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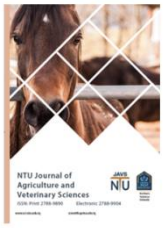

Evaluation of Phytochemical Constituents by GC-MS and Biological Activity of *Eugenia caryophyllus* extract

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Article Informations

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Extract

A B S T R A C T

This research was carried out under laboratory conditions to test the inhibitory ability of the extracts *Eugenia caryophyllus* on pathogenic fungi *Macrophomina phaseolina* Which is diagnosed on the plant Shalek depending on its morphological and microscopic characteristics. The active compounds found in the volatile oil separated from cloves were identified using GC-MS and the following active compounds were obtained (8-Dimethylnona-1, phenol ,Naphthalene, alpha-Farmesene, 1-Lsopropyl-4**,** 7-hexahyro-1**,** Caryophyllene oxide.) .Of these compounds, 1-Lsopropyl-4 gave the highest concentration at 2. 01 mg.gm^{-1} with a retention time of 8.537, and the separated fatty acids were identified from the clove petroleum ether extract using GC-MS, and the following fatty acids were obtained:(Palmatic, Undecanoic, Lauric, Arachidonic, Lenolinic, Linoleic, Oleic, Stearic) .Among these compounds is Linoleic Which gives the highest concentration at 27.52 mg. gm^{-1} With a holding time of 15.280 minutes. The inhibitory effect of clove extracts against *Macrophomina phaseolina* was tested. The results showed that the extracts of petroleum ether and eucalyptus oil, which were used at concentrations of 5, 10 and 15 mg/ml in the PDA medium, had inhibitory effects on the growth of the aforementioned fungus. Both extracts caused a significant effect on the growth of this fungus at all concentrations used. As these fungi failed to form mycelium on PDA.

Introduction

The Clove is a plant from the Myrtaceae family[1]. The aromatic flower buds are fragrant, dried, unopened flower buds. An evergreen tree with a conical shape, a four-part flower, with a strong aromatic scent. The average height of the clove tree is 10 to 12 meters[2][3]. Clove oil contains many active ingredients, such as:(Eugenol, Acetyl eugenol, β-caryophyllene, Vanillin, Crategolic acid, Tannin such as bicornin, Gallotannic acid, Methyl salicylate (Painkiller), Flavonoids eugenin, Kaempferol, Rhaumnetin, Eugenitin, Triterpenoids such as oleanolic acid, Stigmasterol, , Campesterol [4]. Clove is used as an antibacterial, antiviral, anticancer and antifungal agent. It is often used as a topical antiseptic in cases of toothache and is used in the manufacture of toothpastes and mouthwashes[5]. *Fragaria X ananassa Duch* belongs to the *Rosacea* family, and it is a fruit with small fruits that is widespread in regions of the world. Its fruits are characterized by their high nutritional value and good flavor because they contain many substances and nutrients in addition to vitamin C [6]. Decline or death of strawberry plants is a serious challenge to strawberry production, as crown and root diseases are among the most detrimental determinants, especially in the production season Little is known about associated fungal pathogens[7].Soil-borne fungal pathogens play a major role in root death of many important field and horticultural crops and often lead to plant death [8]. Diseases of plant crops negatively affect the agricultural economy of many countries, depending on the severity of the diseases caused by the organism that causes the disease. Among the fungal pathogens that are transmitted through the soil is the fungus *Macrophomina phaseolina*.Infection of the roots with this fungus causes a limitation of nutritional absorption and results in rotting of the roots which leads to the death of plants. The fungus infects more than 500 species of plants [9]. The aim of the study is to isolate the active compounds from cloves and test their bioactivity against *M. phaseolina.*

MATERIALS AND METHODS

The working methods used in the study included two main tracks: separation and determination of the active compounds from clove flower buds and testing the viability of different concentrations of clove plant extracts against the fungus under invistegation.

The first path is the chemistry of the clove plant:

The flower buds of *Eugenia caryophyllus* L. were obtained from the local market in the city of Mosul from reliable sources and classified by taxonomists [10].

Preparation of clove extracts

The extracts were prepared using a continuous extraction apparatus based on the method of the researcher [11] using petroleum ether solvent.

Extraction of volatile oil from clove buds by steam distillation: The volatile oil extracted from cloves was distilled using the Cliffhanger axial steam distillation method [12][13].

