Formulation and evaluation of *Syzygium aromaticum* essential oil nanoemulsion: Effects on *Tribolium castaneum*, wheat growth, and molecular docking for pest control

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Abstract

The nanoemulsion was prepared using ethanol (3%), the biosurfactant Tween 80 (5%), and water (80%), which together constituted 20% (*v/v*) of the nanoemulsion. The toxicity of *Syzygium aromaticum* (clove) essential oil nanoemulsion was evaluated against the population of *Tribolium castaneum* in terms of LC_{50} (lethal concentration), which was determined to be 112.93 ppm. The impact of the clove essential oil nanoemulsion formulated at LC_{50} on wheat germination and seedling growth was assessed using a pot test. Results showed that the treatment inhibited wheat seedling growth and reduced the overall growth rate. Additionally, the metabolites of adult *T. castaneum* beetles were analyzed following exposure to a sub-lethal concentration (LC_{20}) of the clove oil nanoemulsion. The sub-lethal dose significantly decreased glycogen and glucose levels in all adult beetles while increasing invertase activity and total protein in resistant populations throughout the exposure period. These metabolic changes highlight the biochemical impact of the nanoemulsion. A molecular docking study was conducted to predict the mode of action of the major components of the essential oil and nanoemulsion, namely eugenol and α-humulene, at the binding site of the enzyme alkaline acid phosphatase of *Tribolium castaneum*. The results provide insights into the molecular interactions between insect-plant compounds and their effects at the biochemical level. These findings suggest the potential of clove essential oil nanoemulsion as a natural, eco-friendly solution for sustainable pesticide management in stored grain facilities. Furthermore, the study emphasizes the need to understand the side effects on both animals and humans to ensure safe applications.

Keywords: eugenol, invertase, lethal concentration, *Syzygium aromaticum* essential oil, *Tribolium castaneum*.

Formulação e avaliação da nanoemulsão de óleo essencial de *Syzygium aromaticum*: Efeitos sobre *Tribolium castaneum*, crescimento do trigo e encaixe molecular para o controle de pragas

Resumo

A nanoemulsão foi preparada utilizando etanol (3%), o biossurfactante Tween 80 (5%) e água (80%), que juntos constituíram 20% (*v/v*) da nanoemulsão. A toxicidade da nanoemulsão do óleo essencial de *Syzygium aromaticum* (cravo-da-índia) foi avaliada contra a população de *Tribolium castaneum* em termos de CL_{50} (concentração letal), determinada em 112,93 ppm. O impacto da nanoemulsão do óleo essencial de cravo formulada na CL_{50} sobre a germinação do trigo e o crescimento das plântulas foi avaliado por meio de um teste em vasos. Os resultados mostraram que o tratamento inibiu o crescimento das plântulas de trigo e reduziu a taxa de crescimento geral. Além disso, os metabólitos dos besouros adultos de *T. castaneum* foram analisados após exposição a uma concentração subletal (CL_{20}) da nanoemulsão de óleo de cravo. A dose subletal reduziu

significativamente os níveis de glicogênio e glicose em todos os besouros adultos, enquanto aumentou a atividade da invertase e os níveis de proteína total nas populações resistentes ao longo do período de exposição. Essas alterações metabólicas destacam o impacto bioquímico da nanoemulsão. Um estudo de acoplamento molecular foi conduzido para prever o modo de ação dos principais componentes do óleo essencial e da nanoemulsão, nomeadamente eugenol e α-humuleno, no sítio de ligação da enzima fosfatase alcalina ácida de *T. castaneum*. Os resultados fornecem insights sobre as interações moleculares entre compostos inseto-planta e seus efeitos no nível bioquímico. Esses achados sugerem o potencial da nanoemulsão do óleo essencial de cravo como uma solução natural e ecologicamente correta para o manejo sustentável de pragas em instalações de armazenamento de grãos. Além disso, o estudo enfatiza a necessidade de compreender os efeitos colaterais em animais e humanos para garantir aplicações seguras.

Palavras-chave: eugenol, invertase, concentração letal, óleo essencial de *Syzygium aromaticum*, *Tribolium castaneum*.

1. Introduction

The red flour beetle (*Tribolium castaneum*) is one of the most destructive insect pests to grains and flours, primarily due to its high reproductive rate. This beetle is especially problematic during the wet season, when its fourth instar larvae are highly active and cause severe infestations (Klingler, Bucher, 2022). *Tribolium castaneum* is commonly associated with human-stored food, where it has been found in large numbers, often alongside “several hundred individuals.” It infests a wide range of products, including flour, grains, peas, beans, cacao, almonds, dried fruits, and spices, though milled grain products remain its preferred food source (Alam et al., 2017).

Tribolium castaneum poses a significant economic burden on global food storage and production systems. The pest is responsible for approximately 10–15% of annual losses in stored products worldwide, impacting grains, flours, and other foodstuffs. In the cocoa industry, *T. castaneum* infestations can damage up to 50% of cocoa beans, leading to annual losses of approximately $3.16 billion due to reduced production and quality (Herndon et al., 2020). Overall, T. castaneum and similar pests contribute to billions of dollars in global economic losses each year, emphasizing the urgent need for effective pest management strategies.

Despite its damaging impact, *T. castaneum* has one of the fastest population growth rates among stored-product beetles due to its high reproductive capacity and long lifespan. However, its population growth can be limited by adult and larval cannibalism (Upadhyay et al., 2019). The beetle's ability to disperse across the landscape and infest new storage sites facilitates rapid population growth, compounding its threat to food security (Atta et al., 2020).

In storage conditions, *T. castaneum* can cause losses exceeding 18%, contributing to billions of dollars in damage globally. For example, in Pakistan, the beetle infests 6–10% of the country's wheat during storage each year, resulting in significant economic losses (Hassan et al., 2021). Additionally, the infestation leads to contamination of food grains, which can result in food adulteration, customer complaints, and increased costs associated with product returns and treatment. This contamination is a major source of economic impact on businesses (Stathas et al., 2023).

Tribolium castaneum undergoes six larval instars, with the sixth instar developing into a pre-pupa, a crescent-shaped stage before metamorphosis into a pupa (Arthur et al., 2020). The pharate adult stage, which occurs after pupation, involves the development of wing covers and legs. During this stage, the insect is preparing for its adult life, a process that takes several days.

To manage *T. castaneum* populations, synthetic insecticides and fumigants have traditionally been used, but these methods are becoming less effective due to environmental concerns, rising application costs, pesticide resistance, and harm to non-target organisms. Fumigation has been identified as one of the most effective methods for reducing stored grain pests (Gao et al., 2022). However, there is growing interest in plant-based alternatives, especially in developing countries where plants are abundant year-round. Plant extracts and essential oils have shown promise as safer, environmentally friendly alternatives to synthetic chemicals (Aboelhadid, Youssef, 2021).

Essential oils (EOs), such as those derived from clove (*Syzygium aromaticum*), offer a natural method of pest control. Clove oil contains Eugenol and has shown significant insecticidal and antimicrobial properties, making it an effective treatment against *T. castaneum* and other pests (Hashem et al., 2020). Clove essential oil is particularly effective against various insect pests, including *Pediculus capitis* and *Anopheles mosquitoes*, and can

prevent the emergence of *Culex pipiens* larvae. In addition, essential oils like clove oil are less toxic to humans and pets compared to synthetic pesticides, and they leave no harmful residues, making them a more sustainable option (Sharma et al., 2019).

Plant-based insecticides, such as citronella oil and other essential oils, offer a dual-action defense. They kill insects on contact and provide long-lasting repellency. This dual action ensures extended protection, making these plant oils an effective and safer alternative to synthetic chemicals (Afshar et al., 2017). Compared to conventional pesticides, essential oils are more environmentally friendly and leave behind fewer harmful residues, offering a promising solution for pest control in food storage systems.

This study aimed to develop a formulation and evaluate a nanoemulsion based on *Syzygium aromaticum* essential oil with effects on *Tribolium castaneum*, wheat growth, and molecular docking for pest control.

2. Materials and Methods

2.1 Culture preparation of Tribolium castaneum

The red flour beetles (*Tribolium castaneum*) were collected from the insectary at the University of Central Punjab in Lahore, where they were maintained under controlled conditions of 30 °C and 60% relative humidity (RH) (Al-Zereini et al., 2023). These insects were reared in 300 mL glass jars, each filled with a mixture of flour, rice, and broken wheat to provide an adequate food source. To prevent escape and to protect the beetles from contamination by external pests, muslin cloths covered the jars and were secured with rubber bands.

For experimental consistency, different larval stages (second, fourth, and sixth) were raised in separate jars. Males were differentiated from females based on their coat color and size. Males had a deeper brown coat and were larger, while females were smaller. Each jar was labeled with the population name and the date of preparation. Adult beetles were allowed to mature, with daily checks to confirm the emergence of first instar larvae from the growing medium (Hafiz et al., 2017).

2.2 GC-MS analysis of essential oil

Syzygium aromaticum (clove) essential oil (EO), sourced from Chiltan Pure (Pakistan), was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). This technique was chosen due to its ability to provide a detailed and reliable profile of volatile compounds in essential oils, which is essential for determining the chemical constituents of complex mixtures. The GC-MS system used a silica capillary column and was coupled to a mass spectrometer. The essential oil was diluted in ethyl acetate before analysis to ensure proper resolution of peaks.

