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Article

# Study of the inhibitory effect of aqueous extract of thyme leaf powder on alpha-amylase enzyme produced by insect larvae (*Trogoderma granarium*)

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## ABSTRACT

The study included evaluating the effectiveness of aqueous extract of Thyme leaf powder on the inhibition of the alpha-amylase enzyme produced by insect larvae (Trogoderma granarium), as well as studying the most important active compounds to know their biological effect on the enzyme by using HPLC technology. Optimum conditions of temperature and pH were determined to measure the inhibitor activity and stability toward the enzyme. The results of HPLC cleared that the thyme aqueous extract had a high level of tannin, 9 micrograms/ml. The chemical tests for this study indicated that thyme leaves contain saponins, flavonoids, glycosides, resins and alkaloids. The results showed that the aqueous lyophilization process gave the best extraction rate of 68% and also preserved the active compounds without heat. It was noted from the study results that the best inhibition of the enzyme was when using a concentration of 10%, which means that the higher the concentration of the inhibitor is, the higher its effectiveness. By studying the various factors, we find that the best temperature for inhibition is 30 °C. As for PH, it was at pH = 7. It has been concluded in this study that the possibility of using the extract of thyme to inhibit the alpha-amylase enzyme is produced by Insect larvae (Trogoderma granarium) as a paradigm.

**Keywords**: a-amylase, Trogoderma granarium, thyme, lyophilization, HPLC.

## INTRODUCTION

Pesticides are of great agricultural importance because of their use in eliminating a wide range of different pests, but they are very dangerous to humans and animals. Sudden exposure (for a short period) or chronic alike many risks, the most important of which is damage to (the nervous system, digestive system, skin, respiratory system, reproductive system, endocrine and immune system), as well as the risk of pesticides may reach cancer [1]. Despite all the risks mentioned above, because it is not possible to leave the use of pesticides in agriculture, in the last six decades, losses in crops have doubled due to various pests, which made pesticides a key role in the production of different crops and facing global famine in general and the rate of food production in particular [2]. Research centers and agricultural

institutions resorted to finding safer alternatives and new types of pesticides. Some recent studies have demonstrated the possibility of using plant extracts as successful alternatives to combating some insects [3]. Thyme is an aromatic plant that is a good source of powerful antioxidants such as phenols and flavonoids. Thyme leaves are medically active, as the percentage of volatile oils reaches (5-25%). The volatile oils of thyme contain 55% of the active substances such as thymol and carceral [4]. Thyme is characterized by its high content of chemically active compounds (27 compounds) that inhibit many enzymes, particularly alpha-amylase [5]. Alpha-amylase is an enzyme that breaks down complex carbohydrates and starches into simple sugars such as glucose, maltose and dextrin [6]. Thus, it is of huge importance in the growth and development of insect larvae to facilitate food metabolism. This study aimed to survey the aqueous extract of thyme plant and its role in inhibiting the alpha-amylase enzyme product by (Trogoderma granarium), as well as the optimal conditions for inhibition in terms of temperature and pH.

## MATERIALS AND METHODS

*Preparing The Raw Materials:*

Dried thyme leaves were collected from local markets and ground in a laboratory mill. Sift the powder with a laboratory sieve of size (5 mm) in the laboratory of the Plant Protection Department / the National Center for Pesticide Control.

*Preparation of Phenolic Extracts:*

The aqueous extraction was done by lyophilization [7].

*Estimation of The Final Outcome of The Extraction:*

The final yield (gm/100gm) of phenolic extracts extracted from thyme leaves was estimated by lyophilization aqueous extraction method [8].

*Determination of The Concentrations of Phenolic Compounds by Spectrophotometric Method:*

The concentration of phenolic compounds was estimated by taking 100 µl for each dilution with 3 mL of distilled water and (100 µl) of undiluted pure folin-ciocalteu reagent with (600 µl) of aqueous sodium carbonate [9].

*The Active Ingredients Chemical Detection of Thyme Leaf*:

*Detection of Tannins:*

10g of dried leaf powder of plants (wild thyme, mint, rosemary, green tea) was boiled in 50 ml distilled water. Filter the solution and leave it to cool. The solution was divided into two parts. First, a 1% solution of lead acetate was added to infer the presence of tannins in the appearance of a gelatinous precipitate. I n thesecond part, 1% ferric chloride solution, where the appearance of the bluish-green color indicates the presence of tannins [10].