Gas Chromatograph-Mass Spectrometer (GC-MS):

Fatty acids were diagnosed in science and technology laboratories in Baghdad. As for the active compounds of clove oil, they were diagnosed in the Food Research and Consumer Protection Laboratories - University of Basra, by means of a gas chromatography-mass spectrometer type GC-MS QP210 Ultra from the Japanese company SHIMADZU [14].

Separation of fatty acids by saponification process:

To separate the saponified substances present in the petroleum ether extract, (2) grams of clove extract were taken and 100 ml of KOH solution (7.5 M) was added to it, then a thermal sublimation was conducted for 90 minutes at 100 °C The mixture was cooled to room temperature and 100 ml of KOH and distilled water were added to become an emulsion. The solution was placed in a separating funnel and (25 x 2 ml) of ether was added to it to remove the unsaponified fat. The saponified aqueous solution was taken and an acid of 20% of concentrated sulfuric acid H_2SO_4 until reaching PH = (2) and the solution became clear. The fatty acids were extracted By ether in a 2×25 ml separating funnel to obtain the fatty acids dissolved in the ether. The upper two layers consist of ether with fatty acids and the lower layer is aqueous. The separated fatty acids are neglected and kept inside sterile and darkened glass bottles in the refrigerator. To perform diagnostic operations, the fatty acids have been esterified (methylated) to make them less polar and more volatile when diagnosed with the GC-MS [15][16].

Determination the vitality of different concentrations of clove plant extracts against M. phaseolina used under study:

Isolation and identification of infected plants:

The fungi associated with the roots of Fragaria were isolated, on which symptoms of infection were observed, such as weak growth, yellowing of the vegetative system, and rotting of the root system. The infected samples were collected from some greenhouses from the College of Agriculture and Forestry / University of Mosul. The sample was taken in a closed bag to the laboratory of postgraduate studies / natural products in the College of Agricultural Technology / Northern Technical University to conduct the process of isolating fungi. The roots were washed well with running water to remove dust and impurities from them, and then cut into small pieces (0.5-1 cm). They were sterilized with

sodium hypochlorite solution at a concentration of 1% NaOCl for two minutes, then washed with sterile distilled water and dried using sterile filter paper. The infected samples were transferred to a Petri dish containing sterile (PDA) medium to which the antibiotic was added and incubated at a temperature of 25 ± 2 °C. for five days. The fungi were purified by transferring part of the end of the fungus colony to another plate containing the food medium (PDA) and it was done under the same conditions [17][18]. Macrophomina phaseolina isolated based on taxonomic keys Ellis, [19][20][21] Pitt and Hocking, Summerell and Leslie.

Preparation of different concentrations of clove buds extracts:

The stock solution was prepared using the Mitscher method using the dilution method by dissolving (4 g) of the prepared extract in the organic solvent 100 ml Dimehtyl Sulphoxide (DMSO), then the following concentrations (10,15,5) mg/ml were prepared for each of the extracts of *Eugenia caryophyllus* L. I kept sterilized bottles in the refrigerator until use [22].

$$\text{Inhibition percentage} = \frac{\text{Colony growth in control plate} - \text{Colony growth in control plate intersecting plate}}{\text{Colony growth in control plate}} \times 100$$

Test of inhibitory efficacy of *Eugenia caryophyllus* L extracts on *M. phaseolina*:

Concentrations of 10, 5, 15 mg/ml were prepared for each of the studied plant extracts, and they were added to dishes containing PDA medium that were sterilized and cooled at 50°, by three replicates for each extract, except for the control treatment, where sterile distilled water was added. Inoculate the center of each plate with a disc from the edge of a fresh culture of *M. phaseolina* with a diameter of 0.5 mm, the dishes were incubated at a temperature of 25 ± 2 °C, and after the growth of the fungus in the comparison treatment reached the edge of the dish, the average diameters of the fungal colonies were calculated by measuring the average of two orthogonal diameters, and the percentage of inhibition was calculated as in the equation [23].

