle blockage and respiratory impairment.

Further support is provided by Ali *et al.,* (2014), who observed significantly lower flour consumption at higher concentrations of *Allium sativum* and *Curcuma longa* extracts. The active compounds, including methyl allyl disulfide and diallyl trisulfide, were reported to reduce egg hatchability and progeny development, thereby indirectly minimizing grain damage.

In the present study, Neem-treated grains (0.93%) exhibited the least weight loss, followed by DB (0.5 g) and PN (1.5 g) treatments, indicating their superior protective efficacy. This reduction in weight loss can be attributed to the combined effects of insect mortality, reduced adult emergence, and inhibition of feeding activity.

Thus, the findings confirm that botanical treatments effectively reduce grain weight loss through multiple modes of action, including repellency, antifeedant activity, growth inhibition,

and physiological disruption. These effects collectively suppress insect population buildup and feeding damage, thereby preserving grain quality during storage

4.4 *In silico* studies

4.4.1. Functional Annotation of Detoxification Genes Using KEGG Pathway Analysis

The KEGG is a comprehensive bioinformatics resource that integrates genomic, chemical, and systemic functional information to facilitate the understanding of biological processes at the molecular level. KEGG provides curated pathway maps representing metabolic networks, cellular processes, and organism-level systems, enabling researchers to interpret gene functions within a broader biological context. By linking genes to specific biochemical pathways, KEGG serves as a powerful tool for functional annotation and pathway-based analysis, particularly in studies involving metabolism, signalling, and detoxification mechanisms.

In the present study, KEGG pathway analysis was employed to functionally validate the selected detoxification genes and to understand their roles within biological pathways associated with xenobiotic metabolism. Since the genes under investigation CYP6BQ9 and CYP6BQ11 were selected based on their reported involvement in insecticide resistance, it was essential to confirm their biochemical functions and pathway associations. KEGG analysis enables the identification of metabolic pathways in which these genes participate, thereby providing mechanistic insight into their role in detoxification processes. Unlike enrichment-based tools that require large gene datasets, KEGG is particularly suitable for targeted gene studies, allowing direct mapping of individual genes or enzyme families to specific pathways. This approach ensures that the selected genes are not only structurally relevant but also biologically significant within the context of detoxification and resistance mechanisms.

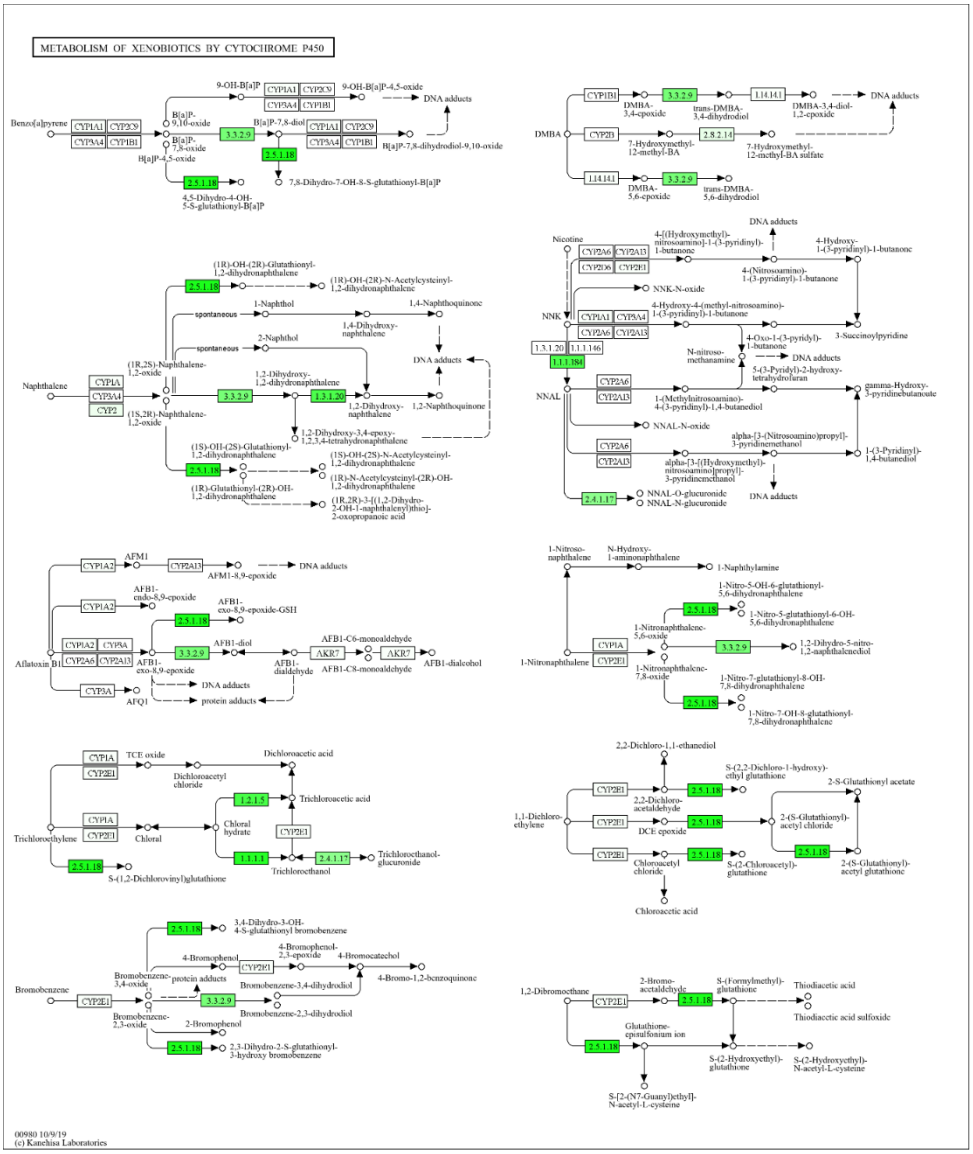
4.4.1.1. KEGG pathway mapping of cytochrome P450

KEGG pathway analysis revealed that both CYP6BQ9 and CYP6BQ11 are classified under the cytochrome P450 enzyme family (KO: K14999) and are functionally associated with the “Metabolism of xenobiotics by cytochrome P450” pathway. This pathway (Fig.11) represents a critical component of Phase I detoxification, where cytochrome P450 enzymes catalyse oxidative reactions that convert hydrophobic xenobiotic compounds into more polar and reactive intermediates.

The pathway illustrates multiple enzymatic reactions, including hydroxylation, epoxidation, and dealkylation, through which cytochrome P450 enzymes introduce oxygen atoms into xenobiotic substrates. These reactions increase the solubility of toxic compounds and facilitate their subsequent metabolism. The presence of numerous CYP enzyme nodes

within the pathway highlights their broad substrate specificity and ability to metabolise structurally diverse compounds, including insecticides and plant-derived allelochemicals.

Fig.11 KEGG pathway of xenobiotic metabolism by cytochrome P450 highlighting oxidative detoxification reaction



Both CYP6BQ9 and CYP6BQ11, being members of the CYP6 family, are involved in these oxidative processes and contribute to the initial step of detoxification. The pathway further demonstrates that the products of these reactions, such as epoxides and hydroxylated intermediates, may either be detoxified further or converted into more reactive forms. This dual nature of cytochrome P450-mediated metabolism underscores its importance in both detoxification and bioactivation processes.

The shared classification of CYP6BQ9 and CYP6BQ11 within the same KEGG pathway indicates functional similarity and suggests that these genes may act cooperatively in

detoxification mechanisms. The redundancy and overlap in their enzymatic activity enhance the efficiency of xenobiotic metabolism, thereby contributing to the organism's ability to withstand exposure to toxic compounds.

The findings of the present study align with previous reports indicating that cytochrome P450 enzymes are key determinants of insecticide resistance across multiple insect species. Studies by Feyereisen (2012) have emphasized the role of P450 enzymes in the metabolic detoxification of insecticides, while Li *et al*., (2007) highlighted their involvement in resistance to a wide range of xenobiotic compounds. The KEGG pathway analysis presented here is consistent with these studies, as it clearly demonstrates the biochemical pathways through which CYP enzymes mediate detoxification.

Thus, the KEGG pathway analysis confirms that CYP6BQ9 and CYP6BQ11 play a central role in Phase I detoxification processes, providing a mechanistic basis for their involvement in xenobiotic metabolism. The pathway not only validates the functional significance of these genes but also highlights their contribution to a broader detoxification network that enables the organism to metabolise and eliminate harmful substances. the KEGG pathway analysis not only validates the functional significance of CYP6BQ9 but also provides a mechanistic framework for understanding its role in insecticide resistance. When combined with CRISPR-Cas9 sgRNA design, this analysis offers a powerful approach for identifying and targeting key detoxification genes.

4.4.1.2. KEGG pathway mapping of GSTd1

KEGG pathway analysis (Fig.12) revealed that GSTd1 is functionally associated with the glutathione metabolism pathway, where glutathione S-transferase enzymes (EC: 2.5.1.18) catalyse the conjugation of electrophilic xenobiotic metabolites with glutathione. This process represents Phase II detoxification, facilitating the conversion of reactive intermediates into water-soluble compounds that can be readily excreted.