*Detection of Glycosides:*

Two equal parts of Fehling's reagent were mixed with the aqueous extract of dried leaf powder (wild thyme, mint, rosemary, green tea). Shuffle (3 ml of distilled water + 2 ml of plant extract + 2 ml of Fehling's reagent) and leave it in a boiling water bath for 10 minutes. Glycosides are indicated by the appearance of a red precipitate [11].

*Detection of Alkaloids:*

10g of dried leaf powder was boiled with 50 ml of 4% hydrochloric acid. The solution was cooled and filtered. (0.5 ml) the filtered solution was tested in a (Watch glass) by each of the following reagents [12]: Mayer's reagent: The appearance of a white precipitate indicates the presence of the alkaloid, and Dragendorff's reagent: An orange precipitate indicates the presence of the alkaloid. Picric acid: the appearance of a yellow precipitate indicates the show of alkaloids.

*Detection of Resins:*

100mg of 95% ethyl alcohol was added to 5g of powdered leaves and left in a boiling water bath for two minutes. The mixture has been filtered. 100 ml of distilled water with hydrochloric acid was added to the filtrated solution. The resulting turbidity indicated the presence of resinous materials. [13] with some modification in alcohol volume.

*Detection of Resins:*

Two solutions were prepared: (A) by dissolving 10g of powdered leaves (wild thyme, mint, rosemary, green tea) in 5 ml 95% ethyl alcohol, then filtering the solution. Solution (B) was prepared by adding 10ml of 50% ethyl alcohol to 10ml of potassium hydroxide 50% solution. Equal amounts of both solutions have been mixed. The presence of flavones was confirmed by the appearance of the yellow color resulting from mixing [11].

*Detection of Sapindales:*

The method presented in [14] was adopted. Because of the toxicity and danger of mercury chloride, in addition to being an internationally banned substance, the extracts have been shaken. It was observed that soap-like foam (bubbles) was formed on the upper surface of the tube, which means that soaps are present. The foam residence time has been recorded.

*Diagnostics of Phenolic Compounds Using HPLC Technology*:

The phenolic compounds were diagnosed by high-performance liquid chromatography-type *SHIMADZU* of Japanese origin in the laboratories of the Plant Protection Department (National Center for Pesticide Control) [15]. The general working conditions of the device were: The mobile phase consisted of (75% acidified water with 3% Acetic acid + 25% Acetone nitrate. Column type (C18), Furnace temperature 40 degrees Celsius, wavelength 280 nm. The flow rate in the mobile phase was 1 ml / Minute. The concentrations of standard phenolic compounds were calculated. The phenolic compounds in the extracts were identified based on the matching of the detention time RT (emergence time) to the specific peak of the standard compounds.

*Extraction of Crude Alpha-Amylase Enzyme From (Trogoderma Granarium) Insect Larvae*:

Wheat infested with an insect (Trogoderma granarium) and its larvae were obtained. 200 live larvae were collected from larval instars. The larvae were ground by (Morter) with the addition of 20 milliliters of sodium phosphate buffer. The powder was placed in a test tube, then centrifuged at 5100 for 5 minutes at 4 °C, PH 6.9. The solution has been filtered to get the impurities removed, such as molting skins or the remains of larvae. The resulting saliva is a source of amylase enzyme.

*Determination of Thyme Aqueous Extract Inhibitor Against the Alpha-Amylase Enzyme*:

The ability of phenolic extracts to inhibit the activity of the enzyme alpha-amylase (Trogoderma granarium) [16]. The percentage of alpha-amylase inhibition was calculated according to the equation mentioned in [17].

*Determination of The Optimum PH for The Activity of The Inhibitor*:

1 ml of the buffer solutions prepared in the above paragraph were placed in test tubes. Each buffer is repeated for each buffer. (0.5) ml of inhibitor solution (aqueous extract of thyme leaf powder) was added to each tube. (0.5) ml of alpha-amylase solution (Trogoderma granarium) was added to each tube containing the inhibitor and buffer. All tubes were incubated in a water bath at (37) °C for (30) minutes. The enzymatic activity of all previous models was estimated at different pH

numbers. In a single experiment, (0.5) ml of the enzyme solution (Trogoderma granarium) was incubated with (0.1) ml of all the previous buffers prepared in the above paragraph without the presence of an inhibitor and replaced with an equal volume of (0.2) molar phosphate buffer solution, using the same method and conditions. The enzymatic activity was estimated according to the steps mentioned above to monitor the amylase activity (Trogoderma granarium) at different pH. The relationship between the enzymatic activity and the optimum pH for the activity of the amylase enzyme, as well as for the amylase inhibitor, was drawn.