Results and discussion:

Identification of active compounds present in volatile oil separated from flower buds of clove plant *Eugenia caryophyllus L*. using GC-MS:

The main active compounds were identified after the esterification of the oil and the production of turbines using mass spectrometry GC-MS, where seven compounds were identified out of 16 compounds. Among these compounds 1-Lsopropyl-4, which gave the highest concentration at 2.01 mg / g^{-1} with a retention time of 8.537. It was followed by alpha-Farnesene, with a concentration of 1.41 mg/g-1, with a small-time difference, with a retention time of 8.351, As for the Naphthalene compound, it appeared at a concentration of 1.09 mg/g^{-1} with a retention time of 8.280. and compounds phenol, Caryophyllene oxide, 7-hexahyro-1, The concentrations were respectively (0.64, 0.57, 0.40) mg/g^{-1}, with times of 7.447, 8.974, 8.619, The lowest concentration was 0.08 mg/gm^{-1} with a retention time of 6.144. of the compound 8-Dimethylnona-1. Some compounds did not appear in the chart due to their low boiling points or they were present in low concentrations according to the results of the analysis. As shown in the figures(1:8) .

This study was similar to what the researcher[24] did. he found the following compounds:(Eugenol, alpha-Cubebene, Phenol, 2-methoxy-4-(2-propenyl)- acetate, Caryophyllene oxide, Naphthalene, 1,2,3,5,6,8ahexahydro-4,7-dimethyl-1-(1- methylethyl)-, (1S-cis)- , Bicyclo[3.1.1]heptane, 6,6- dimethyl-2-methylene-. The researcher's results also showed[25] The results of the approach to our current study were as follows:(Caryophyllene oxide, Alpha-Bisabolol, NAPHTHALENE, 1,2,3,5,6,7,8,8A-OCTAHY, 2-Heptanol, acetate, Eucalyptol, Carinol, PHENOL, 2-METHOXY-4-(1-PROPENYL)-. and researcher[26] Which found the following compounds:(Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6- dimethyl-4-(1- methylethyl), Caryophyllene oxide, p-Eugenol, Eugenol acetate).

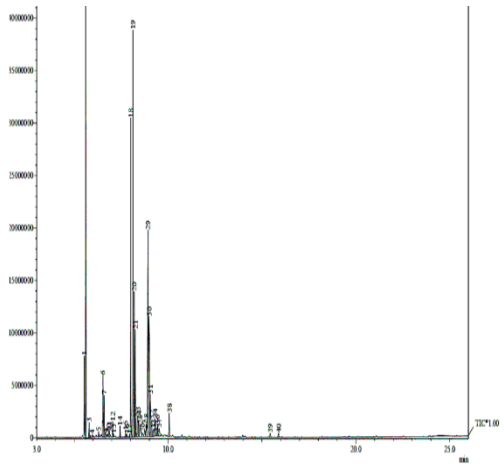


Figure1. Diagram of the active compounds of clove volatile oils identified by GC-MS