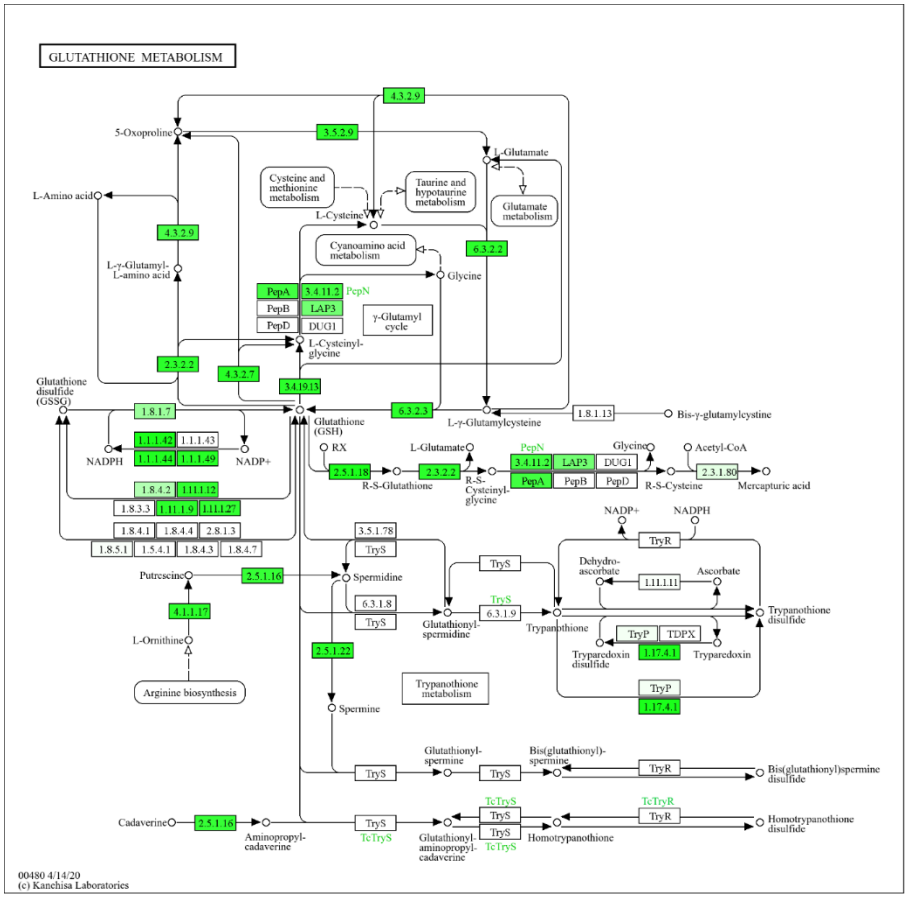


Fig. 12 KEGG pathway of glutathione metabolism illustrating GST-mediated conjugation and detoxification

Although GSTd1 was not directly annotated in KEGG under its specific gene name, its classification within the glutathione S-transferase enzyme family confirms its role in detoxification pathways. The pathway illustrates multiple conjugation reactions mediated by GST enzymes, highlighting their importance in neutralising toxic metabolites generated during Phase I oxidation.

The KEGG pathway for glutathione metabolism illustrates a comprehensive network of biochemical reactions centred around the tripeptide glutathione (GSH), which plays a pivotal role in cellular detoxification and redox homeostasis. Within this pathway, glutathione S-transferase (GST; EC: 2.5.1.18) catalyses the conjugation of reduced glutathione to electrophilic xenobiotic compounds, resulting in the formation of glutathione conjugates (R–SG). These conjugates are subsequently processed into more water-soluble derivatives such as mercapturic acids, facilitating their excretion from the organism.

The pathway clearly demonstrates that GST enzymes act downstream of cytochrome P450-mediated oxidation reactions. Toxic compounds initially undergo Phase I metabolism, generating reactive intermediates such as epoxides and hydroxylated derivatives. These

intermediates are then detoxified through GST-mediated conjugation, which neutralises their electrophilic nature and prevents interaction with cellular macromolecules such as DNA and proteins. In the context of GSTd1, although the specific gene is not explicitly annotated in KEGG for *T. castaneum*, its functional classification within the GST enzyme family confirms its role in this pathway. The highlighted GST reaction nodes in the pathway validate that GSTd1 participates in Phase II detoxification processes, contributing to the conjugation and elimination of toxic metabolites.

The KEGG pathway analysis of glutathione metabolism provides strong functional evidence supporting the role of GSTd1 as a critical Phase II detoxification enzyme in *T. castaneum*. The pathway clearly illustrates that glutathione S-transferases catalyse the conjugation of glutathione to electrophilic substrates, thereby reducing their reactivity and enhancing their solubility. This mechanism is essential for detoxifying harmful intermediates generated during Phase I metabolism, particularly those produced by cytochrome P450 enzymes such as CYP6BQ9 and CYP6BQ11.

The findings of the present study align with the investigations by Enayati et al., who reported that GSTs play a central role in insecticide resistance by catalysing the conjugation of toxic compounds, thereby reducing their bioavailability and toxicity. Similarly, Hayes et al. highlighted the structural and functional diversity of GST enzymes, emphasizing their ability to detoxify a wide range of xenobiotics through glutathione conjugation. The KEGG pathway observed in the present study supports these findings by demonstrating multiple GST-mediated reactions involved in detoxification processes.

Furthermore, Ranson and Hemingway reported that elevated GST activity is a common mechanism of resistance in insects, particularly against organophosphates and other electrophilic insecticides. This observation is consistent with the KEGG pathway, where GST enzymes are positioned at key nodes responsible for neutralising reactive intermediates. The functional mapping of GSTd1 to this pathway therefore confirms its potential involvement in resistance mechanisms in *T. castaneum*.

Furthermore, the KEGG pathway reveals the involvement of glutathione metabolism in maintaining cellular redox balance. GST enzymes not only participate in detoxification but also protect cells against oxidative stress by regulating glutathione levels. This dual role has been extensively discussed by Sheehan et al., who demonstrated that GSTs contribute to both detoxification and antioxidant defense systems. The present findings align with this concept, suggesting that GSTd1 plays a multifunctional role in maintaining cellular homeostasis in addition to detoxification.

Thus, the KEGG pathway analysis confirms that GSTd1 functions as a key enzyme in Phase II detoxification, facilitating the conjugation and elimination of toxic metabolites in *T. castaneum*. The findings provide strong functional validation for its selection as a target gene and highlight its potential role in resistance mechanisms.

The coordinated interaction between Phase I and Phase II detoxification pathways, as illustrated by the KEGG maps for cytochrome P450 and glutathione metabolism, underscores the complexity of insect resistance mechanisms. When integrated with CRISPR-Cas9 sgRNA design and docking studies, this analysis contributes to a comprehensive understanding of detoxification pathways and supports the development of targeted and sustainable pest control strategies

4.4.2. CRISPR-Cas9 sgRNA Design and Target Validation

The rationale behind performing CRISPR sgRNA design in this study, despite the absence of experimental genome editing, lies in its predictive capability to evaluate gene editability and functional vulnerability. This step establishes a mechanistic bridge between structural inhibition and genetic disruption, thereby strengthening the overall inference of target validity. The identification of high-quality sgRNAs confirms that these detoxification genes can be effectively silenced in future experimental studies, which is particularly relevant in resistance management strategies. The use of three sgRNAs per gene further ensures robustness and reproducibility, as it accounts for potential variability in sgRNA performance due to chromatin accessibility and sequence context.

The present study employed an *in silico* CRISPR-Cas9 approach to evaluate the targetability of key detoxification genes, CYP6BQ9, CYP6BQ11, and GSTd1, in *T. castaneum*. The genes (Table-11) were selected based on their established roles in Phase I and Phase II detoxification pathways, where cytochrome P450 enzymes mediate oxidation of xenobiotics, and glutathione-S-transferases facilitate conjugation and excretion of toxic metabolites.

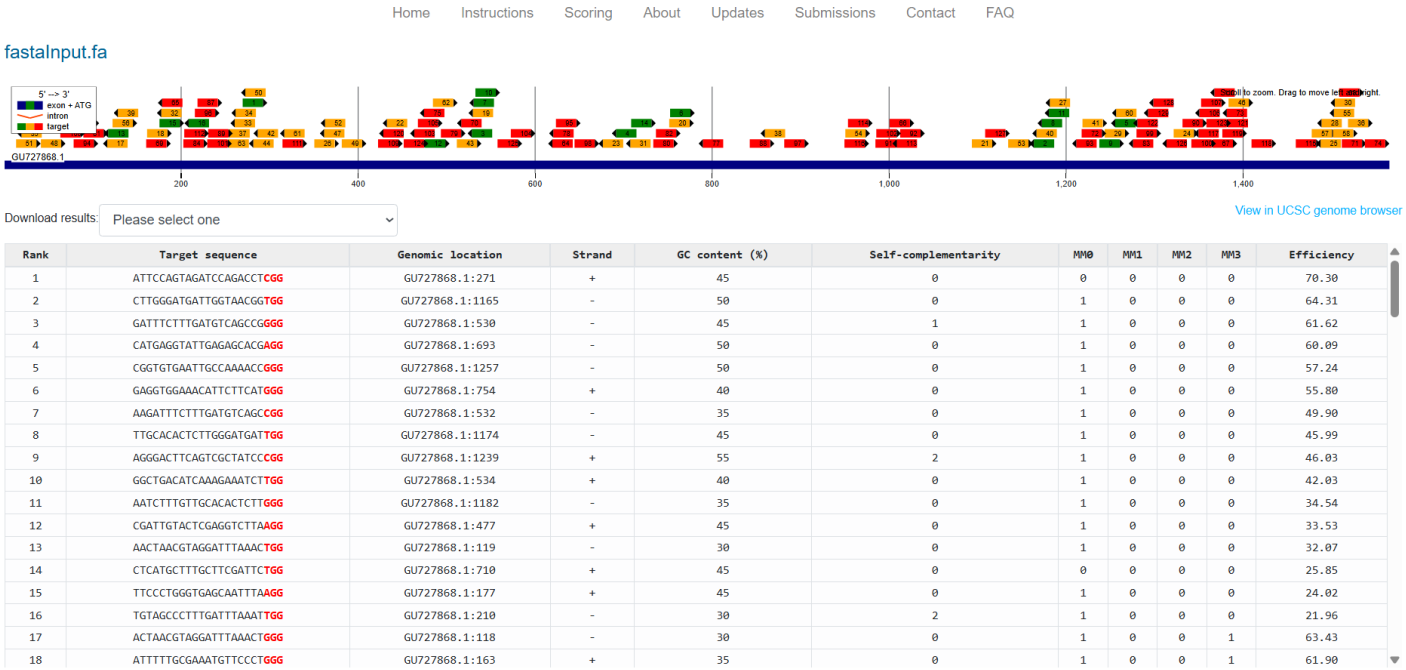
Table -11 sgRNA candidates selected for CRISPR Cas9 targeting