The temperature program started at 40 °C, which was increased gradually to 260 °C over 3 min, enabling the separation of the volatile components. Helium was employed as the carrier gas. The mass spectrometer operated within a range of 30–500 atomic mass units (amu), allowing for the identification of a wide variety of compounds. Retention indices of the detected peaks were compared with those of known standards to confirm the identity of individual chemicals. The composition percentages of the essential oil were determined by calculating the peak area and retention time.

2.3 Preparation of Nanoemulsion

Nanoemulsions can be prepared using either low-energy or high-energy methods. In this study, a high-energy method, specifically high-pressure homogenization, was used to prepare the clove essential oil nanoemulsion (Kumar et al., 2019). The preparation steps are as follows:

Surfactant and Oil Mixture: A beaker was filled with 5 mL of Tween 80 (a non-ionic surfactant) and 15 mL of pure clove essential oil. The mixture was stirred for 30 minutes to ensure complete blending.

Water Addition: 80 mL of distilled water was added, resulting in a final solution volume of 100 mL.

Homogenization: The mixture was then homogenized for 30 minutes to reduce droplet size and form a uniform emulsion.

Sonication: The solution was placed in a sonicator for five minutes to further reduce the droplet size and stabilize the nanoemulsion.

Storage: After preparation, the nanoemulsion was stored at room temperature (approximately 25 °C) for five days to allow for stabilization (Gupta et al., 2023).

The oil phase of the nanoemulsion consisted of the essential oil (14% *v/v*), ethanol (3%), and biosurfactant (Surfactin, Tween 80, with a final concentration of 3%), which made up 20% of the total emulsion (Hashem et al., 2020).

2.5 Figures, diagrams, and other non-originals

Non-original figures (authored by the author) must include, after the title, the source from which they were extracted; the sources must be referenced. Credit to the author of photographs is mandatory, as is credit to the author of drawings and graphics that required creative action in their creation. The image source must be cited in the References topic.

2.6 Nano emulsion as an insecticide

Acetone was used as a solvent to create stock solutions and subsequent dilutions of the *S. aromatum* nanoemulsion. Insects (fourth and sixth instar larvae and newly emerged adult beetles) were exposed to nanoemulsion using the FAO-recommended filter paper impregnation method to determine the LC_{50}, by WHO (2012) recommendations. Several doses, including 1000, 750, 500, 250, 125, 62.5, 31.5, 15.3, and 7.5 ppm, were used in triplicate to determine the LC_{50}. To prepare the doses, a glass pipette was used to apply 1 mL of each concentration of insecticide solution to the center of a 130 cm-by-130 cm filter paper, and the solution was evenly spread out (Ambreena et al., 2017).

The preparation of control *Petri* plates was identical, but acetone was applied to the filter papers. Ten healthy insects were added to each labeled *Petri* plate after air-drying filter sheets had been placed inside of them and covered. According to Lloyd (1969), mortality was noted after 24 h, and larvae that did not move after being pressed against a brush were deemed dead. The mortality data of T. castaneum was subjected to probit analysis (statistical technique used to model binary or ordinal outcomes by linking the independent variable to the probability of an event using the cumulative distribution function (CDF) of a normal distribution) using SPSS software to calculate the LC_{50} values of the 4th and 6th instars larvae and adult beetles. The results were expressed in ppm. Insecticidal toxicity was determined against adult beetles of *T. castaneum* and their effective nanoemulsion was evaluated. Comparison was made in 3 replicates to check the efficacy of clove oil essential oil and its Nano emulsion (Tanzeela Riaz et al., 2021).

2.7 Exposure of LC_{20}

The sub-lethal dose, or LC20, was found to be an effective way to determine the harmful effects of pesticides because, at this dose, beetle physiological and biochemical responses were significant enough to readily understand the mode of action, and mortality response was low due to the low toxic dose of insecticides. Approximately 500 adult beetles and their homogenous population controls were exposed individually to their respective dose of LC_{20} at 35 ± 2 °C and 60 ± 2 °C relative humidity for a 24-hour period. Adult beetles, both treated and untreated, were immediately exposed to biochemical content analysis.

2.8 Seed germination

The impact of nanoemulsion on seed germination and seedling growth was assessed through a laboratory experiment using disposable pots filled with fertile sand. Each 9-cm-diameter pot received 5 milliliters of the respective nanoemulsion treatment solution. Twenty wheat seeds were then placed in each pot, which was subsequently sealed with aluminum foil. The pots were incubated for seven days in the sunlight followed by placement in the sunlight at 30 ± 2 °C, with a 12/12-h light/dark cycle and approximately 80% humidity. A solution containing acetone and water was used as the control. After the seven days, germination rates and the lengths of roots and shoots (measured in centimeters) were recorded.

Figure 1. Seed germination of wheat by nano emulsion of clove essential oil. Source: Authors, 2024.

2.9 Biochemical analysis

Using the LC_{20} of the nanoemulsion, biochemical analysis was estimated for the contents of carbohydrates, including total lipids, total proteins, trehalose, soluble proteins, free amino acids, glucose, and glycogen. Enzymatic activity, including trehalase, amylase, catalase, and invertase against adult *T. castaneum*, was also estimated.

2.10 Estimation of soluble protein

Estimation of contents of soluble proteins of extract of adult beetles was dogged by the method of Naseri & Borzui (2016). This procedure depends on the reaction of ions of copper with the strong chemical bond that is the peptide bond in basic solution, called the Biuret test, which cause the process of oxidation in residues of specific protein (aromatic) of the Bradford reagent, and with any other phenol reagent that looks in blue color. Intensity of blue color and the concentration of protein in the test sample are both directly proportional to each other, and this is measured by recording the absorbance of blue color with the help of spectrophotometer at 750 nm (Naseri et al., 2020).

Biological extract was prepared by first taking 20 beetles separately in test tubes from both treated and untreated groups of adult beetles, and then maceration was done by adding 2 mL of 89% saline solution with the help of motor-driven Teflon glass homogenizer. After this, this homogenate was centrifuged for 30 min at a speed of 3000 x g. Supernatant and pellet were cleared after this.

Take 0.4 mL of tissue supernatant of treated sample, 0.4 mL of tissue supernatant of untreated sample and 0.4 mL of distilled water (blank) in test tubes with three replicates of each sample separately and then add 1 mL of Bradford reagent in all test tubes. Incubate these test tubes 1 at a temperature of 37 °C for almost 5 min. After that, recorded the absorbance of the blank and test samples that were showing a blue color at the wavelength of 595 nm. To calculate the soluble proteins of the sample proteins, the following formula was used:

$$\text{Soluble protein contents } (\mu g/mg) = \frac{\text{C.V} \times \text{total extract} \times \text{dilution}}{0.4 \times \text{weight of beetles (mg)}}$$

Where: C.V = Optical Density Converted value from standard graph.

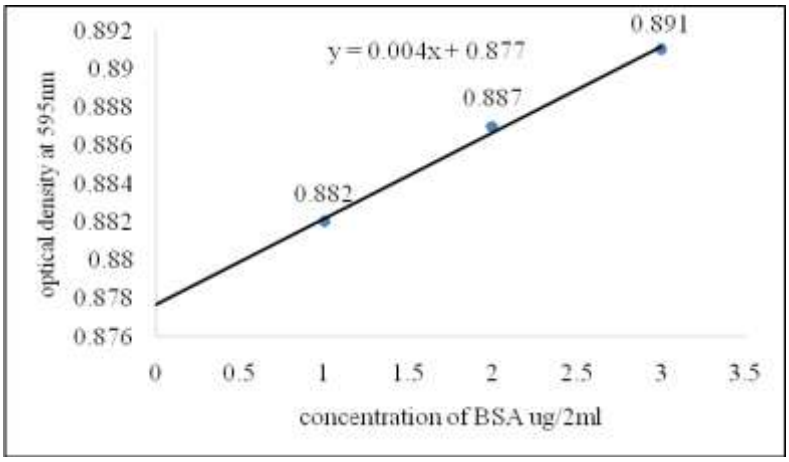


Figure 2. Standard curve for the estimation of soluble protein. Source: Authors, 2024.

