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Research Article

Extraction and evaluation of thyme oil and detection of its heredity effect on the fungus *Aspergillus amstelodami*

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Abstract
Thymus vulgaris is considered one of the herbal plants rich in active compounds that have attracted the attention of many scientists and researchers. However, the genetic safety of these compounds must be investigated, as food safety is one of the topics that has recently topped the field of scientific research.The study objectives included obtaining Thyme Essential Oil (TEO) by hydrodistillation extraction , and then analysing by Gas Chromatography technic (GC), all tests were done on the A1 (Wa1) strain of *Aspergillus amstelodami* . Thyme was collected from local nurseries . The mutagenic effect of four sub-lethal concentrations (0.01 -0.016 – 0.02 – 0.05) ml/ml of thyme oil was evaluated by using mutagenesis methods. The results showed that the highest phenolic compounds in (TEO) were Thymol and Carvacrol , with a percentage of 58.89% and 4.11%, respectively. The study also included investigating the ability of thyme oil to cause genetic effects on eukaryotic cells by testing the ability of this oil to induce genetic mutations in the conidia of fungus *A. amstelodami* by using the mutagenesis methods of pretreatment, plate incorporation and growth-mediated, as the study did not record the presence of any mutagenic effect for the four sub-toxic concentrations (Sub lethal) (0.01 -0.016 – 0.02 – 0.05) ml/ml of thyme oil, this confirms its safety genetically and can be used as a herbal food and medicinal plant.

Keywords: *Aspergillus amstelodami* , Gas Chromatography, Mutagenic effect , Thyme oil.

INTRODUCTION

The use of plant extracts and their essential oils in prevention and treatment has witnessed wide interest from many international organizations to prove the validity and safety of their use in the combination of many medicines (Nasri and Shirzad, 2013). In recent times, the trend of heading to medicinal herbs and their aromatic oils has been considered environmentally friendly due to their safety in use and low toxicity compared to chemically manufactured drugs (Chouhan *et al*., 2017). The essential oils have been used in the medical field to treat a variety of diseases in humans, such as migraine, depression and stroke (Flores *et al*., 2021). A World Health Organization (WHO) report stated that 80% of the world's countries use plant extracts or their medically effective ingredients in treating many diseases (Ansari *et al*., 2023 ; Cedillo-Cortezano *et al*., 2024). Thyme is an evergreen perennial herb that belongs to the Labiatae family and is spread in the Mediterranean regions, Europe and East Asia (Anžlovar *et al*., 2014; Mohanad *et al*., 2020). It is commonly used in cooking due to its distinctive aromatic flavor (Vassiliou *et al*., 2023) , it is important for treating digestive and respiratory disorders (Serrano *et al*., 2020) and for preserving food, medical and cosmetic materials and used as a treatment for skin diseases, analgesic and carminative (Nieto, 2020), it is also considered an antifungal, antibacterial , antiviral (Guimarães *et al*., 2019), antioxidant and anti-mutagen (Miraj and Kian, 2016; Alsaraf *et al*., 2020).Thyme contains essential oils, which are composed of phenols, the most important of which are Thymol, Carvacrol, and other compounds such as Linalool, Flavones, Tannins, Terpinene, Caffic acid (Guimarães *et al*., 2019), so its wide use in traditional medicine (Anžlovar *et al*., 2014; Alsaraf *et al*., 2020).These compounds are pharmaceutically important, so the present study aimed to investigate the safety of using the essential oil of this plant genetically by investigating its ability to cause genetic mutations in *Aspergillus am-*

stelodami.

MATERIALS AND METHODS

Test organism

All tests were done on the A1 (Wa1) strain of *A. amstelodami* which was obtained from the Prof. Dr. Sahi J. Dhahi, University of Mosul\ College of Science \ Department of Biology.

Culture media

In the present study, Minimal medium (M) was used and all tests were performed based on the method (Caten, 1979) plus test materials . The Malt extract- salt medium was also used to get the largest number of conidia.

Extraction of thyme oil

Samples of thyme were collected from local nurseries and then plants were classified in the Museum of Science College / Department of Biology / University of Mosul as (*Thymus vulgaris*).

Thyme oil was extracted by mixing 50 gm of *T. vulgaris* leaves with 750 ml of distilled water by Hydrodistilation using Clevenger (El Kady and El-Maraghy, 1993), then 1ml of oil was dissolved in 9 ml of Ethylene glycol and steriled by using membrane filters with diameter 0.22 microns . Distillated sterile oil was put in dark vials to prepare concentrations that were used in the present study (Shareef, 1998).