Gene	Rank	sgRNA sequence (5′–3′)	PAM	Position	Strand	GC (%)	Efficiency
CYP6BQ9	**1**	ATTCCAGTAGATCCAGACCT	GGG	271	+	45	70.30
	2	CTTGGGATGATTGGTAACGG	TGG	1165	–	50	64.31
	3	GATTTCTTTGATGTCACGCC	GGG	530	–	45	61.62
CYP6BQ11	**1**	GGTTTAGGCTTGGGTACACCT	AGG	108	–	50	68.13
	2	TTTAGCTTGGGTACACTAGC	CGG	105	–	50	69.48

	3	TAAACTAAGTCGAGGTACTC	GGG	1224	+	40	62.40
GSTd1	**1**	GCAACATACGATCCCGACCT	AGG	150	+	55	70.73
	2	ATCCCGACCATGGTCGACCA	CGG	160	+	55	69.28

The sgRNA design results revealed that all three genes exhibited high targetability, as evidenced by the identification of multiple sgRNAs with high predicted efficiency (above 60%), optimal GC content (40–60%), minimal self-complementarity and low off-target potential. Importantly, the selected sgRNAs were predominantly located in the 5′ coding regions, which significantly increases the likelihood of generating functional knockouts by inducing early frameshift mutations and premature termination of translation. This strategic targeting enhances the probability of complete loss-of-function phenotypes, thereby ensuring effective disruption of detoxification pathways. This indicates that the selected detoxification genes are highly amenable to CRISPR-mediated editing, thereby validating their suitability as molecular targets for genetic intervention. Moreover, the low self-complementarity observed among the selected sgRNAs indicates reduced likelihood of hairpin formation, thereby improving target recognition and cleavage efficiency.

A key observation in the present study is the localization of sgRNA target sites predominantly within the 5′ coding regions of the genes. This strategic targeting is crucial, as mutations introduced in early exons are more likely to result in frameshift mutations and premature stop codons, leading to complete loss of gene function. This approach significantly increases the probability of generating effective gene knockouts, which is essential for studying gene function and disrupting detoxification pathways in insect systems.



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fastaInput.fa

5' --> 3'
exon + ATG
intron
target

GU727868.1

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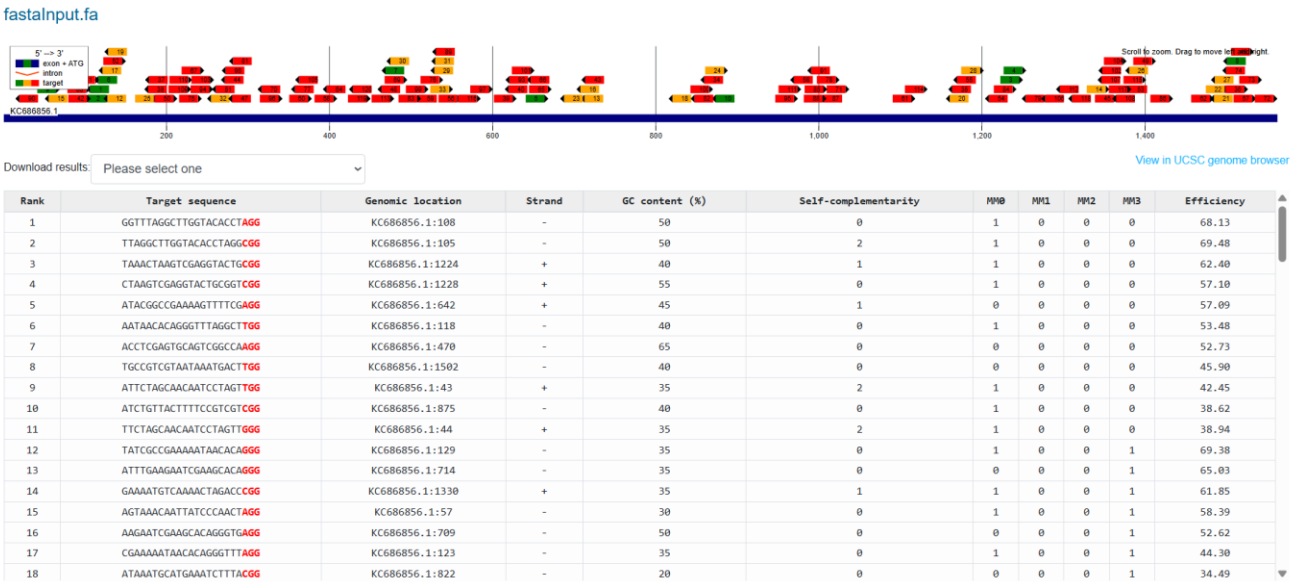
Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	ATTCCAGTAGATCCAGACCTCGG	GU727868.1:271	+	45	0	0	0	0	0	70.30
2	CTTGGGATGATTGGTAACGGTGG	GU727868.1:1165	-	50	0	1	0	0	0	64.31
3	GATTTCTTTGATGTCAGCCGGGG	GU727868.1:530	-	45	1	1	0	0	0	61.62
4	CATGAGGTATTGAGAGCACGAGG	GU727868.1:693	-	50	0	1	0	0	0	60.09
5	CGGTGTGAATTGCCAAAACCGGG	GU727868.1:1257	-	50	0	1	0	0	0	57.24
6	GAGGTGGAAACATTCTTCATGGG	GU727868.1:754	+	40	0	1	0	0	0	55.80
7	AAGATTTCTTTGATGTCAGCCGG	GU727868.1:532	-	35	0	1	0	0	0	49.90
8	TTGCACACTCTTGGGATGATTGG	GU727868.1:1174	-	45	0	1	0	0	0	45.99
9	AGGGACTTCAGTCGCTATCCCGG	GU727868.1:1239	+	55	2	1	0	0	0	46.03
10	GGCTGACATCAAAGAAATCTTGG	GU727868.1:534	+	40	0	1	0	0	0	42.03
11	AATCTTTGTTGCACACTCTTGGG	GU727868.1:1182	-	35	0	1	0	0	0	34.54
12	CGATTGTACTCGAGGTCTTAAGG	GU727868.1:477	+	45	0	1	0	0	0	33.53
13	AACTAACGTAGGATTTAAACTGG	GU727868.1:119	-	30	0	1	0	0	0	32.07
14	CTCATGCTTTGCTTCGATTCTGG	GU727868.1:710	+	45	0	0	0	0	0	25.85
15	TTCCCTGGGTGAGCAATTTAAGG	GU727868.1:177	+	45	0	1	0	0	0	24.02
16	TGTAGCCCTTTGATTTAAATTGG	GU727868.1:210	-	30	2	1	0	0	0	21.96
17	ACTAACGTAGGATTTAAACTGGG	GU727868.1:118	-	30	0	1	0	0	1	63.43
18	ATTTTTGCGAAATGTTCCCTGGG	GU727868.1:163	+	35	0	1	0	0	1	61.90

Fig.13. CHOPCHOP output showing sgRNA target sites for CYP6BQ9

CHOPCHOP output displaying predicted sgRNA target sites across the CYP6BQ9 gene, including efficiency scores, GC content, and off-target predictions.



fastaInput.fa

5' --> 3'
exon + ATG
intron
target

KC686856.1

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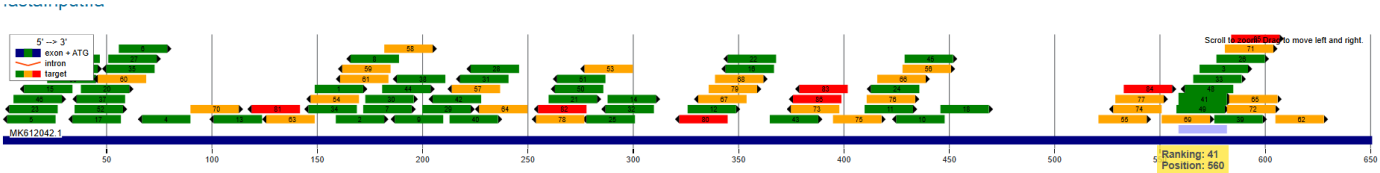
Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	GGTTTAGGCTTGGTACACCTAGG	KC686856.1:108	-	50	0	1	0	0	0	68.13
2	TTAGGCTTGGTACACCTAGGCGG	KC686856.1:105	-	50	2	1	0	0	0	69.48
3	TAAACTAAGTCGAGGTACTGCGG	KC686856.1:1224	+	40	1	1	0	0	0	62.40
4	CTAAGTCGAGGTACTGCGGTCGG	KC686856.1:1228	+	55	0	1	0	0	0	57.10
5	ATACGGCCGAAAAGTTTTCGAGG	KC686856.1:642	+	45	1	0	0	0	0	57.09
6	AATAACACAGGGTTTAGGCTTGG	KC686856.1:118	-	40	0	1	0	0	0	53.48
7	ACCTCGAGTGCAGTCGGCCAAGG	KC686856.1:470	-	65	0	0	0	0	0	52.73
8	TGCCGTCGTAATAAATGACTTGG	KC686856.1:1502	-	40	0	0	0	0	0	45.90
9	ATTCTAGCAACAATCCTAGTTGG	KC686856.1:43	+	35	2	1	0	0	0	42.45
10	ATCTGTTACTTTTCCGTCGTCGG	KC686856.1:875	-	40	0	1	0	0	0	38.62
11	TTCTAGCAACAATCCTAGTTGGG	KC686856.1:44	+	35	2	1	0	0	0	38.94
12	TATCGCCGAAAAATAACACAGGG	KC686856.1:129	-	35	0	1	0	0	1	69.38
13	ATTTGAAGAATCGAAGCACAGGG	KC686856.1:714	-	35	0	0	0	0	1	65.03
14	GAAAATGTCAAAACTAGACCCGG	KC686856.1:1330	+	35	1	1	0	0	1	61.85
15	AGTAAACAATTATCCCAACTAGG	KC686856.1:57	-	30	0	1	0	0	1	58.39
16	AAGAATCGAAGCACAGGGTGAGG	KC686856.1:709	-	50	0	0	0	0	1	52.62
17	CGAAAAATAACACAGGGTTTAGG	KC686856.1:123	-	35	0	1	0	0	1	44.30
18	ATAAATGCATGAAATCTTTACGG	KC686856.1:822	-	20	0	0	0	0	1	34.49

Fig. 14. CHOPCHOP output showing sgRNA target sites for CYP6BQ11

CHOPCHOP output illustrating sgRNA distribution and ranking for CYP6BQ11 based on efficiency, GC content, and specificity.