2.11 Estimation of total proteins

A total protein estimation was done to determine the amount of total protein in the adult beetles' extracts that was affected by a very low dose (LC_{20}) of nano emulsion. This test specifically measures the amount of globulin and albumin. In this estimation, 20 adult beetles from both groups (treated and untreated) were taken in a test tube for the preparation of tissue homogenate, and then maceration (grinding process) was done by adding 2 mL of IM NaOH (sodium hydroxide) with the help of a homogenizer. After it, this homogenate was centrifuged for almost 30 minutes at a speed of 3000 x g. Supernatant and pellet were cleared after this. Take 0.4 mL supernatant from both treated and untreated samples in three replicates of each sample in different test tubes and add 1 mL of Bradford reagent to it and incubate it for 5 min. Same procedure was done with a blank containing 0.4 mL of distilled water. After this, record the absorbance after the appearance of blue color at 630 nm. To calculate the concentration of total proteins in samples, the following formula was used:

$$\text{Total protein contents } (\mu g/mg) = \frac{\text{C.V} \times \text{total extract} \times \text{dilution}}{0.4 \times \text{weight of beetles (mg)}}$$

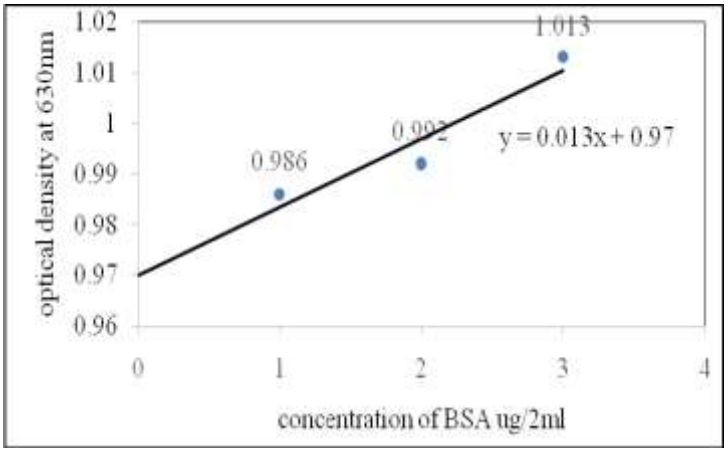


Figure 3. Standard curve for the estimation of total protein. Source: Authors, 2024.

2.12 Estimation of free amino acids

Estimation of contents of FAA (Free Amino Acids) was done by the procedure of more and Stein's described in 1969 (Stein et al., 2022). This estimation is based on the reaction of amino acid with Ninhydrin that results in the production of Ruhemann's purple, which is a dark purple color.

The following procedure was used for the determination of free amino acid content in samples. 20 adult beetles

were taken separately in test tubes from both treated and untreated groups to prepare extracts, and then maceration was done by adding 2 mL of 80% ethanol by the Teflon glass homogenizer. After it, this homogenate was subjected to centrifugation for 10 min at a speed of 5000 x g. Supernatant and pellet were cleared after this. Add 1 mL of ninhydrin reagent in 0.4 mL tissue supernatant of treated group, 0.4 mL tissue supernatant of untreated group (control), 0.4 mL of cholesterol standard and in 0.4 mL blank (distilled water) separately in three replicates of each sample separately. After it, boil all samples in a boiling water bath for 15 minutes at 100 °C, followed by cooling on crushed ice. Then add 1 mL of 50% ethanol in all test tubes and incubate these samples for 10 min at room temperature. Record the intensity of light with the help of a spectrophotometer at a specific wavelength of 570 nm.

Formula that was used to estimate the amount of free amino acids is shown below:

$$\text{Free Amino Acid contents } (\mu g/mg) = \frac{OD^t \times 100 \times \text{total extract} \times \text{dilution}}{OD^{st} \times \text{weight of beetles (mg)}}$$

Where: OD = Optical density (test sample); ODst = Optical density of standard solution.

2.13 Estimation of glycogen contents

Anthrone's approach was used to determine the concentration of glycogen in beetles' extract. This estimation is used for the analysis of carbohydrates present in test samples. This was done under acidic environment and based on the reaction of glycogen with Anthrone's reagent, and at the end point a blue-green color was seen. For this, preparation of tissue isolation was done by first taking treated and non-treated 20 adult beetles in a test tube separately, then maceration was done by adding 2 mL of KOH (Potassium hydroxide) with the help of homogenizer and then boil it in a hot water bath for almost 30 min and add 1.5 mL pure ethanol. After it, this homogenate was centrifuged for 10 min at a speed of 1180 x g.

Supernatant and pellet were cleared after this. 0.1 mL of distilled water was added to the pellet while the supernatant was discarded to proceed with results. This estimation was done in three replicates. Add 3 mL of Anthrone's reagent in each replicate that is 0.1 mL of tissue extract, 0.05 mL of 1% standard glucose and 0.1 mL blank solution (distilled water). Then, place these for boiling at 100 °C on a boiling water bath for almost 10 min and then quickly cool them. This showed a color of blue-green. After it, the absorbance of the test sample and glucose standard solution was observed against all samples by the spectrophotometer at a specific wavelength of 620 nm. Glycogen contents of all test samples were measured by the following formula:

$$\text{Free Amino Acid contents } \left(\frac{\mu g}{mg}\right) = \frac{OD^t \times \text{concentration of standard} \times \text{total extract} \times 0.9}{OD^{st} \times \text{weight of beetles (mg)}}$$

Where: 0.9 = Standard factor (for conversion of glycogen into glucose).

2.14 Estimation of glucose contents

Estimation of glucose contents was preceded according to (Riaz et al., 2021). This method is done under high temperatures, a strong acidic condition, and based on the mixing of color reagent (ortho-toluidine) with glucose, and this mixing reaction, because the formation of a green colored Schiff's base by the production of glycosylamine. In this estimation, 20 adult beetles from both groups (treated and untreated) were taken in a test tube for the preparation of tissue homogenate, and then maceration was done by adding 2 mL ethanol by a homogenizing machine. After it, this homogenate was run for centrifugation for 15 minutes at a speed of 461 x g. Supernatant and pellet were cleared after this. Take 0.5 mL of supernatant and add 2.5 mL of ortho-toluidine colored reagent in this and boil it at 100 °C for 15 min. After this, record the absorbance after the appearance of green color at 590 nm. Same was done with glucose standard (0.5 mL) and blank (0.5 mL distilled water). This estimation was done in three replicates. To calculate the concentration of glucose in the test sample, the following formula was used:

$$\text{Free Amino Acid contents } \left(\frac{\mu g}{mg}\right) = \frac{OD^t \times \text{concentration of standard} \times \text{total extract} \times 0.9}{OD^{st} \times \text{weight of beetles (mg)}}$$

Where: 0.9 = Standard factor (for conversion of glycogen into glucose)

2.15 Estimation of trehalose contents

Estimation of trehalose content was done by the method of Paul. This method is done under high temperature, strong acidic condition, and based on the mixing of Anthrone reagent with sulfuric acid at the point of boiling, and this mixing reaction causes the formation of blue-green color at 620 nm. This method is also used to determine the concentration of reducing and non-reducing carbohydrates because of the oxidizing sulfuric acid present in it (Islam; Mohammad, 2009).

In this estimation, preparation of tissue homogenate was done by first taking 20 beetles in a test tube from both treated and untreated groups, and then maceration was done by adding 2 mL of 89% saline solution by a homogenizer. After it, this homogenate was centrifuged for 45 min at a speed of 4900 x g. Supernatant and pellet were cleared after this. Take volume of 0.05 mL of supernatant of tissue extract, 0.05 mL of standard solution of trehalose and 0.05 mL of blank (containing distilled water) in all test tubes separately and add 2.5 mL of Anthron's reagent in this and boil it at 100 °C for 15 min. After this, record the absorbance after the appearance of blue-green color at 620 nm. Absorbance was recorded with the help of a spectrophotometer. This estimation was done in three replicates. To calculate the concentration of trehalse in the test sample, the following formula was used:

$$\text{Trehalose contents (IU/mg)} = \frac{OD^{t} \times \text{concentration of standard} \times \text{total extract} \times 0.9}{OD^{st} \times \text{weight of beetles (mg)}}$$

2.16 Estimation of trehalase activity

Estimation of trehalase activity was done by the method of Dahlqvist to determine the quantity of glucose that is produced with the help of trehalase present in the extract by the breakdown of trehalose. Disaccharide trehalose is the insect's main hemolymph sugar that is hydrolyzed by trehalose enzyme into two glucose units, which is used as an energy source and helps to run metabolic pathways. This method is done under high temperature, strong acidic conditions, and depends on the mixing of color reagent (Ortho-toluidine) with a solution of glucose, this mixing reaction causes the development of glycosyl amine that makes additional rearrangement and produces colored Schiff's base. Intensity of color was recorded by the help of a spectrophotometer at 590 nm. This method is also used to determine the concentration of glucose produced (Kluch et al., 2020).

Tissue homogenate was prepared by first taking 20 beetles separately in test tubes from both treated and untreated groups, and then maceration was done by adding 2 mL of 89% saline solution with the help of a homogenizer. After it, this homogenate was centrifuged for 45 min at a speed of 4900 x g. Supernatant and pellet were cleared after this. Take 0.3 mL of tissue supernatant, 0.3 mL of glucose standard solution and 0.3 mL of distilled water (blank) in test tubes separately and then add 0.5 mL of trehalose substrate solution followed by adding 0.02 M of 0.6 mL citrate buffer by maintaining at pH 5.6 in all test tubes. Incubate these test tubes at a temperature of 37 °C for almost 30 min. Then add 3 mL of colored reagent (*O*-Toluidine) separately in all test tubes, that is, the tissue extract, the standard solution, and blank. The mixture solution was shaken vigorously and boiled for 8 min in a boiling water bath. Green color was developed in the test sample. After that, record the light intensity of the standard solution, blank, and all test samples that showed green color at a wavelength of 590 nm.

$$\text{Trehalase activity (IU/mg)} = \frac{OD^{t} \times \text{concentration of standard} \times \text{total extract} \times 30}{OD^{st} \times \text{weight of beetles (mg)} \times 180}$$

Where: 30 = total time (in minutes) for enzymatic action; 180 µg of glucose.