*Determination of The Optimum PH for The Inhibitor Stability:*

The inhibitor was incubated for (16) hours with a different range of pH (9-4) and at a temperature of 4 ° C. Inhibitors (aqueous extracts of dried leaf powder of thyme plants) were mixed (0.5-1) (V/V) with previously prepared buffer solutions. Phosphate buffer (0.5ml/ 0.2 Molar) was added with pH (7). The inhibitory activity was estimated in each model. The relationship between pH and percentage of inhibition was plotted.

*Determination of the optimum temperature for the inhibitor stability:*

To determine the inhibitor activity optimum temperature, the inhibitor was incubated in equal proportions (1:1) (v/ v) with amylase enzyme (Trogoderma granarium) at a different temperature range (30-70) °C / 30 min. The relationship between temperature and inhibitory activity was plotted. The enzyme activity without inhibitor was estimated at the same conditions. The enzymatic activity, then the inhibitory activity, then the enzymatic activity and the inhibitory activity were estimated together. The enzymatic reaction was conducted in the presence and absence of inhibitors at different temperatures ranging from (30-70) degrees Celsius to determine the real effect of temperature in the interaction of the inhibitor with the enzyme.

## RESULTS AND DISCUSSION

*Extraction percentage:*

The results showed that the amount of loss in the aqueous extract of thyme was small when using the freeze-drying technique and applying the extraction efficiency equation (Table 1), and the results were close to that of [7]. The weight of dry thyme leaf powder before drying was 5 g, and after drying, 3.4 g. The advantage of the freeze-drying technology (freeze-drying) is to preserve the properties and stability of the composition of the material.

| plant name | Extraction type | Sample Weight Before Extraction (gm) | Sample Weight After Extraction (gm ) |
|---|---|---|---|
| **Thyme** | Lyophilization | 5 gm | 3.4 gm |

**Table 1. Show Difference in Weight of Dried Leaf Powder of Thyme**

The final yield (1g/100gm) of phenolic extracts extracted from dried leaves of thyme plant by hydrolytic extraction method was 68%. The amount of phenolic compounds varies according to the extraction method used, and this is also due to the polarity of phenolic compounds.

*The Phenolic Compounds Concentration:*

The results indicated that the final result of the concentration of phenolic compounds was (14.91504916) mg/gm. The variation of the phenolic content in the studied plants can be explained by the different extraction conditions and the polarity of phenolic compounds, as water is considered highly polar [18].

*Chemical Detection of Active Compounds:*

The study's results were adapted to a set of tests. It gave a positive result for detecting tannin by the appearance of a gelatinous precipitate with lead acetate, as well as the appearance of a green color with ferric chloride. Each of these two detections indicates the presence of a tannin compound in thyme. The results showed the presence of glycosides in the aqueous extract of the dried powder of thyme leaves. The detection gave a red precipitate (reddish brown) (Table 2). This compound is found in 70% of these medicinal plants, including thyme. These compounds are very important to plants, as they represent a means of protecting plant leaves from harmful insects. The aqueous extract of thyme gave saponins a positive result, with the appearance of a thick foam that lasted for several seconds. The dried powder of the leaves of the thyme plant gave a positive result for detecting alkaloids, with the appearance of a white and orange precipitate (Table 2). Alkaloids are found in medicinal aromatic plants in different parts of the plant cell, such as mitochondria, vesicles, chloroplasts, and vacuoles. Alkaloids are considered a defense method in the plant against predators and insects. They are produced through metabolic pathways in plants to facilitate the survival of plants in the ecosystem.