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Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	GCAACATACGATCCCGACCA**TGG**	MK612042.1:150	+	55	0	1	0	0	0	70.73
2	ATCCCGACCATGGTCGACAA**CGG**	MK612042.1:160	+	55	2	1	0	0	0	69.28
3	TGCGGCGCCTGGGTATGAGG**AGG**	MK612042.1:570	+	70	0	1	0	0	0	64.26
4	TAAATTGAGCTCCACACCGA**CGG**	MK612042.1:68	-	45	0	1	0	0	0	62.84
5	GGAAGATAGTACAGGTCGAT**GGG**	MK612042.1:4	-	45	0	1	0	0	0	60.22
6	GGCGGCGAAAGCCGTCGGTG**TGG**	MK612042.1:57	+	75	1	1	0	0	0	60.67
7	TCGACAACGGCTTTGCCCTG**TGG**	MK612042.1:173	+	60	1	1	0	0	0	60.57
8	GGCAAAGCCGTTGTCGACCA**TGG**	MK612042.1:167	-	60	1	1	0	0	0	60.54
9	TATGGCCCTGCTCTCCCACA**GGG**	MK612042.1:188	-	60	0	1	0	0	0	58.79
10	CAACAAAGTCCTGACCCTCA**AGG**	MK612042.1:426	-	50	0	1	0	0	0	58.79
11	GTTCCTGGAGATTTTCCTTG**AGG**	MK612042.1:411	+	45	0	1	0	0	0	58.75
12	ATTTGCCGATTATTACTACC**CGG**	MK612042.1:327	+	35	0	0	0	0	0	58.31
13	TTAGGTGTTCGCCTTTCATG**AGG**	MK612042.1:102	-	45	1	1	0	0	0	58.94
14	CAGAGATTGTACTTCGACAT**TGG**	MK612042.1:289	+	40	0	1	0	0	0	57.89
15	CACTTCCAGGAAGATAGTAC**AGG**	MK612042.1:12	-	45	2	1	0	0	0	59.09
16	CAGCGCCTCCAAAAATCACC**GGG**	MK612042.1:345	-	55	1	1	0	0	0	56.85
17	CAAAAGGACGGCCCTGCATG**GGG**	MK612042.1:35	-	60	2	1	0	0	0	57.82
18	TGCGGGAAATCAGCTCACTT**TGG**	MK612042.1:447	+	50	0	1	0	0	0	55.33

Fig. 15. CHOPCHOP output showing sgRNA target sites for GSTd1

CHOPCHOP output representing sgRNA candidates for GSTd1, highlighting target positions and predicted efficiencies.

The representative CHOPCHOP output pages for CYP6BQ9, CYP6BQ11, and GSTd1 are presented as separate figures in Fig.13,14,15 and to illustrate gene-specific sgRNA design. Each screenshot displays ranked sgRNA candidates along with key parameters such as target position, GC content, predicted efficiency, and off-target scores. These visual outputs enable clear comparison and selection of optimal sgRNAs for each gene. Together, they confirm the precision and reliability of the computational sgRNA design process.

The findings of the present study align with the work of Labun *et al.*, (2019), who emphasized the importance of computational sgRNA design tools in predicting editing efficiency and minimizing off-target effects. Similarly, Hsu *et al.*, (2013) demonstrated that sgRNAs with balanced GC content and low secondary structure exhibit higher cleavage efficiency and specificity. The prioritization of target sites within early exonic regions is also consistent with the observations of Mali *et al.*, (2013), who reported that targeting the 5′ region of genes enhances the probability of generating null mutations.

Furthermore, studies on insect systems have highlighted the applicability of CRISPR-Cas9 in functional genomics and resistance studies. For instance, Zhu *et al.*, (2010) reported that knockdown of CYP6BQ genes significantly increased susceptibility of *T. castaneum* to insecticides, indicating that disruption of these genes can impair detoxification mechanisms.

This supports the present findings, where CYP6BQ9 and CYP6BQ11 were identified as highly targetable genes with efficient sgRNA candidates.

In addition, Kalsi and Palli (2015) demonstrated that transcriptional regulation of cytochrome P450 genes plays a crucial role in insecticide resistance, suggesting that precise genetic targeting of these genes can provide valuable insights into resistance mechanisms. The selection of GSTd1 as a Phase II detoxification gene further strengthens the study, as glutathione-S-transferases are known to conjugate toxic metabolites and facilitate their excretion. Zhu *et al.*, (2007) reported elevated GST activity in resistant strains of *T. castaneum*, indicating that disruption of GST genes can compromise detoxification efficiency. The present study corroborates these findings by demonstrating that GSTd1 is highly amenable to CRISPR targeting, thereby highlighting its potential as a candidate for functional validation.

The integration of CRISPR-based target validation with computational analysis provides a forward-looking framework for future experimental studies. The designed sgRNAs can be utilized in RNA interference (RNAi) or CRISPR-Cas9 mediated knockout experiments to validate gene function and assess their role in insecticide resistance. Furthermore, combining CRISPR-mediated gene disruption with biochemical or molecular assays can provide deeper insights into detoxification pathways and their regulation. The approach adopted in the present study not only facilitates the identification of target genes but also bridges the gap between computational prediction and experimental validation.

Thus, the CRISPR-Cas9 sgRNA design results confirm that CYP6BQ9, CYP6BQ11, and GSTd1 are not only biologically significant but also genetically targetable and functionally significant genes involved in detoxification processes in *T. castaneum*. The identification of efficient and specific sgRNAs provides a strong foundation for future gene-editing studies aimed at disrupting resistance mechanisms. The present findings highlight the potential of CRISPR-based approaches in insect pest management and contribute to the development of innovative, targeted, and sustainable control strategies.

4.4.3. Molecular docking analysis of selected phytocompounds against the detoxifying proteins in *T. castaneum*

Molecular docking analysis was carried out to evaluate the interaction potential of selected phytocompounds with key detoxifying proteins, Cytochrome P450 enzymes and Glutathione-S-Transferase (GST from *T. castaneum*. These proteins play a crucial role in metabolic detoxification and insecticide resistance, making them important molecular targets for insecticidal intervention. The study aims to assess the binding affinity, interaction stability,

and inhibitory potential of the selected compounds in order to understand their possible role in disrupting detoxification pathways and enhancing insecticidal activity.

4.4.3.1. Evaluation of insecticide-likeness using Tice rule parameters

The Tice rule is a physicochemical screening tool used to predict the insecticide-likeness of compounds based on certain parameters. It helps in identifying compounds with suitable bioavailability and membrane permeability for effective insecticidal activity and subsequent molecular docking studies. All the selected compounds, eugenol, piperine and ar-turmerone were subjected to physicochemical evaluation using the Tice Rule (Tice, 2001) and depicted in Table -12.

Table – 12 Insecticide-likeness properties of selected phytocompounds using tice rule

Compounds	Molecular weight KDa/g/mol	Hydrogen bond donar	Hydrogen bond acceptor	No. of rotatable bonds	Log P
eugenol	164.20	1	2	3	2
piperine	285.34	0	3	3	3.5
ar-turmerone	216.32	0	1	4	4

The tice rule provides a framework for predicting the insecticide-likeness of small molecules based on five key parameters: molecular weight (MW ≤ 500 g/mol), Hydrogen Bond Donors (HBD ≤ 3), Hydrogen Bond Acceptors (HBA ≤ 12), number of rotatable bonds (≤ 12), and partition coefficient logP (≤ 5). These criteria are critical in evaluating the absorption, bioavailability, membrane permeability, and overall compatibility of compounds with insect biological systems, particularly in the context of whole-organism activity and environmental persistence. An examination of the physicochemical properties of the selected compounds revealed that all the compounds Eugenol, Piperine, and Ar-turmerone fall within the acceptable limits of the Tice rule parameters, indicating favorable characteristics for insecticidal activity and membrane permeability.

Eugenol (MW = 164.20 g/mol, HBD = 1, HBA = 2, rotatable bonds = 3, logP = 2) exhibited a well-balanced physicochemical profile. The presence of one hydrogen bond donor and two hydrogen bond acceptors, along with moderate rotatable bonds, suggests adequate molecular flexibility and interaction potential. Its moderate lipophilicity supports effective passive diffusion across insect cuticular membranes. Piperine (MW = 285.34 g/mol, HBD = 0, HBA = 3, rotatable bonds = 3, logP = 3.5) demonstrated no hydrogen bond donors but a moderate number of acceptors, along with limited rotatable bonds. These properties indicate strong lipophilicity and good membrane permeability, which may enhance its ability to penetrate lipid-rich insect cuticles. The relatively rigid structure may also favor stable binding interactions with target proteins. Ar-turmerone (MW = 216.32 g/mol, HBD = 0, HBA = 1,

rotatable bonds = 4, logP = 4) showed high lipophilicity with minimal hydrogen bonding capacity. The absence of hydrogen bond donors and the presence of a single acceptor, combined with moderate rotatable bonds, suggest efficient membrane diffusion and sufficient conformational adaptability for biological interactions respectively.

The screening of the majority of the compounds validated their structural and pharmacokinetic compatibility with insect physiological systems. Moreover, the inclusion of compounds allowed for a broad comparative framework to evaluate the correlation between physicochemical traits and binding performance in subsequent docking analysis. This virtual screening pipelines, ensuring that only molecules with viable bio-distribution profiles proceed to target-specific interaction studies.

The application of the Tice rule in agrochemical discovery has been widely recognized as a foundational approach for identifying compounds with favorable physicochemical properties for insecticidal activity. Originally adapted from Lipinski's Rule of Five, Tice's framework specifically addresses the requirements of insecticide and herbicide permeability, environmental mobility, and biological activity. In agrochemical research, these rule-based filters are routinely employed during early-stage screening to reduce large chemical libraries into testing organisms. Similarly, studies on pesticide-likeness have demonstrated that molecular weight, logP, hydrogen bonding capacity, and molecular flexibility are critical determinants governing the absorption and transport of agrochemicals within insect systems.