2.17 Estimation of amylase activity

Main function of the amylase enzyme is the breakdown of starch (complex sugar) into maltose and glucose (simple sugars) by hydrolyzing the glycosidic bonds present in starch molecules. Starch produces a blue to black color with iodine. In this procedure, some starch was used to convert complex carbohydrate into simple sugars,

and the remaining starch produced a blue color after mixing with iodine after incubation period of 15 min at 37 °C.

The following procedure was used for the determination of amylase activity. 20 adult beetles were taken separately in test tubes from both treated and untreated groups to prepare tissue homogenate, and then maceration was done by adding 2 mL of saline solution (89%) by a homogenizer. After it, this homogenate was centrifuged for 45 min at a speed of 4900 x g. Supernatant and pellet were cleared after this. Add 0.1 mL volume of buffer-starch substrate into 0.1 mL tissue extract supernatant and volume of 0.1 mL in blank test tubes separately and incubate for 15 min at 37 °C. Then 0.4 iodine solution (0.1N) was added in it and dilution was done in each mixture with 8.5 mL distilled water. Absorbance was recorded as the blue color developed at 660 nm against all samples. Conversion of absorbance to units of enzyme activity was done by the formula given below:

$$\text{Amylase activity (IU/mg)} = \frac{OD^t \times 0.8 \times \text{total extract} \times 1000}{\text{weight of beetles (mg)}}$$

2.18 Estimation of invertase activity

Ishaya and Swiriski described the method for the approximation of activity of invertase in the adult extract of khapra beetles (Sonar et al., 2021). Enzyme that is invertase hydrolyzes the residues of non-converting sucrose, which is Beta-d-fructofuranoside, to produce invert sugar. This sugar (invert) that is released was countered with 3.5 DNS. Color that was produced in this procedure was proportional to the quantity of invert sugar that is produced in this reaction, which further depends on the invertase enzyme activity present in the sample. Intensity of color that is changed is recorded with the help of a spectrophotometer at the wavelength of 550 nm.

In this procedure, preparation of tissue homogenate was done by taking 20 beetles in test tubes separately from both groups of treated and untreated adult beetles, and then maceration was done by adding 2 mL of 89% saline solution with the help of a homogenizer. After it, this homogenate was centrifuged for 45 min at a speed of 4900 x g. Supernatant and pellet were cleared after this. Take 0.02 mL of tissue supernatant (tissue extract), 0.2 mL of sucrose substrate solution (4%) and 0.02 mL of distilled water (blank) in test tubes separately. Incubation of all test tubes was done for 60 min at 37 °C temperature. After this, add 0.8 mL of 3.5 DNS (dinitro salicylic acid) reagent in all test tubes. Then placed the reaction mixture in a boiling water bath at 100 °C for 5 min and then cooled the mixture in an ice bath. Absorbance was measured at a wavelength of 550 nm against the test sample and the blank sample in a spectrophotometer. This estimation was done in three replicates. To determine the invertase activity (in IU/mg), the standard curve graph was used as different concentrations were prepared from the stock solution of 0.001M glucose as shown in the graph below. Below formula was used to calculate the invertase activity:

$$\text{Invertase activity (IU/mg)} = \frac{\text{C.V} \times \text{dilution}}{\text{tmin x total extract x2(ml) x weight of beetles (mg)}}$$

Where: C.V = OD converted values from standard graph; tmin= Incubation time for hydrolysis of substrate (in minutes); 1 = Factor of conversion for 1 µmole of hydrolyzed sucrose into glucose and fructose.

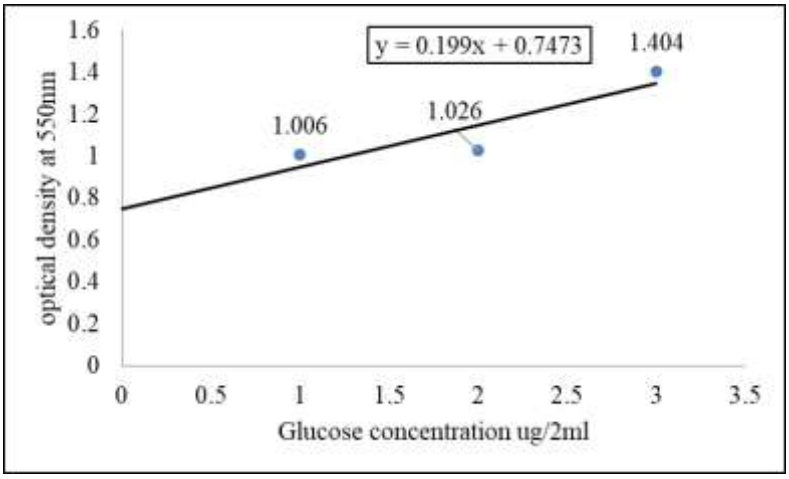


Figure 4. Standard curve for the estimation of invertase activity. Source: Authors, 2024.

2.19 Estimation of catalase activity

Sizer and Beer's presented the method for the estimation of catalase enzyme. Catalase enzyme does catalytic decomposition of hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O). In this estimation, the sample was prepared by taking 20 adult beetles in test tubes separately from both groups of treated and untreated adult beetles, and then grinding was done by adding 2 mL of 0.05 M phosphate buffer by a homogenizing machine with continuous cooling. After it, this homogenate was centrifuged for 10 min at a speed of 1300 x g. Supernatant and pellet were cleared after this. Pellet was used in this procedure while the supernatant was discarded and 0.1 mL of distilled water was added to the pellet. Estimation was done in three replicates. Take 0.3 mL of tissue supernatant (tissue extract) and blank having distilled water and add 1.2 mL of hydrogen peroxide buffer in test tubes separately. Record the absorbance at 240 nm. The following formula was used to calculate the catalase activity:

$$\text{Catalase activity}\left(\frac{\text{IU}}{\text{mg}}\right) = \frac{\text{Total Volume} \times 1000\ \text{xhomogenate}}{43 \times 10 \times \text{Vol. of supernatant} \times \text{weight of beetles(mg)}} \times \frac{\Delta A}{\Delta t}$$

2.20 Estimation of alkaline phosphatase activity

Activity of alkaline phosphatase depends on the conversion (enzymatic) of the phosphate substrate (*p*-nitrophenyl) into p-phosphoenolate and phosphoric acid. Termination of this reaction occurred when sodium hydroxide (NaOH) was added to it. Yellow color in the mixture showed the presence of *p*-phosphoenolate and this depends directly on the activity of alkaline phosphatase.

$$p\text{-nitrophenyl phosphate} + H_2O \xrightarrow{AkP} p\text{-phosphoenolate} + \text{phosphoric acid}$$

Tissue homogenate was prepared by taking 20 beetles separately in test tubes from both treated and untreated groups of adult beetles, and then maceration was done by adding 2 mL of 0.89% saline solution by a homogenizer. After this, this homogenate was centrifuged for 15 min at a speed of 13500 rpm. Incubate 0.5 mL of Glycine-NaOH buffer at a temperature of 37 °C for 5 min and then add it in 0.5 mL of tissue supernatant and 0.5 mL blank solution separately. Incubate these test tubes at a temperature of 37 °C for almost 30 min after shaking well. After it, add 5 mL of sodium hydroxide in the test sample to stop the enzymatic reaction. Record the absorbance of the blank and the test samples that were showing a yellow color at the wavelength of 405 nm by the spectrophotometer. Alkaline phosphatase activity was calculated by the given formula:

$$\text{Alkaline phosphatase activity}\ \ (\text{IU/mg}) = \frac{\text{OD}^t \times \text{TRV} \times 200 \times \text{D.F}}{\text{weight of beetles (mg)}}$$

2.21 Estimation of acid phosphatase activity

Activity of acid phosphatase depends on the conversion (enzymatic) of the phosphate substrate (*p*-nitrophenyl) into *p*-phosphoenolate and phosphoric acid. Termination of this reaction occurred when sodium hydroxide (NaOH) was added to it. Yellow color in the mixture showed the presence of p-phosphoenolate and this depends directly on the activity of acid phosphatase.

$$p\text{-nitrophenyl phosphate} + H_2O \xrightarrow{AKP} p\text{-phosphoenolate} + \text{phosphoric acid}$$

Biological sample was prepared by taking 20 beetles separately in test tubes from both treated and untreated groups of adult beetles, and then the mortar thaw method was done by adding 2 mL of 0.89% saline solution by a homogenizer. After this, this homogenate was centrifuged for period of 15 min at a speed of 13500 rpm. Incubate 0.5 mL of citrate buffer substrate at a temperature of 37 °C for 5 min and then add it in 0.5 mL of tissue supernatant and 0.5 mL blank solution separately. Incubate these test tubes at a temperature of 37 °C for almost 30 min after shaking well. After it, add 5 mL of sodium hydroxide in the test sample to stop the enzymatic reaction. Recorded the optical density of blank and test samples that were showing a yellow color at the wavelength of 405 nm by a spectrophotometer. Acid phosphatase activity was calculated by the given formula:

$$\text{Acid phosphatase activity (IU/mg)} = \frac{OD^t \times TRV \times 101 \times D.F}{\text{weight of beetles (mg)}}$$

2.22 Estimation of glutathione-S-transferase activity

This estimation depends on the enzymatic conjugation to CDNB and Glutathione. Conjugation level was determined using a spectrophotometer by measuring the intensity of light at a wavelength of 340 nm.