Alkaloids can be a natural herbicide. The results showed positive detection of resins (secondary metabolites) of the extract of dried leaf powder of thyme plant through the appearance of turbidity (Table 2). Resins are found in the trunks, twigs, and leaves of plants. Resins are shiny, brittle, and hard materials, usually orange, yellow, or brown (Table 2). Resins are insoluble in water but dissolve in organic solvents. The study's results indicated the presence of flavonoids in the aqueous extract of dried leaves of thyme through the appearance of a yellow color (Table 2). Flavonoids are found in green plants and in a very abundant proportion, as well as in fruits and seeds, and are responsible for the color, aroma and flavor characteristics of plants.

| Num. | Chemical Compound | User Detection | Detection Guide | Detection Result |
|---|---|---|---|---|
| 1 | Tannins | A- lead acetate 1% | Appearance of a gelatinous precipitate | + |
| 2 | | B- ferric chloride 1% | bluish-green color appearing | + |
| | Glucosizdase | Fahlink detector | red precipitate (reddish brown) | + |
| 3 | Saponins | Shake the aqueous extract. | The appearance of thick foam | + |
| 4 | Alkaloids' | Dragendorf detector | orange precipitate | + |
| | | Meyer's detector | white precipitate | + |
| | | C- Picric acid | white precipitate | + |
| 5 | Resins | Ethyl alcohol 95% and boiling distilled water | Turbidity | + |
| 6 | Flavonoids | ethyl alcohol 95% +KOH | Yellow color appears | + |

**Table 2. Chemical Detection of Active Compounds**

*Identification of Phenolic Compounds by High-Performance Liquid Chromatography (HPLC):*

The phenolic compounds were diagnosed by high-performance liquid chromatography technique.

| Extraction type. | Plant | Thymol µg/ml | Tannic acid µg/ml | Coumarin µg/ml | Caffeine µg/ml | Gallic acid µg/ml |
|---|---|---|---|---|---|---|
| **Lyophilization** | Thyme | 0.28 | 9 | 0.01 | 0.01 | 1.92 |

**Table 3. The aqueous extract of thyme-dried leaves Phenolic Compounds identified by High-Performance Liquid Chromatography (HPLC).**

*Inhibitory Activity of The Thyme Aqueous Extract Against the Crude Alpha-Amylase Enzyme (Trogoderma Granarium Larvae)*:

The current study (Fig. 1) showed the inhibitory ability of aqueous extract of dried leaves of thyme plant against alpha-amylase enzyme. It was noted that the

inhibition ability increases with the increase in the concentration of the extract used. The results indicated that the percentage of inhibition of the aqueous extract of thyme at the concentration (1%) was (79) mg/ml, while at the concentration (5%) the inhibition was (81) mg/ml. When the concentration (10%) was given (87) mg/ml. Thus, the inhibitory effect of the concentration (10%) compared to the concentration (5% and 1%) is the most influential on the enzyme. It should be noted that thyme contains major compounds, including thymol, which has a high inhibitory ability against the alpha-amylase enzyme [21]. Some studies have shown that several herbs, including thyme, contain biologically active compounds that have direct effects on diabetics and blood sugar action. Amylase -α & glucosidase.

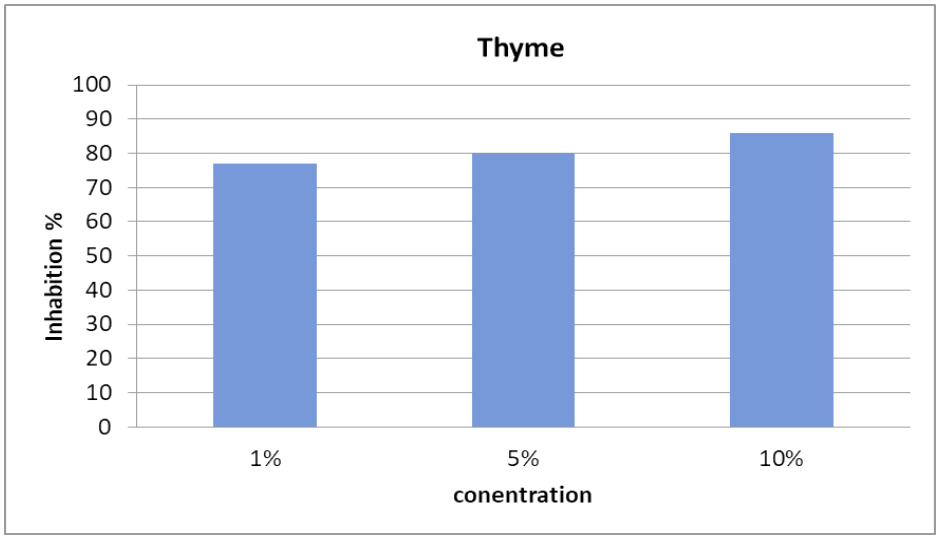


**Figure 1. Inhibitory Activity of The Thyme Aqueous Extract Against the Crude Alpha-Amylase Enzyme (Trogoderma Granarium Larvae).**