Thyme oil analysis

Gas Chromatography (GC) analysis was performed using a gas chromatography apparatus model Shimadzu 2010, Japanese origin, using an ionizing flame detector (FID). A capillary separation column (DB-1) with lengths (0.25mm, 0.25 um, 30m) was also used , where the temperature of the injection area and the reagent, respectively, was 280 - 330 °C, while the temperature of the separation column was gradual, starting from 100 - 300 °C, with a rate of 5 °C\min . Inert nitrogen gas was used as a carrier gas at a rate of 105 kpa.

Preparation of the conidial suspension

The conidial suspension of the fungus *A. amstelodami* was prepared from 4-day modern culture incubated on CM medium, and then the concentration of conidial suspension was determined on 10^7 by using Haemocytometer (Al-Hyaly, 2011).

Preparation of the storage solution for Benomyl

The storage solution of Benomyl was prepared by dissolving 0.02 g of the fungicide in 500 ml of distilled water(DW) to obtain a solution of a concentration of 40 micrograms of Benomyl ml or 20 micrograms of the active substance Benomyl ml. Sterilized the solution with the buffer at 121 ° C for a period of 15 minutes and kept in the refrigerator at a degree 4-8 ° C until use (Welker and Williams, 1980).

Minimum Inhibitory Concentration(MIC)

The MIC of thymus oil was determined by using the medium (M), which contained concentrations (0-0.2) ml/ml separately. The inoculation method was followed: three pricks of strain A1 were performed in a petri dish containing the medium (M) and a specific concentration of the studied substance, with three replicates (R1, R2, R3) for each concentration. After 4 days of incubation, the diameters of colonies were measured around the acupoint, and then they were compared to the diameter of the colonies growing on the minimum medium (M) free of the test substance (control) (Al-Hyaly and Hadi, 2019), the diameters of colonies and the percentage -(%) of inhibition for each concentration were measured by:

Percentage of inhibition = Average spontaneous treatment – Average treatment of material/ Average spontaneous treatment X 100 Eq. 1

Isolation of mutations and calculation of their frequencies

We isolated spontaneous and induced mutations by thyme oil, which was resistant to the pesticide Benomyl with a final concentration 0.6mg /ml from the growth medium because of its the inhibitory concentration for the fungus *A. amstelodami* 100% (Al-Hyaly, 2011), the frequency of mutation was calculated based on a number of live conidia in the conidial suspension (Al-Hyaly, 2011).

Mutagenic effect

The mutagenic effect of four sub-lethal concentrations of thyme oil was studied (0.01 -0.016 – 0.02 – 0.05) ml/ml using pretreatment, plate incorporation and growth mediated (Al-Taee, 2006).

Positive control

Nitrous acid HNO2 was used as a chemical mutagen (positive control) (Justin *et al*., 2010) to ensure the ability of the A1 strain to mutate when treated with a known mutagen, by treating *A. amstelodami* with the chemical mutagen HNO2 nitrous acid (Al-Rawi, 2011). The suspension was incubated at a temperature of 30 C ° for an hour with repeated shaking, then stopped the reaction was by placing the sample in an ice bath and 100 ml of a phosphate buffer solution was added to it. The conidia were washed several times to get rid of nitrous acid, after that the mutants were isolated and their frequency calculated (Azevedo, 1970).

Statistical analysis

The mean Mutant Frequency (MF) and Standard Error

(SE) were calculated for each treatment. The results were analyzed statistically using the T-test at a significance level≤ 0.05 (Daoud and Alyaas, 1990).

RESULTS AND DISCUSSION

Evaluation of thyme oil

The results of Gas Chromatographic (GC) analysis showed that thyme oil consisted of 9 main compounds in terms of the appearance of peaks with different retention times , which ranged between 1.664 minutes for the first peak and 6.1 minutes for the last peak , (Figure 1). Arrais *et al*. (2023) confirmed that the analysis of thyme oil using the gas chromatography technique gave five basic compounds (Linalool, Thymol, Carvacrol , Eucalyptol and Trans-Caryophyllene, where the percentage of Carvacrol reached 6.6 %.