$$\text{Glutathione-SH} + \text{CDNB} \xrightarrow{GST} \text{Glutathione-S-CDN}$$

In this estimation, 20 adult beetles from both groups (treated and untreated) were taken in a test tube for the preparation of tissue homogenate, and then maceration was done by adding 2 mL of 50 mM phosphate buffer with the help of a homogenizer. After it, this homogenate was centrifuged for almost 20 min at a speed of 11200 x g. Then add 0.2 mL 1-chloro-2, 4-dinitrobenzene, 0.2 mL GS and phosphate buffer (0.1 mL) in tissue supernatant (0.01 mL) in all test tubes of treated, untreated and blank. Incubate these test tubes at a temperature of 25 °C for almost 2-3 minutes after shaking well. Optical density was recorded against the blank and the samples at the wavelength of 340 nm by a spectrophotometer. Activity of GST was calculated by the given formula:

$$\text{GST activity } (\mu\text{mole min}^{-1}\text{ mg}^{-1}) = \frac{\frac{\Delta OD}{\Delta t} \times D.F \times 101 \times D.F \times 3.1 \times 10^6}{0.2 \times 9600 \times \text{weight of beetles (mg)}}$$

Where: ΔD = Change in absorbance; Δt = 2 minutes; 3.1 = volume of total assay in; 106 = Conversion factor from M to uM; 0.2 = Homogenate volume; 1= path of light in cm; 96000 = Extinction coefficient.

Specific gravity of Glutathione-S-transferase was calculated by the following formula:

$$\text{Specific activity of GST } \frac{IU}{\mu g} = \frac{\mu\text{mole min}^{-1}\text{ mg}^{-1}}{\text{protein content}(\mu g mg^{-1})}$$

2.23 In-silico interaction of the enzymes

For the construction of 3D models, sequences of alkaline acid phosphatase were obtained from the National Center for Biotechnology Information (NCBI) server. These sequences were submitted to the Swiss-Model and Protein Data Bank, tools for protein structure homology modeling, using suitable structural templates to generate reliable theoretical 2D models. The structure models of alkaline acid phosphatase proteins, including their active

sites (pockets), were created using manual docking tools. For ligand selection, the main compounds present in essential oil (EO) and nano emulsions (NE) at high concentrations 60-75% were chosen as ligands for protein modeling. These compounds were retrieved from PubChem and Chemspider databases and prepared in MOL format using the Molecular Operating Environment (MOE) program to create a library of ligands.

Molecular docking was employed to predict the binding sites for the proteins, aiding in understanding the binding mechanism between the proteins and ligands. The docking procedure was performed using Autodock Vina software package, as described previously. The protein and ligand structures were modified by adding hydrogen atoms and minimizing energies using specific parameters. The best model obtained from the modeling process was utilized for docking analysis. The protein structure underwent 2D protonation and energy minimization before being used as receptors in the docking analysis. The active site of the protein was identified using the site discovery studio, and docking was executed with default parameters. Upon completion, a docked structure indicating the corresponding e-values was generated.

2.24 Statistical analysis

Statistical analysis was determined with the help of Minitab 16 software. Data was shown in the form of S.E.M. (standard error of mean), while data obtain from effects of sub lethal dose of nano emulsion and essential oilon metabolites of carbohydrates and enzymatic action was preceded through observations of "t" test un-paired at 95% confident interval and for the estimation of statistical importance, comparison of individual mean was done. Significance level for every experiment was indicated to be non-significant ($p > 0.05$) and significant ($p < 0.05$).

3. Results

3.1 GCMS analysis of essential oil

The results of the GC-MS analysis showed that the 4 metabolites were present in the *S. aromaticum* essential oil. The 56.74% eugenol, propylene glycol 25.41%, Phenol, 2-methoxy-3-(2-propenyl) 5.8% and R-(-)-1,2-propanediol 8.7% (Figure 5). Table 1 showed the chemical composition of essential oil. The GC-MS analysis of *S. aromaticum* essential oil reveals that eugenol is the major bioactive compound, responsible for most of the oil's therapeutic properties, particularly its antimicrobial and anti-inflammatory effects. Propylene glycol and R-(-)-1,2-propanediol are primarily used as stabilizing agents and solvents, ensuring the quality and stability of the oil. Phenol, 2-methoxy-3-(2-propenyl) (allyl eugenol) adds to the antimicrobial and antioxidant activities, but in smaller amounts. The overall composition indicates a balance between bioactive compounds and stabilizers, enhancing both the oil's therapeutic and practical uses.

Figure 5. GCMS of essential oil of *Syzygium aromaticum*. Source: Authors, 2024.

Table 1. Chemical composition of the essential oil of *Syzygium aromaticum*.

Metabolite	Molecular formula	Molecular weight	Peak area %	Retention time min
Propylene Glycol	$C_3H_8O_2$	76.09 g/mol	25.41	3.35

Phenol,2-methoxy-3-(2-propenyl)	$C_{10}H_{12}O_2$	164.11g/mol	5.80	3.48
Eugenol	$C_{10}H_{12}O_2$	164.20 g/mol	56.74	10.27
R-(-)-1,2-propanediol	$C_3H_8O_2$	76.09 g/mol	8.97	3.38

Source: Authors, 2024.

3.2 Insecticide efficacy

The insecticidal effects of *S. aromaticum* essential oil on adult *T. castaneum* beetles were evaluated through surface-film bioassays, where it exhibited the highest toxicity compared to other substances tested. Furthermore, exposure to clove oil vapors resulted in significant mortality across all developmental stages of *T. castaneum*. It was also noted that adult beetles were more susceptible to both direct contact with *S. aromaticum* and its fumigant properties than the larvae.

3.3 Larvicidal activity against Tribolium castaneum

Insecticidal toxicity was determined against adult beetles of *T. castaneum* and their effective nanoemulsion was evaluated. Comparison was made in 3 replicates to check the efficacy of clove oil essential oil and its nanoemulsion. The most effective diluted value of clove oil essential oil nanoemulsion was 750ppm and 1000ppm, where more beetles and larvae showed a mortality rate. Table 2 showed the dilution mortality rate of the beetles and larvae.

Toxicity of *S. aromaticum* essential oil nanoemulsion for the population of *T. castaneum* was determined in terms of LC_{50} at 95% confidence limit by probit analysis. Clove oil nanoemulsion was used to check the toxic effect against red flour beetles. By using different dilution concentrations of nanoemulsion, the most effective concentration was 750. The LC_{50} value of Nano emulsion against larvae is 112.94 ± 0.118 ppm and for beetles was 31.4 ± 0.115 ppm. Essential oil toxicity against adult beetles was 12.52 ± 0.116 ppm of *T. castaneum* and toxicity against larvae is 129.09 ± 0.129 ppm. So, the essential oil showed high toxicity against beetles and nanoemulsion showed high LC_{50} against larvae. Tables 1 and 2 showed the toxicity mortality.

Table 2. Comparison of toxicity of essential oil alone and nanoemulsion against *Tribolium castaneum*.

Dose ppm	Percent mortality after exposure to nanoemulsion		Percent mortality after exposure to essential oil	
	Beetles	Larvae	Beetles	Larvae
1000	91	83	81	79
750	81	92	93	94
500	62	46	44	65
250	51	50	53	47
125	52	34	38	22
62.5	19	25	26	17
31.25	23	3	38	47
15.6	21	0	0	23
7.8	16	2	21	0

Source: Authors, 2024.

The highest percent mortality is observed at higher concentrations, particularly 1000 ppm, where beetles show 91% mortality with nanoemulsion and 81% mortality with essential oil (Table 3). Similarly, larvae exhibit 83%

mortality with nanoemulsion and 79% with essential oil at the same concentration. The mortality rates for larvae tend to be higher in the nanoemulsion treatment compared to the essential oil at most concentrations (e.g., 750 ppm, where larvae show 92% mortality in nanoemulsion vs. 94% in essential oil). At lower concentrations (e.g., 7.8 ppm), mortality drops significantly, especially for larvae exposed to essential oil (0% mortality), indicating that the effectiveness of the treatments decreases as the concentration reduces.

Table 3. Percent mortality of 4th instar larvae and adult beetles after exposure to essential oil and nano emulsion.

Insecticides	Insects	LC_{50}± SEM (ppm)	95% Fiducial limits	X^2	*P*	Df
EO	Larvae	129.09 ± 0.129	0.862-1.369	23.67	0.53	25
	Beetles	12.52 ± 0.116	0.295-0.748	13.03	0.97	25
NE	Larvae	112.94 ± 0.118	0.147-1.009	16.79	0.88	25
	Beetles	31.30 ± 0.115	0.399-0.848	10.82	0.99	25

Source: Authors, 2024.

Nanoemulsion treatment appears more toxic to larvae compared to beetles, as reflected by the lower LC_{50} value (112.94 ± 0.118 ppm for larvae) compared to the essential oil (129.09 ± 0.129 ppm). Beetles show a significantly lower LC_{50} when exposed to essential oil (12.52 ± 0.116 ppm) than when exposed to nanoemulsion (31.30 ± 0.115 ppm), indicating that beetles are more vulnerable to essential oil than to its nanoemulsion form.