A comprehensive study by Avram *et al.,* (2014) established Quantitative models for Pesticide-Likeness (QEP), confirming that descriptors used in the Tice rule such as MW, logP, HBD, HBA, and rotatable bonds are statistically significant predictors of insecticidal activity across large agrochemical datasets. Their analysis of over 59,000 compounds revealed that nearly 68% of insecticides satisfied Tice-like physicochemical constraints, highlighting the robustness of these parameters in agrochemical design. This suggests the importance of such rule-based screening in prioritizing compounds with optimal bio-distribution and interaction potential before computationally intensive docking or experimental validation.

Further support for the relevance of Tice rule in modern pesticide discovery is provided by recent computational studies integrating docking and ADMET filtering. Souza *et al.,* (2025) applied Tice-based thresholds (MW 150–500, logP 0–5, HBD ≤ 2, HBA ≤ 8, rotatable bonds ≤ 12) during virtual screening of insecticidal candidates, demonstrating that compounds satisfying these criteria exhibited better binding affinity and structural stability in docking studies. Similarly, Tahir *et al.,* (2024) incorporated Tice rule-based filtering into computational pesticide discovery pipelines, emphasizing that such physicochemical screening significantly

improves the efficiency of identifying biologically active ligands by eliminating structurally unsuitable molecules at an early stage.

Thus, the integration of Tice rule-based screening in phytochemical studies provides scientifically validated framework for predicting insecticide-likeness, membrane permeability, and target interaction efficiency. The present findings, where eugenol, piperine, and ar-turmerone comply with all Tice parameters, are consistent with previous agrochemical research demonstrating that compounds within these physicochemical limits are more likely to exhibit effective bioavailability and enhanced docking performance. Thus, Tice rule serves as a crucial bridge between chemical structure and biological function, enabling rational selection of phytocompounds for downstream in silico and experimental studies.

4.4.3.2. Ramachandran plot analysis of selected proteins

To ensure the structural reliability of the receptor models employed in the virtual screening of phytocompounds against *T. castaneum*, a detailed stereochemical assessment was conducted using Ramachandran plot analysis through the PROCHECK suite. The crystal structures of Cytochrome P450 enzymes (CYP6BQ9 and CYP6BQ11) and Glutathione-S-transferase (GSTd1) were evaluated to examine the distribution of backbone dihedral angles (φ and ψ) and their conformity within energetically allowed regions. This analysis serves as a critical quality validation step, confirming the structural integrity and suitability of the protein models for subsequent docking studies

4.4.3.3 Stereochemical validation of CYP6BQ9 using Ramachandran plot

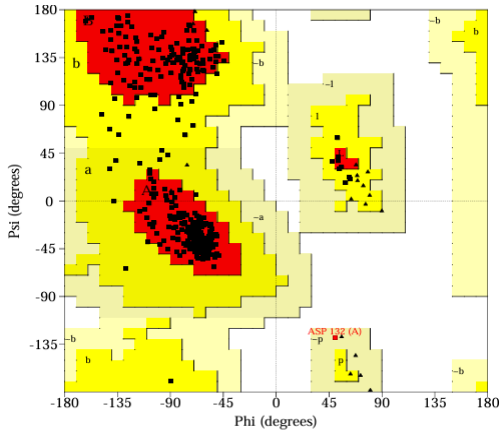

Fig. 16. Ramachandran plot of CYP6BQ9 showing residue distribution in allowed regions

The Ramachandran plot analysis of the Cytochrome P450 (CYP6BQ9) protein model (Fig. 16), generated using PROCHECK, demonstrated that 92.6% of non-glycine and non-proline residues were positioned within the most favored regions, while 7.2% were located in

extra allowed regions and only 0.2% in generously allowed regions. Importantly, no residues were detected in disallowed regions, indicating a high level of stereochemical accuracy in the protein structure.

The predominance of residues in the most favored regions surpasses the standard threshold for model reliability, thereby confirming the structural stability and correct folding of the protein. Moreover, the consistent clustering of dihedral angles within permissible regions suggests the presence of well-defined secondary structural elements, including α-helices and β-sheets. The absence of unfavourable conformations further strengthens the validity of the model, making it highly reliable for subsequent computational analyses such as molecular docking and molecular dynamics simulations, with minimal likelihood of structural deviations influencing the outcomes.

4.4.3.4. Stereochemical validation of CYP6BQ11 using Ramachandran plot

The Ramachandran plot analysis of the Cytochrome P450 (CYP6BQ11) protein structure (Fig. 17), generated using PROCHECK, indicated that 92.6% of non-glycine and non-proline residues were located within the most favored regions, while 6.9% were distributed in extra allowed regions and 0.2% in generously allowed regions. A very small fraction (0.2%) of residues was observed in disallowed regions, suggesting minor deviations in backbone geometry.

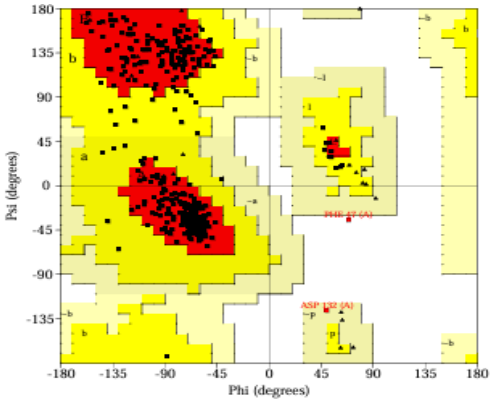

Fig. 17. Ramachandran plot of CYP6BQ11 showing residue distribution in allowed regions

Despite these minimal outliers, the overall distribution reflects a high-quality structural model, as the majority of residues occupy energetically favorable conformational spaces. The predominance of residues in favored regions indicates well-defined secondary structural elements, including α-helices and β-sheets. Although the presence of a negligible number of residues in disallowed regions suggests slight structural inconsistencies, the model remains structurally stable and reliable. Therefore, the CYP6BQ11 protein structure is considered

suitable for further structure-based computational analyses, including molecular docking and molecular dynamics simulations, with minimal impact from conformational irregularities.

4.4.3.5. Stereochemical validation of GSTd1 using Ramachandran plot

The Ramachandran plot analysis of the Glutathione-S-transferase (GSTd1) protein structure performed using PROCHECK is illustrated in Fig. 18

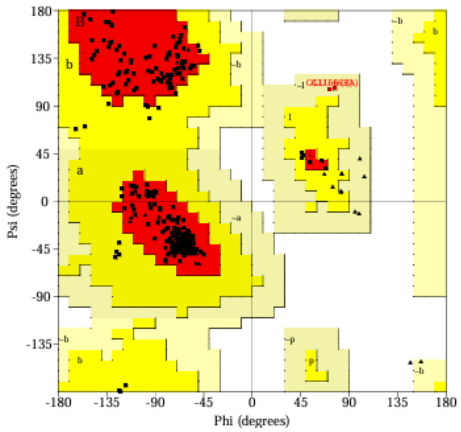

Fig. 18. Ramachandran plot of GSTd1 showing residue distribution in allowed regions

It revealed that 94.7% of non-glycine and non-proline residues were positioned within the most favored regions, while 4.8% were located in extra allowed regions and 0.5% in generously allowed regions. Notably, no residues were detected in disallowed regions, indicating excellent stereochemical quality and optimal backbone geometry. The high proportion of residues within favored regions confirms the structural integrity and proper folding of the protein, with dihedral angles consistently clustered within expected conformational spaces. This distribution corresponds to well-defined secondary structural elements such as α-helices and β-sheets. The absence of outliers further enhances confidence in the reliability of the model, making it highly suitable for downstream computational studies, including molecular docking and molecular dynamics simulations, with minimal risk of conformational artifacts affecting ligand–protein interactions.

4.4.3.6 Binding interaction analysis of selected phytocompounds with certain detoxifying proteins in *T. castaneum*

In the present study, molecular docking analysis was performed to evaluate the interaction potential of selected phytochemicals, ar-turmerone, eugenol, and piperine with known insecticidal properties against the CYP6BQ9, CYP6BQ11 and GSTd1 proteins, with the aim of assessing their influence on its structural and functional integrity. The three-dimensional structure of the validated target proteins were previously subjected to

Ramachandran plot analysis for structural reliability and utilized as receptor models for docking simulations carried out using Auto Dock Vina.

4.4.3.7 Molecular Docking Results of CYP6BQ9

The molecular docking analysis of selected phytocompounds against the CYP6BQ9 protein revealed notable differences in binding affinity and interaction efficiency (Table 13), figure illustrates 2D, 3D interactions (Fig.19,20,21).

Table – 13 *In Silico* docking analysis of selected compounds against CYP6BQ9

S.No.	Compound Name	Pubchem ID	Binding Affinity	Inhibition constant	Ligand Efficiency	VDW Hb	Cluster RMS
1	Ar-turmerone	160512	-5.77	59.17	-0.36	-6.96	0.0
2	Eugenol	3314	-4.57	445.51	-0.38	-5.66	0.0
3	Piperine	638024	-6.93	8.28	-0.33	-7.77	0.0