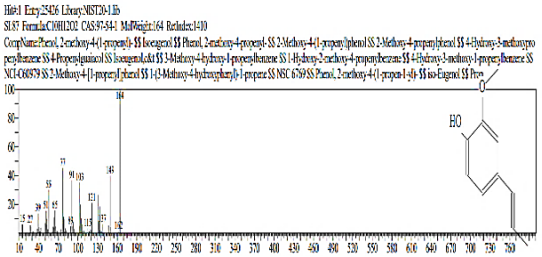


Figure 2. (E)-4,8-Dimethylnona-1,3,7-triene

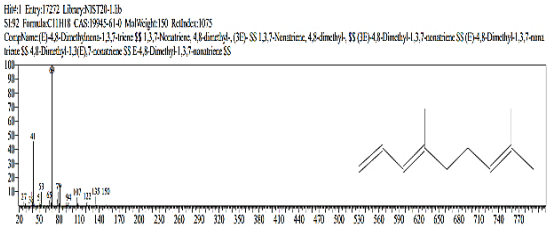


Figure 3: Phenol, 2-methoxy-4-(1-propenyl)-

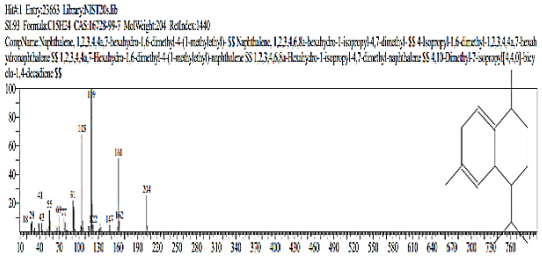


Figure4: shows a compound Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha

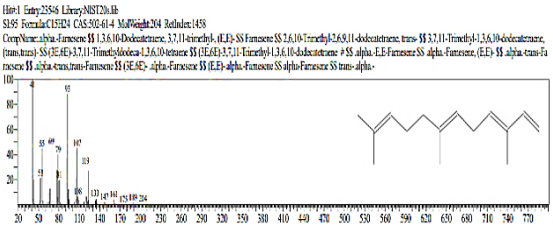


Figure 5 shows the alpha compound. -Farnesene

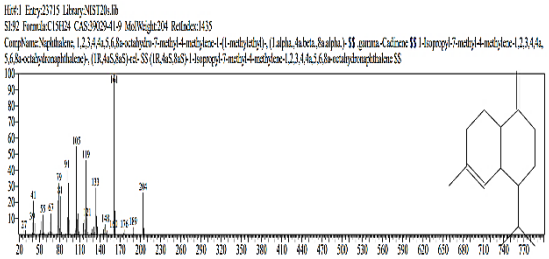


Figure 6: 1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene

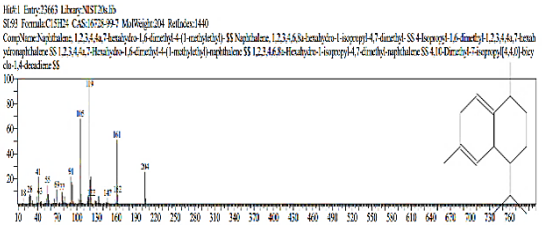


Figure 7: Shows a compound Naphthalene,1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-

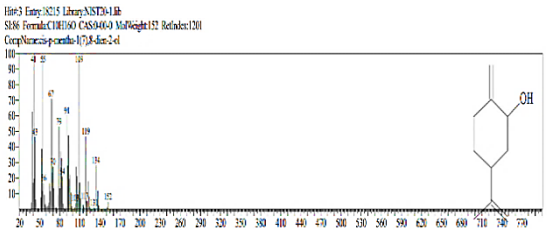


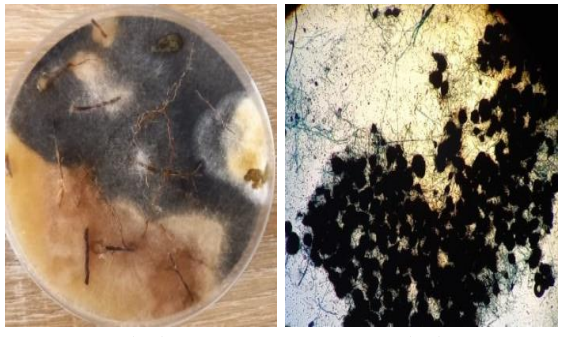
Figure 8: Caryophyllene oxide

Phenotypic diagnosis of *M.phaseolina*: The results of isolation and diagnosis from the roots of the shlik, which showed symptoms of yellowing and rotting, showed the presence of the fungus Microphobia phaseolina, as shown in the figures (9:13). These results agreed with previous studies[7][8].

(9) **(10)**

Figure 9: Symptoms of root rot infection in Shalik plant caused by infection with fungus *M. phaseolina*

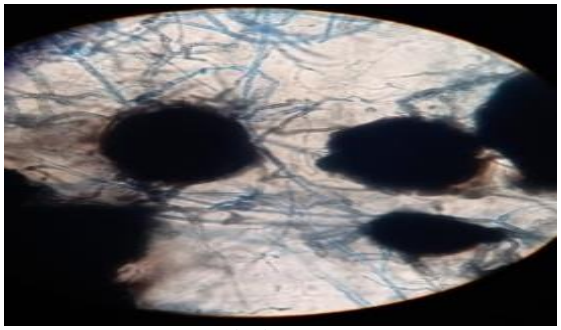
Figure 10: Shalik plant roots infected with *M. phaseolina* on the center of the PDA



(11) **(12)**

Figure 11: The appearance of the fungus *M. phaseolina* on infected roots

Figure 12: *M. phaseolina* isolated from roots of infected Shalik plants at 10X magnification



(13)

Figure 13: ***M****. phaseolina* isolated from roots of infected Shalik plants at 40X magnification power

Identification of separated fatty acids from petroleum ether extract of *Eugenia caryophyllus.L* by using GC-MS:

The identification of the petroleum ether extract of the buds of the clove tree using mass spectrometry GC-MS revealed the presence of fatty acids and terpenes, among these compounds Linoleic which gave the highest concentration at 27.52 mg. gm^{-1} with a holding time of 15.280 minutes, Followed by vehicles (Palmatic‹Oleic) At a concentration of (15.85,15.85) mg. gm^{-1} with holding times of (8.566 and 16.300) minutes, respectively. As for the Stearic compound, it appeared at a concentration of 13.65 mg. gm^{-1} with a holding time of 18,690 minutes. Arachidonic and Undecanoic compounds, the concentrations were respectively (2.51, 1.56) mg. gm^{-1} times (11.190,9.796). minute. The lowest percentages of Lenolinic and lauric compounds, respectively, were (0.56, 0.35) mg. gm^{-1} has a time lag with holding times (13.311, 10.708). minute. Some compounds did not appear in the chart due to their low boiling point or were present in low concentrations according to the results of the analysis. As shown in the figure(14) . This is consistent with the study carried out by the researcher[28] Where the results reached by the researcher showed that it contained the following compounds:(Eugenol, Caffeic acid, Kaempferol, Vallinic acid‹Ferulic acid ‹Ellagic acid ‹Chlorogenic acid‹ Borneal). It also agreed with the study conducted by the researcher[29] The following compounds were separated (Eugenol ‹ Kaempferol ‹Gallic Acid ‹Vanillic Acid). It also agreed with what the researcher found[30] who found the compounds: (Gallic acid‹ kaempferol‹Caffeic acid‹Rutin‹Ferulic acid ‹ Quercetin).

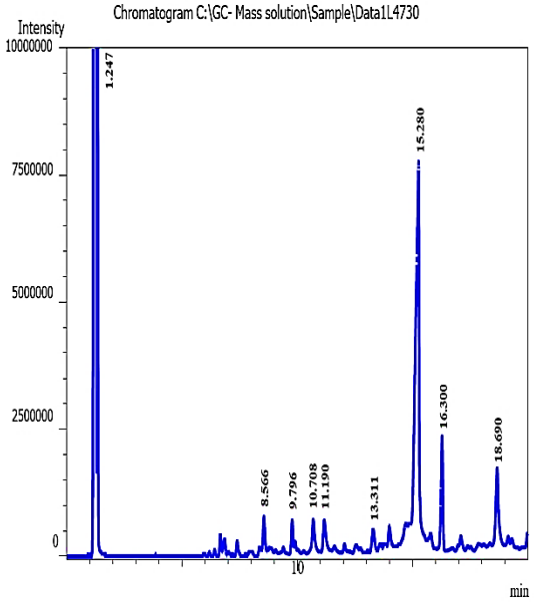


Figure 14: Chromatography-mass spectrometry GC-MS of separated fatty acids from petroleum ether extract of clove buds

Table 1: Identification of fatty acids separated from dendritic clove buds by GC-MS

N	Peak	R. time	Area	Con%	Name
1	2	8.566	3227648	15.85	Palmatic
2	3	9.796	3796976	1.56	Undecanoic
3	4	10.708	4735638	0.35	Lauric
4	5	11.190	4775251	2.51	Arachidonic
5	6	13.311	3292609	0.56	Lenolinic
6	7	15.280	70662698	27.52	Linoleic
7	8	16.300	12374568	15.85	Oleic
8	9	18.690	12221776	13.65	Stearic

Testing the inhibitory effectiveness of different extracts of Eugenia caryophyllus shoots against *M.phaseolina* :

The results showed in Table (2) and Figure (14) that the petroleum ether extract had a significant effect on the growth rate of the fungus, where the percentage of inhibition was 100% for all concentrations used (10, 15, 5) mg/ml.

As for clove oil, it was significantly superior in all concentrations (5, 10, 15) mg/ml over the control treatment. As the inhibitory ability of these extracts increased directly with increasing the concentration of the extract. That both petroleum ether and eucalyptus oil exhibited an inhibitory effect on the fungus in the experiment.

As for the effect of concentrations, there were no significant differences between (5, 10) mg/cm^3, while the concentration (15%) mg/cm3 was significantly higher than the control treatment.