The results shown in Table 1 indicated that Thymol was the main component with a concentration of 58.89% (peak 4) with an area ratio of 25.2328% and a peak height ratio of 24.8982% based on the standard Thymol compound that was injected into the device (Figure 2) , while the percentage of Carvacrol was 4.11% (peak 9) with area ratio 14.5712% and peak height ratio 17.9171% based on the standard Carvacrol compound that was injected into the device Figure (3). This results agree with Nieto (2020), who reported that Thymol is one of the main constituents in thyme essential oil, and its percentage ranged between 40–80% , while Galovičová *et al*., (2021) confirmed that *T. vulgaris* essential oil (TEO) consisted of 5 main components (Thymol, p-cymene , 1,8-cineole, -terpinene and Carvacrol), as the research results indicated that the concentration of Thymol and Carvacrol reached to 48.1% and 5.5%, respectively , using Gas Chromatography technology.

Minimum Inhibitory Concentration(MIC)

The minimum inhibitory concentration of Thyme Oil was determined by using increasing concentrations of it ranging from 0 - 0.2 ml /ml .The results presented in Table 2 showed that the diameters of *A. amstelodami* colonies began to decrease with increasing concentrations of Thyme Oil in the culture medium. The percentage of inhibition ranged between 14.66% and 100% at concentrations 0.01-0.2 ml/ ml respectively. This is consistent with what many researchers have reported on antifungal effectiveness of Thyme oil (Pinto, *et al*., 2020), This effectiveness is due to the Thyme Oil containing many effective compounds such as Thymol and Carvacrol act as an antifungal agent (Lima, *et al*., 2019; Qu, *et al*., 2021).

To isolate the spontaneous and induced mutants with nitrous acid which is possible as a result of treatment with thyme oil, the fungicide benomyl was used at a concentration of 0.6 g / ml, which gave an inhibition rate of 100%, and this concentration was considered a toxic concentration of the fungus and was used to isolate the mutants (Table 3).

Determination of mutagenic effect

Four sub-lethal concentrations of Thyme oil were selected (0.01 – 0.016 – 0.02 – 0.05) ml/ml to test their mutagenic effect. McCann and Ames (1978) confirmed that the expression of mutagenic activity occurs at concentrations below toxic level, as the destruction of DNA accompanies the toxic level.

From Tables 4,5,6 the present study found that Thyme Oil at the concentrations (0.01 – 0.016 – 0.02 – 0.05) ml /ml showed no significant mutagenic effect at 0.05≤ p in *A. amstelodami* conidia by comparing the mean frequencies of benomyl-resistant mutants induced with Thyme Oil with the average frequency of the spontaneous benomyl-resistant mutants by using the three mutagenic methods of pretreatment, plate incorporation and growth mediated . However, the results of comparing the average frequency of mutants induced in Thyme Oil with the average frequency of mutants induced by nitrous acid (positive control) showed that there were significant differences at a probability level of 0.05≤ p and this indicated that strain A1 was a mutagenic strain as it responded to the mutagenic effect of

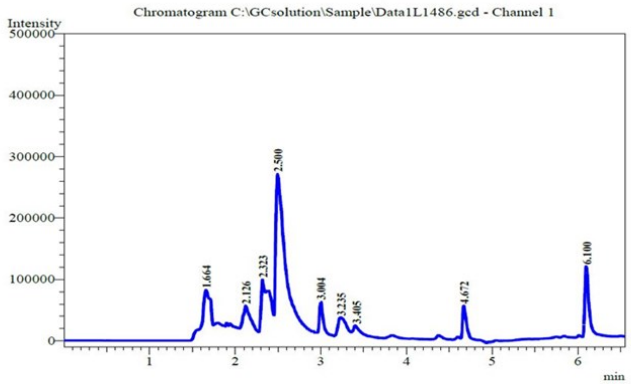

Fig. 1. *Evaluation of thyme oil by using Gas Chromatography analysis*

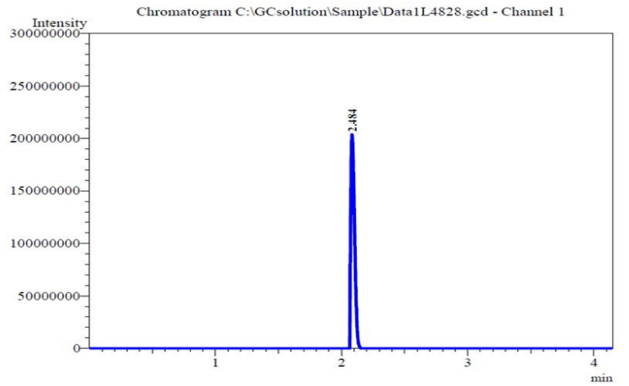

Fig. 2. *Standard Thymol compound performed by Gas Chromatography*

Table 1. Retention and the percentage of area and height of thyme oil compounds performed using gas chromatography