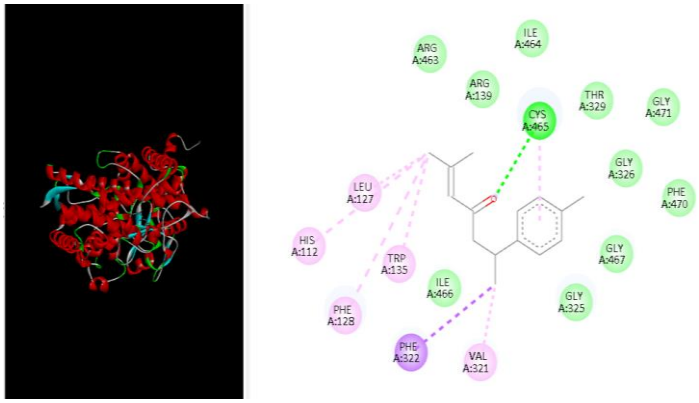

Fig.19.3D visualization and 2D interaction of Tumerone – CYP6BQ9

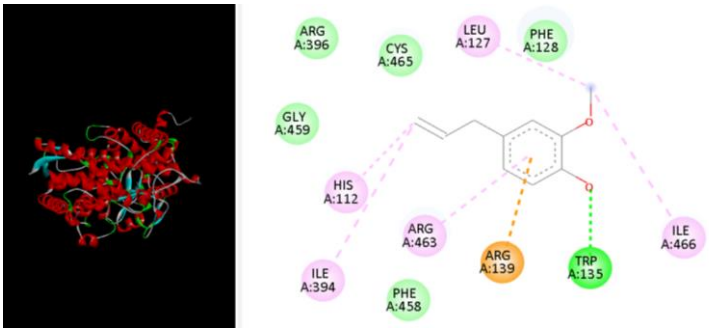

Fig.20 3D visualization and 2D interaction of Eugenol – CYP6BQ9

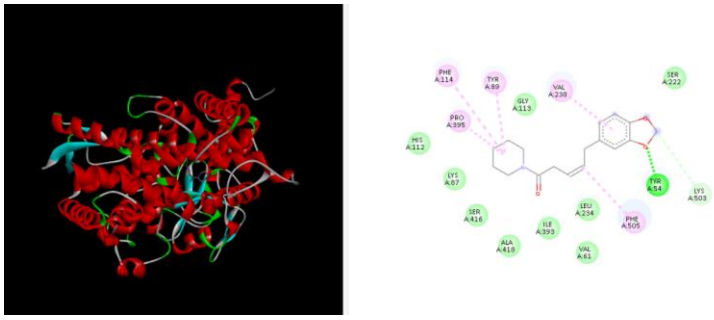

Fig.21.3D visualization and 2D interaction of piperine – CYP6BQ9

Among the three ligands, piperine exhibited the strongest binding affinity (−6.93 kcal/mol), followed by ar-turmerone (−5.77 kcal/mol) and eugenol (−4.57 kcal/mol), indicating that piperine forms the most stable ligand–protein complex. The inhibition constant (Ki) values further supported this observation, with piperine showing the lowest value (8.28 µM), reflecting higher binding potency compared to ar-turmerone (59.17 µM) and eugenol (445.51 µM).Ligand efficiency analysis demonstrated comparable values across the compounds, with eugenol (−0.38) showing slightly higher efficiency than that of ar-turmerone (−0.36) and piperine (−0.33), suggesting effective binding relative to its smaller molecular size. Van der Waals and hydrogen bonding interaction energies were also most favorable for piperine (−7.77 kcal/mol), followed by ar-turmerone (−6.96 kcal/mol) and eugenol (−5.66 kcal/mol), indicating stronger intermolecular interactions in the piperine–protein complex. The cluster RMSD value of 0.0 Å for all compounds reflects the stability and consistency of the predicted docking conformations.

Thus, the docking results suggest that piperine is the most promising inhibitor of CYP6BQ9, exhibiting the highest binding affinity, lowest inhibition constant, and strongest interaction profile, followed by ar-turmerone and eugenol. These findings indicate the potential of piperine as an effective phytochemical candidate for targeting CYP450-mediated detoxification pathways in *T. castaneum*.

4.4.3.8 Molecular Docking Results of CYP6BQ11

The molecular docking analysis of selected phytocompounds against the CYP6BQ11 protein demonstrated distinct variations in binding affinity and interaction potential (Table 14), figure illustrates 2D, 3D interactions (Fig.22,23,24).

Table - 14 *In Silico* docking analysis of selected compounds against CYP6BQ11

S.No.	Compound Name	Pubchem ID	Binding Affinity	Inhibition constant	Ligand Efficiency	VDW Hb	Cluster RMS
1	Ar-turmerone	160512	-5.44	103.17	-0.34	-6.53	0.0
2	Eugenol	3314	-4.36	638.92	-0.36	-5.59	0.0
3	Piperine	638024	-6.27	25.14	-0.33	-7.14	0.0

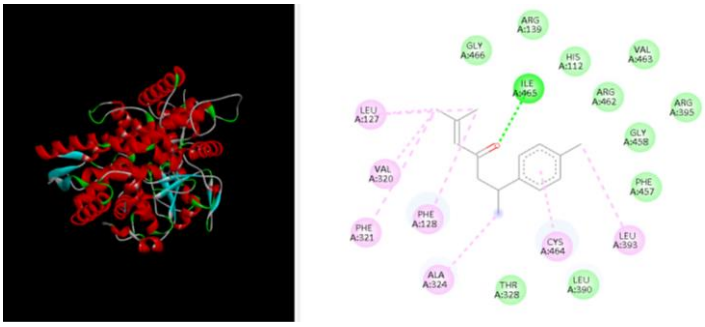

Fig.22.3D visualization and 2D interaction of Tumerone – CYP6BQ11

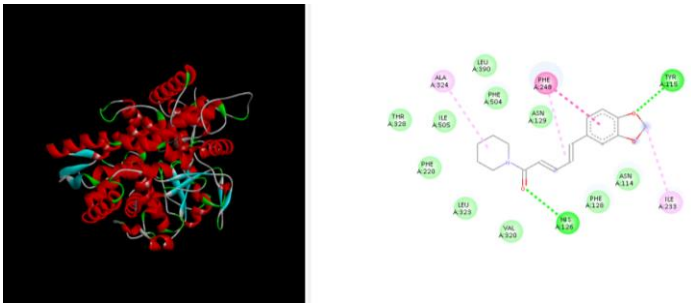

Fig.233D visualization and 2D interaction of Eugenol – CYP6BQ911

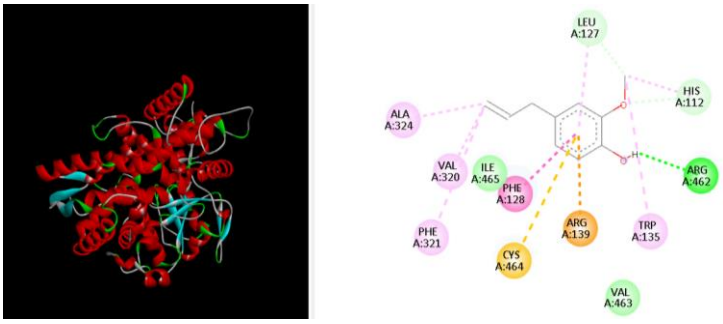

Fig.24. 3D visualization and 2D interaction of piperine – CYP6BQ11

Among the tested ligands, piperine exhibited the highest binding affinity (−6.27 kcal/mol), indicating the formation of relatively stable ligand–protein complex, followed by ar-turmerone (−5.44 kcal/mol) and eugenol (−4.36 kcal/mol). The inhibition constant (Ki) values further supported this trend, with piperine showing the lowest value (25.14 µM), suggesting stronger binding efficiency compared to ar-turmerone (103.17 µM) and eugenol (638.92 µM).

Analysis of ligand efficiency revealed comparable values among the compounds, with eugenol (−0.36) showing slightly higher efficiency relative to ar-turmerone (−0.34) and

piperine (−0.33), indicating efficient binding in relation to its smaller molecular size. The van der Waals and hydrogen bonding interaction energies were most favorable for piperine (−7.14 kcal/mol), followed by ar-turmerone (−6.53 kcal/mol) and eugenol (−5.59 kcal/mol), reflecting stronger intermolecular interactions in the piperine–protein complex. Moreover, the cluster RMSD value of 0.0 Å for all ligands proved the stability and reproducibility of the docking conformations.

Thus, the results indicate that piperine exhibits the most promising inhibitory potential against CYP6BQ11, followed by ar-turmerone and eugenol, suggesting its effectiveness in targeting cytochrome P450-mediated detoxification pathways in *T. castaneum*.

4.4.3.9. Molecular Docking Results of GSTd1

The molecular docking analysis of selected phytocompounds against the Glutathione-S-transferase (GSTd1) protein revealed differences in binding affinity and interaction strength among the ligands (Table 15) figure illustrates 2D, 3D interactions (Fig.25,26,27). Similar to CYP6BQ9 and CYP6BQ11 proteins, Among the tested compounds, piperine demonstrated the highest binding affinity (−5.83 kcal/mol) with GSTD1, indicating the formation of a relatively stable ligand–protein complex, followed by ar-turmerone (−5.30 kcal/mol) and eugenol (−4.15 kcal/mol). The inhibition constant (Ki) values further supported this observation, with piperine exhibiting the lowest value (53.16 µM), suggesting stronger binding potential compared to ar-turmerone (138.08 µM) and eugenol (902.57 µM).

Table – 15 *In Silico* docking analysis of selected compounds against GSTd1

S.No.	Compound Name	Pubchem ID	Binding Affinity	Inhibition constant	Ligand Efficiency	VDW Hb	Cluster RMS
1	Ar-turmerone	160512	-5.3	138.08	-0.33	-6.48	0.0
2	Eugenol	3314	-4.15	902.57	-0.35	-5.24	0.0
3	Piperine	638024	-5.83	53.16	-0.28	-6.71	0.0