Table (2): Effect of clove bud extracts on the growth of *M. phaseolina*

concentration	comparison treatment	Clove oil	Petroleum ether extract	Concentration (mg/mL)
	Average diameter of fungal colonies in the treatment			
3a	8b	0c	0c	5
3a	8ab	0c	0c	10
3a	8a	0c	0c	15
	8a	0d	0d	extract effect

The petroleum ether extract of the clove plant is more efficient in inhibiting the tested fungi. The reason for this may be attributed to the ability of the petroleum ether extract to extract and dissolve the active substances in the used parts of the plant. The most important of which are resins, alkaloids, flavonoids, tannins, and saponins, some of which dissolve in polar solvents without others. Solvents such as alkaloids that cause a defect in the permeability of the cell membrane of the cell, which results in the cell losing small molecules such as amino acids, simple sugars and ions This leads to a decrease in the metabolic activities of the cell including energy metabolism, active transport, and the process of making proteins. This is consistent with two studies , first a study carried out by the researcher[31] as he studied the effect of alcoholic extract of cloves on some bacterial species and second a study done by the researcher[32] who used clove extract as an inhibitor of strawberry gray fungus.

The ability of clove flower buds oil to inhibit the growth of a fungi is due to the fact that it contains some fungal-toxic compounds such as eugenol , 8.01%, engenylacetate , 3.56%, beta caryophyllene. [33] or vehicles Carvaerol , Geraniol , Trans-cinnamaldehyde which has high effectiveness in inhibiting the growth of fungi[34] Researcher [35] using the electron microscope to follow up the effect of clove oil on the growth of fungi found significant changes represented by thinning of the hyphae wall, rupture of the plasma membrane, and deformation of the mitochondria.

Conclusion

Based on the results presented, it can be concluded that the active substances of cloves are remarkable. These active substances have a significant effect on the growth of fungus in all concentrations used.

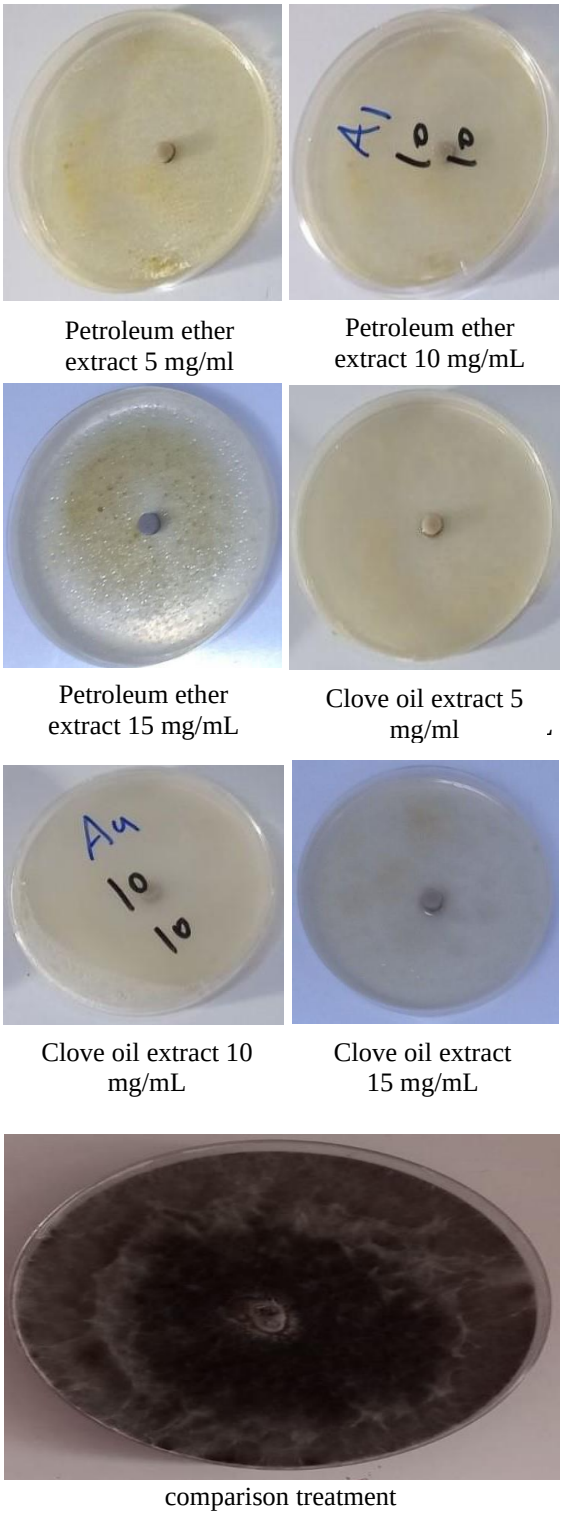


Figure (15): Effect of types of clove extracts on the growth of *Macrophomina phaseolina*

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