3.4 Impact of nanoemulsion on seed germination testing

It was found that all concentrations affected germination with these parameters decreasing as the nanoemulsion concentration increased. A dose-response relationship was observed. Nano emulsions with LC_{20} values showed the least inhibitory effect on germination.

Additionally, the CEO nanoemulsion had an impact on wheat seedling growth. Different levels of inhibition were seen in the shoot length, with the control group displaying the longest shoot length. The results for root length also varied and revealed a dose-dependent response. In conclusion, CEO nanoemulsion showed the greatest inhibitory capability and a dose-dependent effect on the germination of wheat seeds and the growth of seedlings. Table 4 showed the effect of nanoemulsion on wheat seeds.

Table 4. Effect of clove oil nanoemulsion on wheat seed germination.

Replication		Number of seeds	Total germinated seeds	Germination percentage %
Control	1	20	12	60
	2	20	14	70
	3	20	16	80
Sample	1	20	11	55
	2	20	15	75
	3	20	13	65

Source: Authors, 2024.

The study demonstrates that CEO nanoemulsion has a clear dose-dependent inhibitory effect on wheat seed germination and seedling growth, with greater concentrations leading to stronger inhibition. This provides valuable information for understanding the potential side effects of CEO nanoemulsion on plant growth and its use in agricultural or pest control applications.

3.5 Metabolites concentration

The reported percentage changes in enzymes and metabolites of *T. castaneum* adults treated with nanoemulsion highlight significant biochemical responses to stress. An increase in glycogen (57%), glucose (38%), and trehalose (81%) suggests a shift in energy reserves and metabolic adjustments to counteract treatment effects. Trehalose, a critical sugar for energy storage and stress protection in insects, and its elevated enzyme activity (trehalase, 32%) indicate an adaptive response to maintain homeostasis under adverse conditions (Elbein et al., 2003).

The 78% rise in total protein content and 3% increase in soluble proteins suggest increased synthesis of stress-related proteins, such as enzymes or chaperones, essential for maintaining cellular functions under nanoemulsion-induced stress. Proteins play a key role in structural maintenance and metabolic adaptation, making these changes significant for understanding stress physiology in insects (Feder; Hofmann, 1999).

Enzymatic changes, including reduced catalase activity (-48%), reveal impaired oxidative stress management. Catalase is vital for detoxifying hydrogen peroxide, and its decline indicates potential oxidative damage due to treatment. Similarly, the reduction in glutathione S-transferase (GST) activity (-48%), a critical detoxification enzyme, suggests compromised defense mechanisms against xenobiotics (Habig et al., 1974). Decreases in alkaline phosphatase and acid phosphatase (-48%) further suggest disrupted phosphate metabolism and cellular signaling, which could impact nutrient transport and energy production.

Carbohydrate metabolism showed significant modulation, with amylase activity decreasing by 35%, indicating reduced starch digestion, potentially limiting energy supply. However, a 20% increase in invertase activity, which breaks sucrose into glucose and fructose, highlights a compensatory mechanism to ensure energy availability under stress.

These findings are particularly relevant for pest management strategies, as the observed metabolic and enzymatic disruptions may impair survival, reproduction, and resistance of *T. castaneum* populations. Nanoemulsions appear to induce metabolic stress, making insects more vulnerable to oxidative damage and reducing their overall fitness, aligning with goals of sustainable pest control (Koul; Walia, 2009).

Overall, the data provide critical insights into the biochemical adaptations of *T. castaneum* to nanoemulsion-induced stress, revealing potential vulnerabilities that can be exploited in pest control efforts. The reported changes underscore the importance of energy metabolism, detoxification pathways, and oxidative stress management in insect physiology.

By comparing the values after biochemical analysis of different metabolites of treated and untreated group of adults of *T. castaneum* population, it is revealed that total proteins content is increase by 78%, soluble protein contents increased by 3%, contents of glycogen increased up to 57% while contents of glucose concentration in adults of *T. castaneum* increased by 38%, trehalose content of treated group increased up to 81%, activity of trehalse increases in treated group by 32% when compared both groups, contents of amylase decreased and Invertase increases up to -35% and 20% while catalase, alkaline phosphatase, acid phosphatase and GST contents of treated group decreased by -48%. All these percentage values came after comparing both groups of treated and untreated adults' red flour beetles by the nanoemulsion. Tables 5, 6, and 7 showed the percent change in energy reserves and enzyme in *T. castaneum*.

The biochemical analysis revealed that the exposure to the CEO nanoemulsion induced several changes in both energy reserves and enzymatic activities in *T. castaneum*. There was an increase in energy reserves such as total proteins, glucose, glycogen, and trehalose, suggesting metabolic adjustments in response to the treatment. Enzymatic activities, however, showed a mixed pattern: some enzymes (such as catalase and trehalase) were significantly downregulated, while others (like alkaline phosphatase and invertase) were upregulated, possibly reflecting stress responses and metabolic alterations. The findings provide valuable insight into the physiological impacts of CEO nanoemulsion on *T. castaneum* and contribute to understanding its potential use in pest control and its effects on insect metabolism.

Table 5. Change in contents of nutrients of *Tribolium castaneum* after LC_{20} exposure of EO and NE.

Parameters	Treated	unTreatd	Percent change
Total proteins	0.99 ± 0.002	0.21 ± 0.01	78

Soluble proteins	0.88 ± 0.003	0.91 ± 0.02	3
Glucose	0.39 ± 0.02	0.77 ± 0.03	38
Glycogen	2.42 ± 0.004	2.99 ± 0.04	57
Trehalose	11.42 ± 0.03	12.23 ± 0.3	81
Free amino acid	11.21 ± 0.02	9.56 ± 0.02	-165

Source: Authors, 2024.

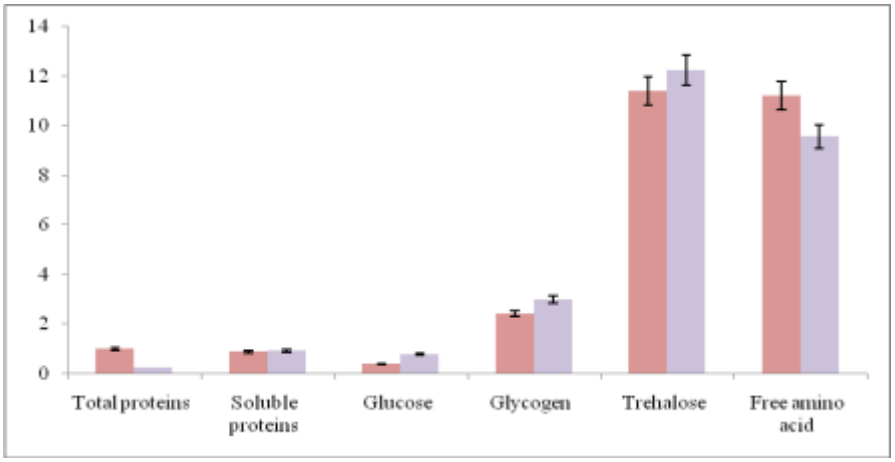


Figure 6. Concentration change in various energy reserves in both groups. Source: Authors, 2024.

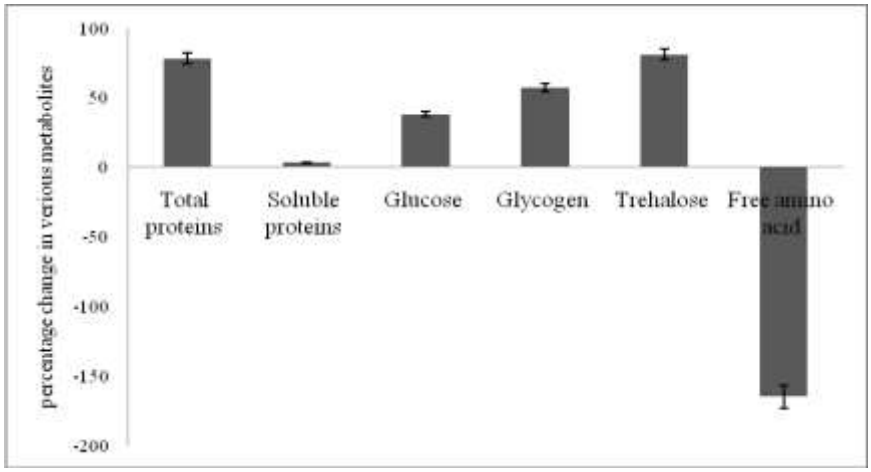


Figure 7. Percentage change in various energy reserves of treated and untreated groups. Source: Authors, 2024.

Table 6. Percentage change in various enzymes in *Tribolium castaneum*.

Parameters	Treated	Untreated	Percent Change %
Catalase	11.43 ± 0.01	9.24 ± 0.02	-219
Trehalse	13.47 ± 0.001	9.11 ± 0.01	-436
Alkaline phosphatase	6.34 ± 0.002	7.23 ± 0.003	89
Glutathione S Transferase	12.69 ± 0.003	12.21 ± 0.01	-48

Amylase	1.29 ± 0.004	1.99 ± 0.004	70
Acid phosphatase	0.53 ± 0.002	0.91 ± 0.03	38
Invertase activity	1.14 ± 0.003	0.95 ± 0.003	-19

Source: Authors, 2024.