Peak Table - Channel 1						
Peak#	**Ret: Time**	**Area**	**Area%**	**Height**	**Height%**	**Name**
1	1.664	52507	12.8187	22763	9.7819	
2	2.126	34578	8.4416	15853	6.8125	
3	2.323	67471	16.4718	38224	16.4258	
4	2.500	103357	25.2328	57941	24.8982	
5	3.004	25895	6.3218	20221	8.6894	
6	3.235	24988	6.1003	9150	3.9320	
7	3.405	10608	2.5898	5718	2.4572	
8	4.672	30524	7.4519	21144	9.0858	
9	6.100	59686	14.5712	41695	17.9171	
Total		409624	100.0000	232709	100.0000	

Table 2. Diameters of *Aspergillus amstelodami* fungi colonies which cultured on (M) medium containing different concentrations of Thyme Oil by point inoculation method

Concentration of the oil ml / ml	**Repeat of colonies diameters (cm)**			**Average**	**Percentage of inhibition**
	R1	**R2**	**R3**		
Zero	7.5	7.9	7.3	7.5	-
0.01	6.4	6.2	6.6	6.4	14.66
0.016	4.6	4.4	4.2	4.4	41.33
0.02	3.3	4	3.9	3.7	50.66
0.05	3.2	3.1	2.9	3.0	60.00
0.14	2.4	2.4	2.6	2.4	68.00
0.2	Zero	Zero	Zero	Zero	100

Table 3. Diameters of *Aspergillus amstelodami* colonies cultured on medium (M) to which different concentrations of the Benomyl fungicide were added

Concentration of the Benomyl fungicide microgram / ml	**Repeat of colonies diameters (cm)**				**Percentage of inhibition**
	R1	**R2**	**R3**	**Average**	
0	4.6	4.1	4.12	4.27	-
0.3	0.41	0.53	0.46	0.46	89.22
0.4	0.25	0.2	0.31	0.25	94.14
0.5	0.1	0.15	0.12	0.12	97.18
0.6	0	0	0	0	100

nitrous acid, and this confirmed the possibility of use of this strain to detect the potential mutagenic effect of the material under study. Therefore, the results showed that there was no mutagenic effect of Thyme Oil under study on *A. amstelodami* conidia under experimental conditions .This result is consistent with many studies that indicated the inability of Thyme Oil to induce genetic mutations in prokaryotic organisms, which were detected using the Ames test based on *Salmonella* strains (De Martino *et al*., 2009). Not only that, but thyme showed antimutagenic ability against genetic mutations caused by the mutagenic agent sodium azide when tested with two strains of *Salmonella*. (Durgadevi and Kalava, 2013), and given that Thyme Oil contains Thymol, Carvacrol, Linalool, Flavones, Tannins, Terpinene, Caffic acid that have biological activity against mutagenesis(Bioantimutagen) (Alsaraf *et al*., 2020), through

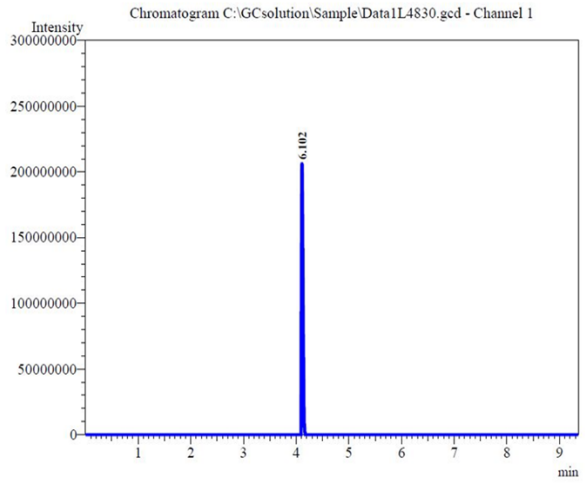

Fig. 3. *Standard Carvacrol compound performed by Gas Chromatography*

Table 4. Average frequency of spontaneous and induced mutant (× 10^{-5})in fungal conidia of *Aspergillus amstelodami* treated by Thyme Oil by pretreatment method