*The Optimum Ph for The Activity of The Inhibitor*:

The optimum pH for the inhibition activity of phenolic extracts was estimated with a range of pH ranging from (4-9) to determine the optimal pH for the binding of these inhibitors with the enzyme at (37) m for (30) minutes. Figure (2) shows the effect of pH on the activity of Phenolic compounds extracted aqueously (lyophilized) from the dried powder of thyme leaves in their role as inhibitors of alpha-amylase enzyme. It was found that the inhibitor is effective with a range of pH (4-9). The inhibitor showed the highest effectiveness at pH (7), where the percentage of inhibition was (37.1%). No significant difference is observed from the percentage of inhibition at pH (8), where the percentage of inhibition was (35.2%). The effectiveness of alpha-amylase enzyme alone without addition showed that it decreases when away from the optimum pH (6.8-7), where the highest activity was (0.97) units/ml at pH (7). There is no significant difference in the enzyme activity at pH (8,6), which was (0.91 and 0.88) units/ml respectively. The enzyme activity gradually decreases at pH higher or lower than the mentioned values [23].

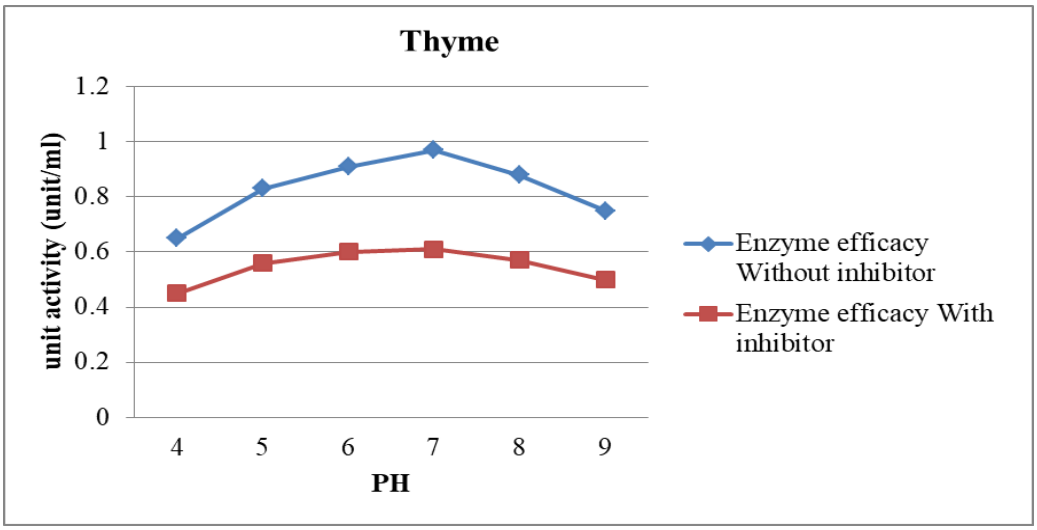


**Figure 2. shows the Inhibitory Activity of The Thyme Aqueous Extract Against the Crude Alpha-Amylase Enzyme (*Trogoderma Granarium* Larvae).**

*The Optimum PH for The Inhibitor Stability Against A-Amylase*:

The current study results (Figure 3) showed the optimum pH of inhibitory stability (active compounds) extracted from dried leaf powder of thyme plant against a-amylase enzyme. The optimum pH of the alpha-amylase enzyme ranges between (6.8-7), which is almost neutral [23]. It was found that the stability of the active compounds in inhibiting alpha-amylase enzyme reached (68.3%) at pH (7). This represents the highest stability of the enzyme's activity. It was noticed that the percentage of inhibition increased (71% and 74.5%) at pH (8 and 9) respectively, and the percentage of inhibition decreased (52.3% and 43.3%) at pH (5 and 6), respectively (Figure 3).