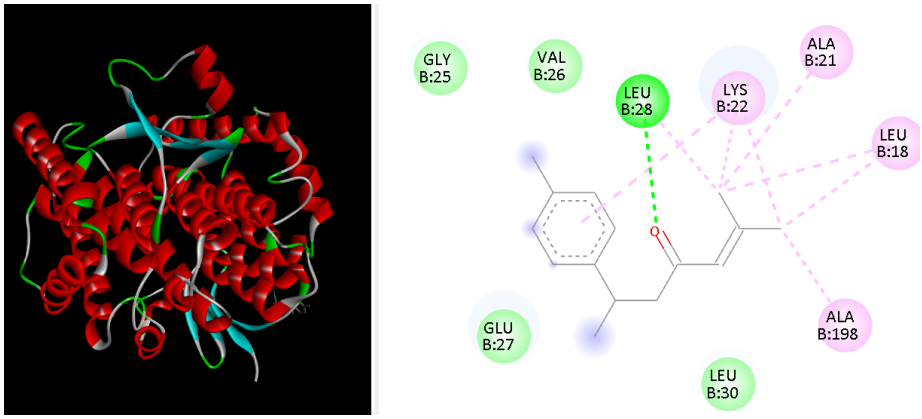

Fig.25. 3D visualization and 2D interaction of Tumerone – GSTd1

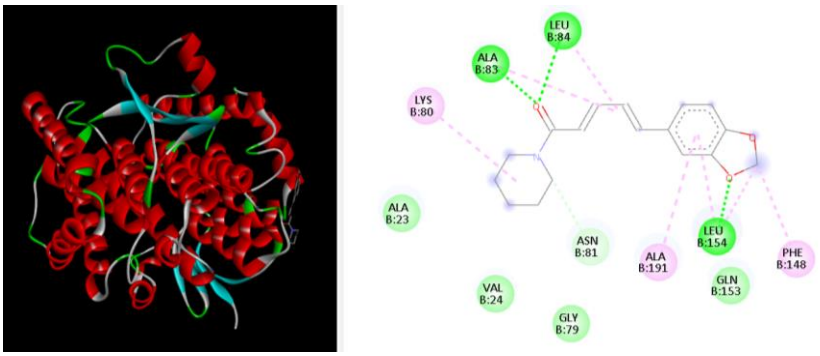

Fig.26.3D visualization and 2D interaction of Eugenol – GSTd1

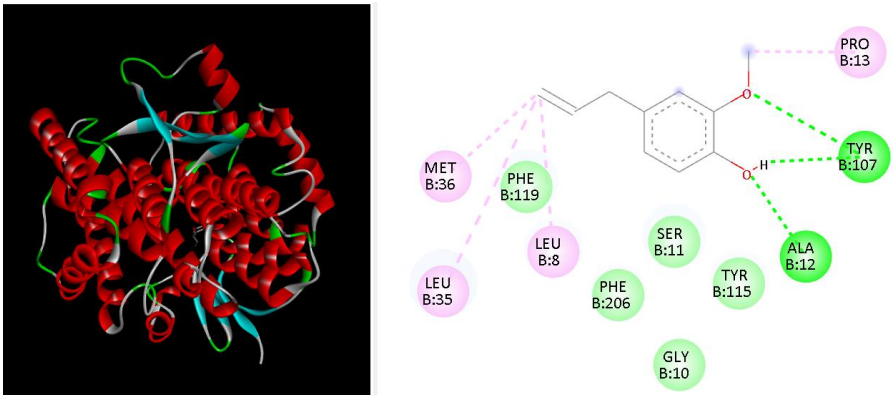

Fig.27. 3D visualization and 2D interaction of piperine – GSTd1

Ligand efficiency values of eugenol (−0.35) showing slightly higher efficiency relative to ar-turmerone (−0.33) and piperine (−0.28), indicating effective binding relative to its molecular size. The van der Waals and hydrogen bonding interaction energies were most favorable for piperine (−6.71 kcal/mol), followed by ar-turmerone (−6.48 kcal/mol) and eugenol (−5.24 kcal/mol), reflecting stronger intermolecular interactions in the piperine–GSTd1 complex. The cluster RMSD value of 0.0 Å for all ligands indicate the stability and consistency of the docking conformations. Thus, the results suggest that piperine exhibits the strongest inhibitory potential against GSTd1, followed by ar-turmerone and eugenol, indicating its effectiveness in targeting detoxification pathways mediated by glutathione-S-transferase in *T. castaneum*.

The molecular docking analysis of the selected phytocompounds, ar-turmerone, eugenol, and piperine against the three target proteins (CYP6BQ9, CYP6BQ11, and GSTd1) demonstrated consistent variations in binding affinity and interaction efficiency. Among all ligands, piperine exhibited the strongest binding affinity with all proteins, reflected by lower binding energy values and inhibition constants, indicating the formation of more stable ligand–protein complexes. Ar-turmerone showed moderate binding interactions, while eugenol

consistently displayed comparatively weaker binding affinity, despite showing relatively good ligand efficiency due to its smaller molecular size.

The van der Waals and hydrogen bonding contributions, followed a similar trend, with piperine exhibiting the most favorable interaction profiles. The consistent cluster RMSD values of 0.0 Å for all ligand–protein complexes confirm the stability and reliability of the docking conformations. The results collectively suggest that the selected phytocompounds are capable of interacting effectively with key detoxification enzymes of *Tribolium castaneum*, particularly cytochrome P450s and glutathione-S-transferase, which play critical roles in insecticide resistance mechanisms.

Thus, piperine emerged as the most promising phytochemical compound, demonstrating superior binding efficiency and inhibitory potential against all the three target proteins, followed by ar-turmerone and eugenol. These findings indicate that piperine may act as a potent inhibitor of detoxification pathways, thereby enhancing insecticidal activity, and highlight its potential as a lead phytochemical for the development of eco-friendly biopesticidal agents.

The molecular docking results obtained in the present study were further compared with previously reported studies to validate the binding efficiency and inhibitory potential of the selected phytocompounds against key target proteins of *T. castaneum.* El Hamdaoui *et al.,* 2025 reported that carvacrol showed strong binding affinity toward acetylcholinesterase (−6.6 kcal/mol) and moderate interactions with GABA and octopamine receptors, indicating a multi-target neurotoxic mechanism. This corroborates with the findings of the present study, where phytocompounds, particularly piperine, demonstrated effective binding with detoxification-related proteins, suggesting that multi-target interactions enhance insecticidal efficiency. Although the target proteins differ, both studies highlight that strong binding affinity is directly associated with increased biological activity and enzyme inhibition.

Similarly, Ercan *et al.,* 2024 observed that hypericin exhibited extremely high binding affinity (−12.5 kcal/mol) toward insect PINK1 protein, indicating strong ligand–protein stability. This aligns with the present study, where piperine exhibited the lowest binding energy among the tested ligands, confirming that compounds with more negative binding energies form more stable complexes and are likely to act as effective inhibitors. The stability of docking conformations observed in both studies further reinforces the reliability of in silico predictions.

Ghaffar *et al.,* 2020 reported that quercetin showed strong binding near the heme-binding domain of CYP450 enzymes, particularly CYP6BK3, leading to inhibition of detoxification pathways. The observed binding of piperine with CYP proteins in the present

study suggests a similar mechanism of blocking metabolic detoxification and enhancing insecticidal efficacy.

In agreement with this, Sahoo and Baliyarsingh, 2024 who demonstrated that compounds such as bumetrizole and cedrene exhibited strong binding affinities (up to −8.8 kcal/mol) with multiple targets including CYP450 and AChE, confirming the effectiveness of multi-target inhibition. The correlation between lower binding energy and higher inhibitory efficiency observed in their study aligns with the present results.

Furthermore, Shunmugadevi *et al.*, 2025 reported that phytocompounds such as ellagic acid (−9.4 kcal/mol), catechin, and quercetin exhibited stronger binding than the synthetic pesticide malathion, indicating superior inhibitory potential. This is in agreement with the present study, where natural compounds, particularly piperine, demonstrated favorable binding affinities and interaction energies, supporting that the plant-derived compounds can outperform synthetic pesticides at the molecular level.

Thus, the present study, confirms the binding affinity, inhibition constant, and interaction stability are key determinants of phytochemical efficacy from the result it was obvious that the compounds with lower binding energy exhibit higher inhibitory potential.

4.5. Integrated analysis of *in silico* studies

The present investigation employed a comprehensive in silico framework integrating KEGG pathway analysis, CRISPR-Cas9 sgRNA design, and molecular docking to elucidate the functional relevance, structural interactions, and genetic manipulability of key detoxification genes, CYP6BQ9, CYP6BQ11, and GSTd1 in *T. castaneum.* This multi-dimensional strategy enabled a systems-level interpretation of detoxification, bridging biochemical pathways with molecular interactions and genome-editing feasibility. The KEGG pathway mapping clearly demonstrated that CYP6BQ9 and CYP6BQ11 are central components of the cytochrome P450-mediated xenobiotic metabolism pathway (Phase I), whereas GSTd1 operates within the glutathione-mediated conjugation system (Phase II). Functionally, Phase I enzymes catalyse oxidative transformations such as hydroxylation, epoxidation, and dealkylation, converting lipophilic phytochemicals into more reactive intermediates. These intermediates are subsequently processed through Phase II conjugation reactions, where GST enzymes facilitate the binding of glutathione, generating water-soluble metabolites that can be efficiently excreted. The structural organization of these pathways, characterized by multiple enzymatic nodes and parallel reaction routes, reflects a high degree of redundancy and metabolic plasticity, enabling the insect to process a wide spectrum of xenobiotic compounds.

Complementing the pathway-level insights, CRISPR-Cas9 sgRNA design analysis revealed that all three detoxification genes are highly amenable to genome editing, with multiple sgRNA candidates exhibiting optimal GC content, high on-target efficiency, and minimal secondary structure constraints. A notable observation is the preferential localization of sgRNA targets within the 5′ coding regions, which enhances the probability of inducing frameshift mutations and generating functional knockdowns The presence of several high-quality sgRNAs per gene underscores the feasibility oof designing robust gene-editing strategies; however, it simultaneously indicates that single-gene disruption may be insufficient due to compensatory mechanisms within the detoxification network mechanisms within the detoxification network. This highlights the importance of multiplex gene targeting approaches to effectively impair detoxification capacity and overcome functional redundancy inherent in cytochrome P450 and GST enzyme families.

At the structural level, molecular docking analyses provided further mechanistic insights into enzyme–ligand interactions, demonstrating that major phytochemicals present in the tested formulations particularly piperine, ar-turmerone, and eugenol bind stably within the sites of CYP and GST proteins. Among these compounds, piperine consistently exhibited the strongest binding affinity, as indicated by more favorable binding energies and stable docking conformations with minimal RMSD deviations. The interactions were predominantly stabilized through hydrogen bonding, hydrophobic interactions, and van der Waals forces, suggesting energetically favorable complex formation. Despite these inhibitory interactions, the persistence of multiple detoxification enzymes with overlapping substrate specificity implies that partial inhibition of individual enzymes may not substantially disrupt the overall detoxification process, thereby maintaining metabolic continuity under phytochemical stress.