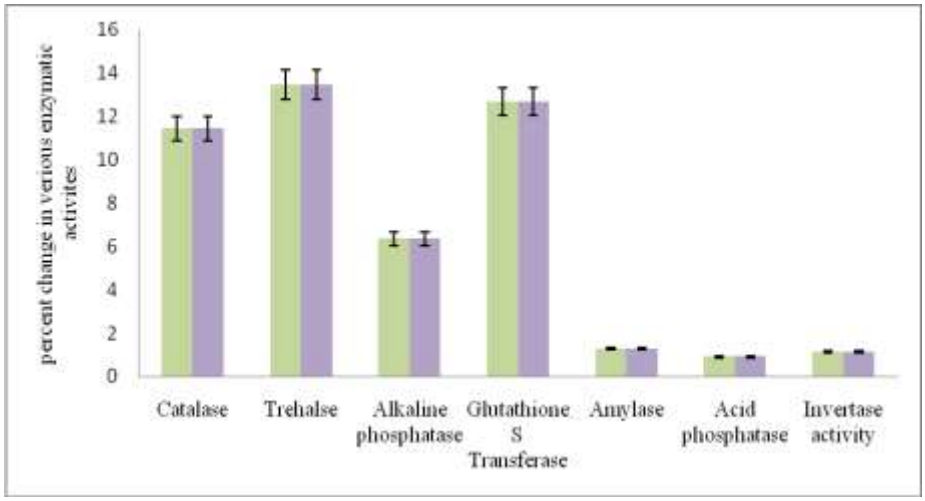


Figure 8. Concentration change in various enzymatic activities in both groups. Source: Authors, 2024.

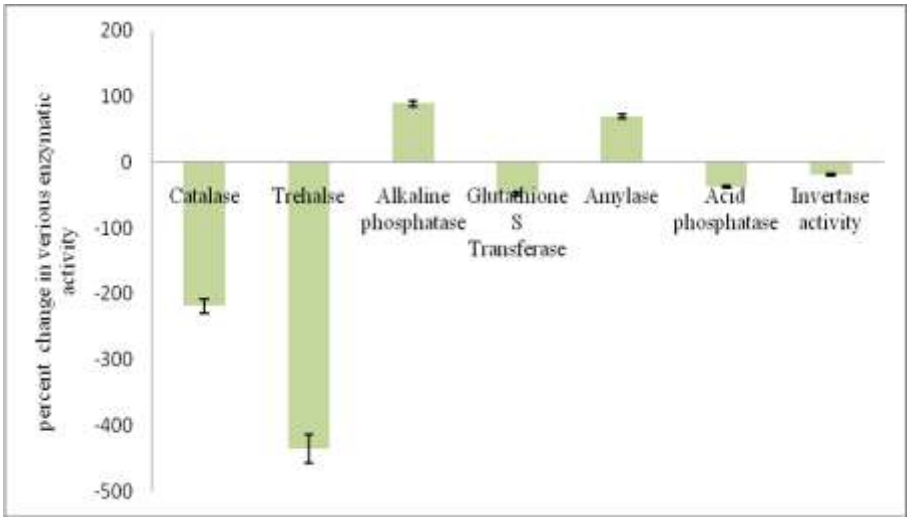


Figure 9. Percent change in various enzymatic activities in both groups. Source: Authors, 2024.

3.7 In-silico interaction of enzymes

Previous studies have consistently reported Phenol, 2-methoxy-3-(2-propenyl) and Eugenol as potent bioactive compounds with strong interactions with enzymes due to their hydroxyl and methoxy functional groups. For example, Shukla et al. (2018) demonstrated binding energies in the range of -28 to -35 kcal/mol^{-1} for Eugenol with other hydrolases, supporting this study's findings of its moderate binding strength. Homology modeling revealed a 73.27% sequence identity between the ALP template used and the modeled enzyme. The high sequence identity lends confidence to the docking prediction. Studies utilizing homology modeling for ALP or similar enzymes have used templates with sequence identities >70% for reliable predictions (Khan et al., 2020). This aligns with the methodology here, affirming the accuracy of the predicted binding interactions.

Molecular docking was employed to forecast the binding location of the key compounds found in *S. aromaticum*

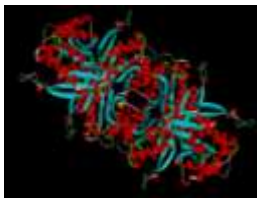
EO on to the ALP enzyme. To streamline the acquisition of fundamental protein structure data, a series of 3D protein structures was developed using homology modeling techniques. The Basic Local Alignment Search Tool (BLAST) via the Swiss Model server and PDB was utilized to construct templates, with only the ALP enzyme showing a significant sequence identity of 73.27% and thus selected as the template. The process of docking protein 3D structures holds significant importance in rational drug design.

Utilizing Auto Dock Vina software, molecular docking was manually conducted to pinpoint the binding sites of the primary compounds with both proteins. Visual representations of the most energetically favorable interactions between the enzyme and ligands in 2D were presented. In terms of the essential oil (EO) form, Phenol, 2-methoxy-3-(2-propenyl displayed the strongest interactions at the lowest energy levels, while eugenol showed the weakest interaction strength in the binding of ALP (-27.5 kcal/mol) (-33.9 kcal/mol). This computational approach involved generating a structural model of the ALP enzyme based on its genetic resemblance to known structures. Subsequently, the EO and NE molecules were simulated to interact with this model, allowing for predictions regarding their binding patterns and strengths with the ALP enzyme. Table 7 showed the molecular interaction.

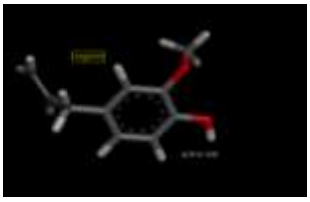
Table 7. Clove interaction with the alkaline acid phosphatase (ALP) enzyme.

Essential Oil Component	Interaction ene kcal/mol	ALP sequence Identity
Eugenol	27.5	73.27%
Phenol, 2-methoxy-3-(2-propenyl)	33.9	73.27%

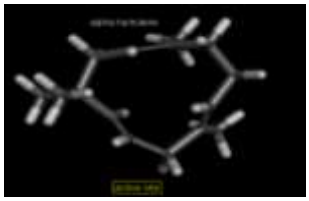
Source: Authors, 2024.



a): selection of protein from NCBI



b) eugenol ligand selection



c) alpha- humelene selection from

d) interaction of eugenol with ALP

e) structure of docking

f) interaction of alpha-humulene with ALP

Figure 10. The EO and NE molecules were simulated to interact with this model, allowing for predictions regarding their binding patterns and strengths with the ALP enzyme. Source: Authors, 2024.

4. Discussion

This study demonstrates the potential of clove essential oil (EO) and its nanoemulsion (NE) formulation as a promising, eco-friendly alternative to synthetic insecticides for the control of *T. castaneum* (red flour beetle), a

major pest infesting stored wheat (Draz et al., 2024). Clove EO and NE displayed significant insecticidal effects against adult *T. castaneum*, and their efficacy was observed to be concentration-dependent, suggesting that the formulation's effectiveness can be adjusted according to pest population densities (Pavoni et al., 2019). The results of the present study provide crucial insights into the toxicity and biochemical effects of clove EO and NE on the beetles, offering a basis for the future development of plant-based, sustainable pest control strategies in agriculture (Sarmah et al., 2024).

Insecticide efficacy is crucial for controlling *T. castaneum*, a pest that significantly damages stored grains. Our findings revealed that both clove EO and nanoemulsion were highly toxic to adult beetles, with toxicity increasing as the concentration of the treatment was raised (Upadhyay; Ahmad, 2011). The optimum lethal concentration (LC) for the nanoemulsion was identified to be between 750 ppm and 1000 ppm. This indicates that at these concentrations, clove EO nanoemulsions could potentially be used for the control of *T. castaneum* populations in agricultural settings, particularly in situations where eco-friendly pest management solutions are preferred over synthetic insecticides. Additionally, the adult beetles showed greater sensitivity to both direct contact with clove EO and its fumigant properties, confirming that adult stages are more vulnerable than larvae. This finding is consistent with other studies on insecticidal EOs, suggesting that adult beetles may be more susceptible due to their larger surface area and longer exposure to the toxic substances (Giunti et al., 2019).

Several studies have also explored the insecticidal potential of essential oils from other plant species, demonstrating their ability to manage stored pest populations effectively. For example, *Coriandrum sativum* (coriander EO) and *Azilia eryngioides* (an Apiaceae species) were found to have LC_{50} values against T. castaneum of 46.48 μL/L and 20.0 μL/L, respectively. These findings are in agreement with the current study, which supports the efficacy of EOs for pest control. Moreover, while research on clove EO specifically is limited, other plant-based nanoemulsions have been studied for their effects on *T. castaneum*. For instance, nanoemulsions containing *Pterodon emarginatus* EO, as well as those based on thymol or *Lippia sidoides*, have demonstrated promising results in controlling other pests like *Sitophilus zeamais* and *Sitophilus oryzae*, further supporting the efficacy of EO nanoemulsions as pest control agents (Golden et al., 2018).