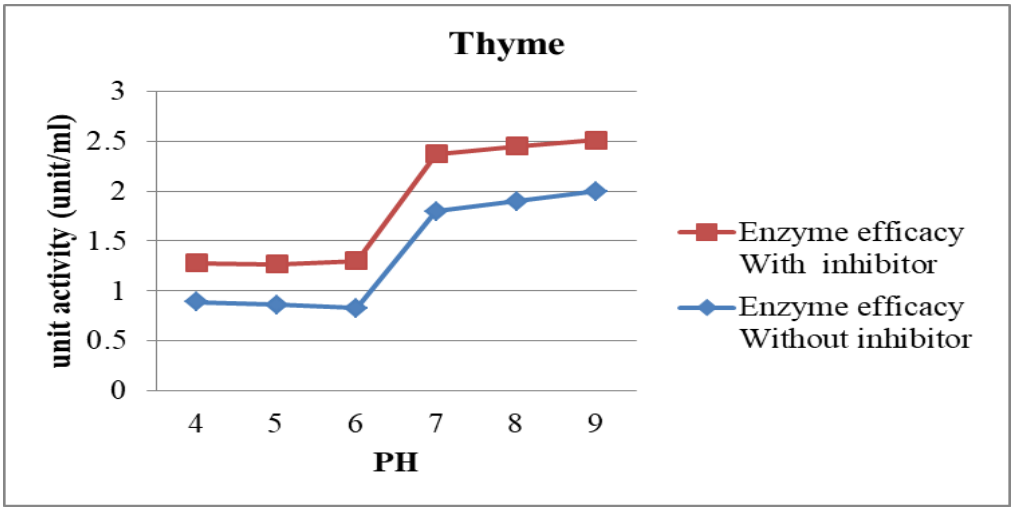


**Figure 3. Show the relationship Between the optimum pH and The Inhibitor Stability Against a-Amylase (*Trogoderma Granarium* Larvae).**

*The Optimum Temperature for The Activity of The Inhibitor*:

The results (Fig. 4) indicated that the highest activity of the alpha enzyme was recorded at a temperature of 30 ° C. It gradually decreased to 70 ° C as the enzyme lost much activity .In the case of the enzyme incubated with the inhibitor under study, the highest decrease in enzyme activity was observed, where the percentage of inhibition was (66.6%). The percentage of inhibition at a temperature of 70 ° C was (42.5%), which differs from the percentage of inhibition at other temperatures,

which were (20.6, 18.7, 28.5) % for each of the temperatures (60, 50, 40) ° C, respectively.

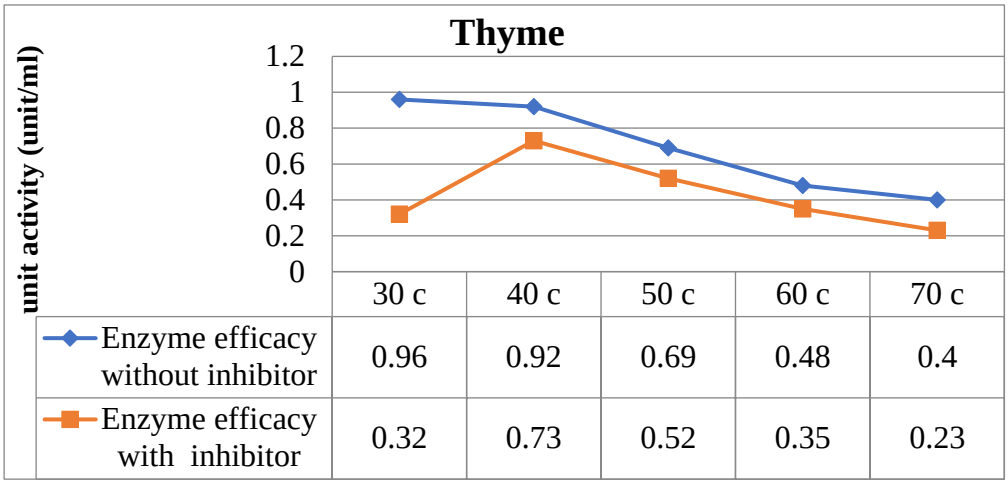


**Figure 4. The Optimum Temperature for The Inhibitor Activity Against a-Amylase (*Trogoderma Granarium* Larvae).**

*The Optimum Temperature for The Inhibitor Stability Against A-Amylase*:

Thermal stability is one of the important factors by which it is possible to determine the temperatures at which the inhibitor maintains its effectiveness. Adequate temperatures are significant for using the damper in scientific applications. This study was carried out by incubating the extracted damper at temperatures ranging from (30-70) °C. The stability of the damper was observed at a maximum temperature of 30 °C (66.2%), then it began to gradually decrease with an increase in temperature from (50-70) °C. The temperatures (50, 60 and 70) C did not differ among themselves in the percentage of inhibition, as they gave close results (55.4%, 51.2%, 44.6%), respectively. The experiment results showed a significant difference in the temperature of 40 °C (62%) from the percentages of temperatures (50, 60 and 70°C). This decrease is due to the effect of temperature on the composition of the active compounds.

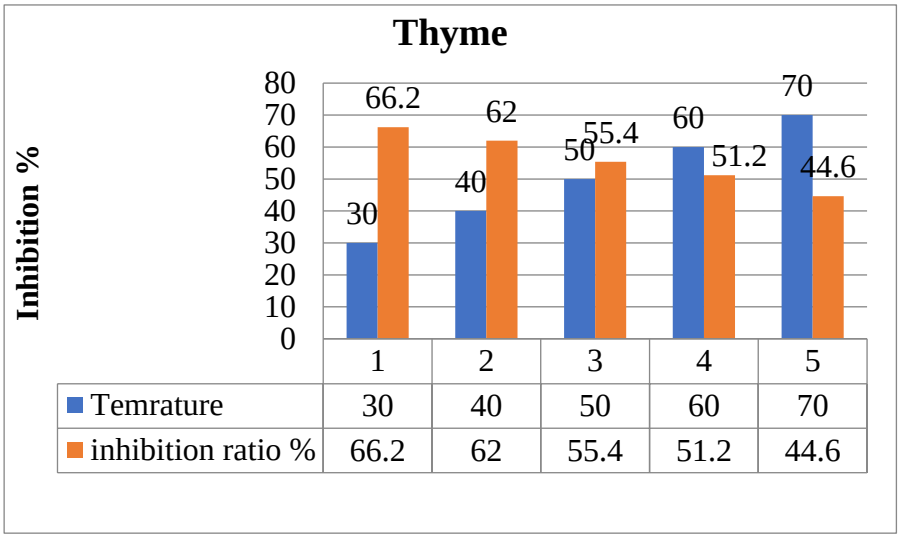


**Figure 5. The Optimum Temperature for The Inhibitor Activity Against a-Amylase (*Trogoderma Granarium* Larvae).**

## DISCUSSION

The high amount of phenols in the aqueous extraction is due to their being highly polar compounds [18]. Through the results, we can refer to the need to find a mixture of solvents suitable for each compound in the process when extracting phenolic

compounds. We should also know that phenols may be polar or non-polar depending on the extraction conditions of these compounds [19]. The proven results in (Table 3) indicate that the aqueous extract of dried thyme leaves contained a high concentration of tannic acid, up to (9) µg/ml, because this compound is known to be one of the (polar) hydrophilic compounds [20]. Thyme extract and a number of aromatic plants have a significant inhibitory effect on the activity of the two main enzymes of carbohydrate digestion, glucosidase and α-amylase. The inhibition process depended on the number of hydroxyl groups (OH) in the phenolic compound. This covalent bonding increases with the decrease in pH, which increases the effectiveness of inhibition [22]. The efficiency of the effectiveness of enzymatic inhibitors depends largely on the pH. Also, it depends on the formation of the complex between the inhibitor and the enzyme at any pH on the type of inhibitor and the type of enzyme. The optimum pH for inhibitor activity may not be constant due to its association with all amylases [24]. The study showed that the increase in the inhibitory activity of high temperature is affected by the type and source of the inhibitor [25].

## CONCLUSIONS

Lyophilization is the best way to preserve the basic material and its quality. By lyophilization, the weight loss is small for the sample. Not being exposed to high temperatures in this way has a key role in reducing the activity of the active compounds. The aqueous extract contains a quantity of polar active compounds with water. The extract showed inhibitory activity against the enzyme alpha-amylase (Trogoderma granarium). All extracts under study contain many effective compounds when diagnosed using HPLC technology. The leaves of the thyme plant contain many effective compounds, such as tannins, saponins, glycosides, resins, alkaloids and flavonoids, which have antioxidant and enzyme-inhibitory effects.

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