The integration of these in silico findings with experimental observations provides a cohesive understanding of the biological responses of *T. castaneum* to botanical formulations. The experimental results demonstrated that all treatments, including Phytonova Balls, Diatomaceous Earth Balls, and Neem Balls, exhibited strong bioefficacy, as evidenced by high repellency rates (80–90%), complete mortality over extended exposure, significant suppression of adult emergence, and reduced grain damage. The repellency patterns indicate immediate behavioral avoidance, while the gradual increase in mortality suggests a cumulative toxic effect rather than acute toxicity. Furthermore, the marked reduction in progeny emergence indicates that these treatments interfere with critical developmental processes, potentially affecting egg viability, larval development, and reproductive success. The observed reduction in grain weight

loss further confirms the effectiveness of these formulations in limiting feeding activity and population proliferation.

However, when interpreted alongside the *in silico* data, it becomes evident that despite the strong phenotypic effects induced by the treatments, the detoxification system of *T. castaneum* remains functionally active and resilient. The coordinated action of Phase I cytochrome P450 enzymes and Phase II GST-mediated conjugation ensures continuous metabolic processing of phytochemicals, even under sustained exposure conditions. The inherent redundancy, broad substrate specificity, and sequential organization of detoxification pathways enable the insect to tolerate and metabolize a diverse array of phytochemical compounds, thereby mitigating the full impact of enzyme inhibition observed in docking studies. This functional robustness highlights a critical adaptive advantage of the insect, allowing it to withstand chemical stress through dynamic metabolic compensation.

Thus, the integrated analysis reveals a complex interplay between phytochemical toxicity and insect detoxification capacity. While the botanical formulations exert strong repellent, toxic, and developmental inhibitory effects, the persistence of an efficient and redundant detoxification network in *T. castaneum* limits the extent of metabolic disruption. These findings suggest that future pest management strategies should focus on multi-target approaches, such as simultaneous inhibition or genetic disruption of multiple detoxification genes, to effectively overcome compensatory mechanisms and enhance the efficacy of botanical insecticides.

Limitations of the study

Despite the comprehensive integration of KEGG pathway analysis, CRISPR-Cas9 sgRNA design, and molecular docking, the present study is subject to several limitations that should be considered when interpreting the findings. First, the *in-silico* nature of the analyses inherently relies on predictive algorithms and database-derived annotations, which may not fully capture the dynamic physiological conditions within *Tribolium castaneum*. KEGG pathway mapping, while informative, represents generalized metabolic frameworks and does not account for tissue-specific expression, temporal regulation, or inducible responses of detoxification genes under phytochemical exposure.

Similarly, the CRISPR-Cas9 sgRNA design results are based on computational predictions of efficiency and specificity; however, actual genome editing outcomes may vary due to factors such as chromatin accessibility, off-target effects, and cellular repair

mechanisms, which were not experimentally validated in this study. The absence of functional gene knockout or gene expression analyses limits the ability to directly confirm the biological roles of CYP6BQ9, CYP6BQ11, and GSTd1 in detoxification processes.

Furthermore, molecular docking provides a static representation of enzyme–ligand interactions and does not incorporate protein flexibility, solvent effects, or in vivo metabolic conditions. Therefore, the predicted binding affinities and interaction profiles may not fully reflect true inhibitory potential within the insect system. The lack of complementary techniques such as molecular dynamics simulations or enzyme inhibition assays further constrains the interpretation of docking results.

From an experimental perspective, although strong bioefficacy of the formulations was observed, the study did not directly quantify detoxification enzyme activity, gene expression levels, or metabolite profiling following treatment exposure. This limits the ability to establish a direct mechanistic link between the observed phenotypic effects and the underlying detoxification pathways predicted *in silico*. Also, the potential synergistic or antagonistic interactions among phytochemical constituents within the formulations were not dissected at the molecular level.

Finally, the inherent redundancy and complexity of detoxification systems in insects suggest that targeting single genes or enzymes may not be sufficient to disrupt metabolic function. However, this study did not experimentally evaluate multi-gene targeting strategies or assess long-term adaptive responses such as resistance development. Therefore, while the findings provide strong foundational insights, further *in vivo* validation and multi-omics approaches are necessary to substantiate the mechanistic interpretations and enhance the translational applicability of the results.

VI. SUMMARY AND CONCLUSION

Stored grains play a vital role in global food security, yet they are highly vulnerable to post-harvest losses, particularly due to insect infestations. Among these, *T. castaneum* is one of the most destructive secondary pests, causing severe quantitative and qualitative deterioration of stored products through feeding, contamination and rapid population growth. Conventional control strategies relying on synthetic insecticides have become increasingly inefficient due to resistance development, environmental hazards and food safety concerns. Consequently, botanical insecticides have emerged as sustainable alternatives offering eco-friendly, biodegradable and multi-target mode of action. However, their efficacy is influenced by insect detoxification mechanisms involving key enzyme systems such as cytochrome P450 monooxygenases and glutathione-S-transferase. Advances in computational biology, including KEGG pathway analysis, CRISPR-Cas9 gene targeting and molecular docking, provide powerful tools to understand these detoxification processes and phytochemical interactions at the molecular level. Integrating experimental bioassays with in silico approaches offers a comprehensive strategy for developing effective pest management solutions. Thus, the present study entitled "Integrated experimental and in silico evaluation of spice-based Phytonova Balls for the management of red flour beetle, *Tribolium castaneum* (Herbst)" was undertaken to develop eco-friendly botanical formulation and to elucidate their molecular interactions with detoxification pathways.

- The test insect, *T. castaneum* collected from households were reared on wheat grains under optimum conditions (temperature 27–30°C, Relative Humidity 65–75% and the F1 generations were used for further studies.
- The botanicals selected for formulating PNB including leaves (bay leaf, spring onion), peels (orange peel, ginger peel, garlic peel) and spices (clove, cinnamon, pepper, turmeric). In addition to PNB, DEB and NB were also prepared using compaction technique. Different concentration of the balls prepared for PNB (0.5g , 1g, 1.5g), DEB (0.5g, 1g, 1.5g) and neem (1g).Simultaneously , the size of the balls were also measured and recorded for PNB (1.20cm for 0.5 g ;1.46 cm for 1g ,1.59 cm for 1.5g) DEB (0.89 cm for 0.5g , 1.22 cm for 1g, 1.41 cm for 1.5 g)NB 1.32cm for 1g.
- The PNB was subjected to Gas chromatography - Mass spectroscopy (GCMS) and Fourier Transform Infrared Spectroscopy (FTIR) analysis.

- The fingerprint region of FTIR analysis indicated the presence of diverse functional groups such as aromatic, phenolic, amine, sulfur-containing and other glycosidic groups corresponding to various secondary metabolites. The diversity of functional groups supports the presence of multi active bio compounds responsible for insecticidal activity.
- GCMS analysis of PNB revealed the presence of multiple bioactive phytochemicals derived from the selected botanicals confirming the chemical richness of the formulation. Major compounds identified were piperine (from pepper), eugenol (from clove), turmerones (turmeric) and cinnamaldehyde (from cinnamon). All the compounds were of which are well-documented for their insecticidal, repellent and growth-inhibitory properties.
- Bioassay studies was laid on a completely Randomised Design (CRD). 20 g of broken wheat grains was taken in a Ziplock cover and treated with 0.5g, 1g, 1.5g of PNB and DEB, 1g of neem with uncontrolled treatment. Five pairs of newly emerged *T. Castaneum* were introduced into each cover and all the treatments and control were maintained in triplicates. The repellent activity of all the formulations were recorded for 48 hours. The parameters observed for the bioassay studies were.
- The 1.5 g PNB formulated balls showed the highest repellent activity, demonstrating high effectiveness, with values reaching 85.81%, which indicates strong behavioral avoidance by insects.
- Adult mortality data revealed a progressive increase in insect mortality over time, reaching 100% at extended exposure periods. DEB 1.5 g achieved complete mortality on the 6th day, followed by neem 1 g on the 7th day and PNB 1.5 g on the 8th day, demonstrating a cumulative toxic effect.
- Adult emergence results revealed a significant reduction, with near-complete suppression at higher concentrations. Among the treatments, PN-1.5 g (0.3nos), DB-1.5 g (0.3 nos), and Neem 1 g (0.3nos) were the most effective, showing the greatest inhibition of adult emergence and indicating disruption of reproductive success.
- Grain damage assessment showed lower weight loss and infestation in treated grains than in the untreated control. Neem 1 g (0.93g), PNB-1.5 g (1.35g), and DB-0.5 g (1.25g) gave the best grain protection, showing strong effectiveness and reduced pest damage in a dose-dependent manner.
- KEGG pathway mapping demonstrated that CYP6BQ9 and CYP6BQ11 are involved in Phase I detoxification, specifically in the cytochrome P450-mediated xenobiotic

metabolism pathway (KO: K14999). Also, it was revealed that GSTd1 functions in Phase II detoxification, catalyzing glutathione conjugation reactions (EC: 2.5.1.18) for detoxification of reactive intermediates.

- CRISPR-Cas9 sgRNA design identified multiple high-quality sgRNAs with efficiency scores ranging from ~61.62% to 70.73% and GC content between 40% and 55%, indicating strong gene-editing potential.
- The sgRNAs were predominantly located in the 5′ coding regions (positions ~105–1224 bp), enhancing the probability of frameshift mutations and effective gene knockout.
- Molecular docking results showed that phytochemicals formed stable complexes with detoxification proteins, with piperine exhibiting the highest binding affinity and lowest binding energy among tested compounds.
- The interactions were stabilized through hydrogen bonds, hydrophobic interactions, and van der Waals forces, indicating strong ligand–protein binding stability.
- Integrated in silico analysis revealed that detoxification operates through coordinated Phase I and Phase II pathways, with multiple enzymes exhibiting functional redundancy and overlapping substrate specificity.
- Thus, the combined experimental and computational results demonstrated that botanical formulations achieved high repellent (85.81%), complete mortality (100%), and strong progeny suppression (≈90–100%), while also targeting detoxification mechanisms at the molecular level.

Thus, this study not only validates the efficacy of botanical formulations as eco-friendly and sustainable alternatives to synthetic insecticides but also advances the understanding of insect detoxification mechanisms through an integrated experimental and *in silico* approach. The findings provide a strong scientific foundation for the development of targeted, multi-modal pest management strategies and open new avenues for the application of functional genomics and molecular modeling in stored grain protection systems.

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