In particular, nanoemulsions offer several advantages over traditional EO formulations, including improved stability, bioavailability, and increased toxicity. These benefits stem from the encapsulation of the active compounds within nano-sized droplets, which enhances their ability to penetrate insect exoskeletons and reach internal physiological targets. For example, a study on pulegone encapsulated in coarse NE reported over 90% mortality in *S. oryzae* and *T. castaneum* over 5 weeks. The stability and extended action of nanoemulsions make them an attractive option for long-term pest management in grain storage (Pavoni et al., 2019).

Beyond mortality rates, this study also examined the biochemical changes induced by clove EO and NE in T. castaneum adults. The results revealed significant alterations in the energy reserves and enzymatic activities of the beetles, providing a deeper understanding of the physiological impacts of the treatments. Notably, exposure to the nanoemulsion resulted in a 48.3% reduction in glucose levels and a 19.3% decrease in glycogen content, indicating a disruption in the beetle's energy metabolism. This reduction in available energy likely contributed to the high mortality rates observed in the treated insects. Trehalose, another energy storage compound, was found to increase by 32%, possibly as a compensatory response to the loss of glucose and glycogen reserves. This finding is consistent with other studies on insecticidal EOs, where the disruption of energy metabolism has been shown to contribute to pest mortality (Kosar et al., 2019).

The increase in trehalose content may also indicate that the beetles are trying to counteract the effects of the stress caused by the EO. Trehalose is a stress-responsive sugar commonly found in arthropods and plays an important role in maintaining energy homeostasis under adverse conditions. Similarly, the increase in free amino acids (17.25%) suggests protein breakdown as a survival mechanism in response to the biochemical stress induced by the nanoemulsion (Shukla et al., 2015).

Several enzymatic activities were also affected by exposure to the EO and nanoemulsion. Trehalase activity increased by 32%, indicating that the beetles may be attempting to mobilize stored trehalose as an energy source. Additionally, acid phosphatase and invertase activity increased by 35% and 20%, respectively, suggesting a response to stress or injury (Riaz et al., 2025). On the other hand, amylase activity decreased, potentially indicating a disruption in the beetles' ability to break down starches into simpler sugars. One of the most noteworthy findings in the biochemical analysis was the increase in catalase activity by 123.7%, which suggests that the beetles were attempting to counteract oxidative stress induced by the nanoemulsion. Catalase is a key enzyme involved in the detoxification of reactive oxygen species (ROS), and its increased activity is indicative of the beetles' attempt to mitigate oxidative damage (Liao et al., 2024). This is consistent with previous studies,

where exposure to plant-based insecticides has been shown to induce oxidative stress in insects. The biochemical changes observed in this study are consistent with previous research on the effects of essential oils and nanoemulsions on insects. For example, the reduction in glucose and glycogen levels following exposure to *C. sativum* EO is similar to the findings in the present study, suggesting that essential oils induce a general metabolic disturbance in *T. castaneum*. Furthermore, the increase in trehalose and free amino acids observed here is comparable to changes seen in other insecticide-treated pests, such as *S. oryzae* and *A. aegypti*, which indicates a conserved physiological response to stress across different insect species (Amin et al., 2019).

In terms of enzymatic activity, the changes observed in the present study are also in line with previous research showing that exposure to insecticidal EOs can disrupt various enzyme systems in insects. For example, an increase in catalase activity has been reported in Aedes aegypti after exposure to plant-derived insecticides, reflecting the insect's attempt to cope with oxidative stress. Additionally, studies on other insect species have demonstrated similar changes in acid phosphatase and invertase activities after exposure to EOs, suggesting that these enzymes may play a role in the insect's response to insecticide-induced stress.

The results of this study offer several important implications for integrated pest management (IPM) strategies, particularly in sustainable agriculture and post-harvest storage (Akami et al., 2019). The demonstrated toxicity and biochemical effects of clove EO and NE on T. castaneum indicate that these formulations could serve as effective alternatives to synthetic insecticides, which often pose risks to human health, non-target organisms, and the environment. Moreover, the eco-friendly nature of clove EO and its nanoemulsion formulation aligns with the growing demand for green chemistry solutions in pest management (Lisi et al., 2024).

However, further research is needed to assess the field applicability of clove EO and NE, particularly in the context of grain storage environments where temperature, humidity, and other factors can influence the efficacy of pest control strategies. Additionally, investigating the environmental impact of clove EO and NE, including their potential effects on non-target organisms, will be crucial for ensuring their safe and sustainable use in agriculture (Gostin; Popescu, 2023). While the results of this study are promising, several limitations must be addressed. The study was conducted under controlled laboratory conditions, and it is essential to evaluate the efficacy and safety of clove EO and NE in real-world agricultural settings. Future research should focus on testing the long-term stability, efficacy, and environmental impact of clove EO nanoemulsions under varying field conditions (Kadoglidou; Chatzopoulou, 2023).

5. Conclusions

This research investigates the potential of clove EO and NE as effective, eco-friendly alternatives to synthetic insecticides for managing *Tribolium castaneum* (red flour beetle), a major pest in stored grain environments. The study demonstrated that both clove EO and its nanoemulsion formulation were effective at killing *T. castaneum* adults and larvae in a concentration-dependent manner, with the nanoemulsion showing superior insecticidal efficacy compared to the essential oil alone.

The results highlight the insecticidal properties of both clove EO and NE, confirming their potential as viable alternatives to traditional synthetic pesticides. While clove EO showed toxicity to *T. castaneum* at varying concentrations, the nanoemulsion exhibited greater effectiveness, likely due to the enhanced bioavailability, stability, and penetration capabilities of the nano-sized droplets. The formulation of nanoemulsions provides several advantages over traditional essential oils, including improved delivery of the active ingredients to target pests, and potentially longer-lasting effects in pest control.

The environmental impact of synthetic insecticides has been a growing concern due to their toxicity to non-target organisms, potential bioaccumulation, and soil and water contamination. In contrast, essential oils like clove EO are biodegradable, non-toxic to humans, and less harmful to beneficial organisms such as pollinators and natural predators of pests. The use of nanoemulsions further enhances the eco-friendliness of the formulation by allowing more efficient use of the essential oil, reducing the need for large quantities of chemical agents.

Moreover, the biodegradable nature of nanoemulsions and their ability to degrade more quickly in the environment minimizes their environmental footprint. Clove EO contains bioactive compounds like eugenol and phenolic compounds, which are known for their insecticidal properties. When encapsulated in nanoemulsions, these compounds can be delivered more effectively to pests while using smaller quantities of the active substance, further reducing environmental impact.

The findings from this study open up new possibilities for the use of clove EO and its nanoemulsion formulation in agricultural pest management, particularly in grain storage and post-harvest pest control. The nanoemulsion's

enhanced stability and extended-release properties make it a cost-effective solution for pest control, as it may require fewer applications compared to traditional insecticides. Additionally, the low toxicity to humans and environmentally friendly nature of the formulation makes it suitable for organic farming and sustainable agricultural practices.

Given the growing concerns about pesticide resistance and the detrimental effects of synthetic chemicals on ecosystems and human health, the development of natural insecticides such as clove EO nanoemulsions is particularly timely. Natural insecticides offer a safer alternative to synthetic compounds, aligning with the global movement towards more sustainable and green pest management strategies. Moreover, nanoemulsions offer the potential for targeted delivery, ensuring that the active ingredients are efficiently transported to pest populations, reducing waste and maximizing efficacy.

Nanoemulsions represent a cost-effective means of enhancing the delivery of natural insecticides. While the initial cost of nanoemulsion formulation may be higher than conventional insecticide formulations, the reduced quantity required for effective pest control and the longer-lasting effects mean that the overall cost-per-application would be lower. This makes nanoemulsions a viable option for small-scale farmers and organic agriculture, where sustainability and cost efficiency are key considerations.

Future research can focus on optimizing nanoemulsion formulations by exploring the synergy between different essential oils, enhancing the stability of the nanoemulsion, and identifying methods to increase shelf life without compromising efficacy. Additionally, studies that examine the long-term effects of nanoemulsions on ecosystems, soil health, and non-target organisms will be essential for ensuring the sustainability of these formulations in the long term.

The use of natural insecticides, such as clove EO, aligns with global goals of promoting sustainable agriculture and reducing the reliance on chemical pesticides. The development of natural and eco-friendly pest control agents is especially crucial in developing countries, where over-reliance on synthetic chemicals is a concern, and there is a growing need for affordable, non-toxic, and environmentally safe alternatives to synthetic insecticides. By developing natural products, such as clove EO-based formulations, pest management practices can be made more accessible and affordable, contributing to food security while also promoting the health of ecosystems.

The findings of this study are relevant in various geographical regions, as *T. castaneum* is a globally distributed pest that infests grains in many countries. However, the efficacy of clove EO and NE formulations may vary depending on factors such as temperature, humidity, and storage conditions. Future studies should investigate how these factors impact the performance of the nanoemulsion in different climatic regions, allowing for regional adaptation of pest control strategies.

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7. Authors' Contributions

Laraib Zafar Iqbal: original research draft writing. *Farhan Ikhtiar*: conceptualization and investigation. *Muhammad Usman Farooq*: data curation. *Faheem Fraz*: software implementation. *Tanzeela Riaz*: supervisor.

8. Conflicts of Interest

No conflicts of interest.

9. Ethics Approval

Not applicable.

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