Concentration of Thyme Oil ml / ml	Replicates Mutation			Average± Standard error	Calculated t value*
	R1	R2	R3		
0	0	0.231	0.118	0.116 0.0665±	
0.01	0	0.290	0	0.290 ±0.0966	1.5263
0.016	0	0	0	0 ±0	1.7575
0.02	0.275	0	0.341	0.205 ±0.1043	0.7523
0.05	0.571	0.439	0	0.336 ±0.1725	1.2154
HNO_2	38.52	30.65	36.927	35.365±1.1776	26.599 *

Without treatment and its repetitions represent the spontaneous repeats (negative control). HNO2: treatment with nitrous acid (positive control). *: Significant at probability level 0.05≤ p.

Table 5. Average frequency of spontaneous and induced mutant (× 10^{-5})in fungal conidia of *Aspergillus amstelodami* treated by Thyme Oil by plate incorporation

Concentration of Thyme Oil ml / ml	Replicates Mutation			Average±Standard error	Calculated t value*
	R1	R2	R3		
0	0	0.261	0	0.087±0.085	
0.01	0.1	0	0	0.033±0.034	0.6352
0.016	0	0	0	0 ±0	1.0259
0.02	0	0	0.170	0.056 ± 0.056	0.3087
0.05	0.180	0	0	0.060 ± 0.0599	0.2611
HNO_2	38.52	30.65	36.927	1.1776 ± 35.365	26.599*

Without treatment and its repetitions represent the spontaneous repeats (negative control). HNO2: treatment with nitrous acid (positive control). *: Significant at probability level 0.05≤ p

Table 6. Average frequency of spontaneous and induced mutant (× 10^{-5})in fungal conidia of *Aspergillus amstelodami* treated by Thyme Oil by growth mediated

Concentration of Thyme Oil ml / ml	Replicates Mutation			Average±Standard error	Calculated t value*
	R1	R2	R3		
0	0.089	0	0	0.029±0.029	
0.01	0	0	0	0± 0	1.000
0.016	0	0.35	0	0.116 ±0.116	0.7304
0.02	0.174	0.335	0	0.169 ± 0.096	1.4000
0.05	0.578	0.32	0	0.299 ± 0.1678	1.5882
HNO_2	38.52	30.65	36.927	1.1776 ± 35.365	26.599*

Without treatment and , its repetitions represent the spontaneous repeats (negative control), HNO2: treatment with nitrous add (positive control). *: Significant at probability level 0.05≤ p

its function as a good antioxidant (Sharifi-Rad *et al*., 2017) and works to reduce the mutagenic action of the mutagen, physical or chemical by disrupting the mutagenesis mechanism or by repairing damage in the DNA (Gautam *et al*., 2014), so it can be considered as an antimutagen and works to protect the DNA (Horvathova *et al*., 2007), and it is considered genetically safe and healthy (EMA, 2010).

Khan *et al*. (2019) reported that Carvacrol possesses a biological anticancer property for prostate cancer cell lines, and its high preventive and therapeutic efficiency for this type of cancer using MTT assay & LDH release assay. As the researchers (Bound *et al*., 2020) confirmed that both Thymol and Carvacrol showed high antimutagenic properties against mutations induced in *Salmonella typhimurium* TA 1538, which was tested by Ames test at the concentration 1 µmol/plate, as the rate of inhibition reached 87.7%. The antimutagenic activities of these phenolic compounds indicated their safe potential application as food preservatives.

Conclusion

Much work has been done to determine the genotoxicity and safety of natural herbal products, but there is little scientifically proven mutagenic information on thyme and its essential oil. Thyme oil, at the concentrations studied, did not have any mutagenic activity on

A. amstelodami conidia compared with the spontaneous benomyl-resistant mutants and the (positive control), using three mutagenic methods. It also had antifungal effectiveness.This was due to the presence of two main bioactive compounds Thymol and Carvacrol , with a percentage of 58.89% and 4.11%, respectively, which have antimutagenic and antifungal activities . So, it is considered genetically safe and healthy. In addition to the potential ability of Thyme oil to repair and protect DNA from mutagenic agents**.** The strain A1 of Aspergillus amstelodami as a biological system to investigate the mutagenic effect of thyme oil showed its response to the mutagenic factor adopted in the current study, nitrous acid. Thus, it can be adopted in subsequent studies as a mutant strain**.**

Conflict of interest

The authors declare that they have no conflict of interest.

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