Control of Blue Mold of Pear Fruit caused by *Penicillim expansum* using Local Plant Extracts and their Derivatives

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Abstract:

Due to the fractionation of *Hypericum triquetrifolium* extract, it was found that the isolated Hypericin gave the inhibition percentage of about 93.76% and the fungus growth diameter average of 0.59 cm. The volatile oil extracted from *Vitus agnus-castus* significant effect on the growth of the fungus about 71.20% inhibition percentage and the fungus growth diameter average about 2.39 cm. However, the extraction of *Nigella sativa* had a significant effect on the fungus growth about 82.03% and the fungus growth diameter average about 1.43 cm due to 0.5 µl ml^{-1}. For detection of secondary compound products for *H. triquetrifolium* by HPLC technique, five compounds (peaks) were detected, the higher percentage of the secondary compound, Hypericin, was 225.073 µg ml^{-1}, this is the active ingredient of this plant. An experiment was conducted on the actual possibility of the highest concentration of the extracts, which gave the highest percentage of inhibition of fungus *Penicillium expansum* by immersing the fruits of pear (cv. Harme naska) alone and mix with each other and found that the mixing process has given impressive results, so mixtures of the last four gave the highest percentage inhibition (100%) and on this basis can be relied upon recommendation, we can say that the mixture (Hypericum + Nigella + Zingiber) gave a 100% . This treatment is better because it does not exist in the composition of a chemical on the other hand give a guarantee for those who work in organic cultivation.

Keywords: Disease Management, Blue Mold, *Penicillium expansum* , Plant extracts , HPLC

I. Introduction:

Blue mold is one of the most important post-harvest diseases of pear fruit and apple and can account up to 50% of stored pear fruit losses [1].This disease is caused by a pathogen called *Penicillium expansum*.,one of the most common ascomycetous fungi involved in the post-harvest decay of numerous fruits such as apples, peaches, pears and cherries, which infects fruits through wounds after harvest as well as through natural openings e.g. lenticel, stem end and calyx. Under cold storage conditions, blue mold lesions (from wound infections) caused by the common species may be expected to be one to one and a quarter inches in diameter eight to ten weeks after infection. In rots caused by other *Penicillium* species, the decayed tissues are firmer.

It may lack surface growth under cold storage conditions, and are slow-

growing compared to *P. expansum*. The importance
of these species should not be ignored, it is probable that under certain conditions, they may assume considerable importance. Also *Penicillium* is worldwide known for Production of secondary metabolites and extra cellular enzymes of commercial value including the pectinases, utilized in fruit juice industry carried out during the stage of pulp maceration [2] and [3].

Many methods are used for controlling *Penicillium expansum*, such as biological control by using natural product and non-invasive bacterium, yeast, synthetic fungicides, and atmosphere storage, additional control may be achieved by subjecting the fruit to a hot water bath immediately after harvest.

Plant materials contain many compounds called secondary compound. This use has been supported by the isolation of active antifungal compounds from plant extracts [4]. Among aqueous extracts of the *Strychinos nux-vomia*; *Zingiber officinalie* and *Allium sativum* against *Trichoconigella padwickii* it was found that the first one was more effective against *Trichoconigella padwickii* [5].
Oils extracted from the seeds of black cumin *Nigella sativa* were found to have antifungal activities against eight seed borne fungi viz, *Aspergillus niger, A. flavus, Fusarium oxysporum F. moniliforme, F. nivale, F. semitectum Drechslera hawiinesis* and *Alternaria alternate*. All the oils extracted showed fungicidal activity of varying degree against these species. The essential oil of many plant and their constituents have been found effective as anti-fungal agent [6]. [7] found that ethanolic and water extracts of *Hypericum triquetrifolium* , *Vitex agnus_castus*, *Eryngium billardieri, Chrozophors tinctoria, Heliotropium circinatum* and *Prosopis fracta* were more effective against *Ascochyta rabiei* and *Fusarium oxysporum* fungi compared to other plant extracted , and the fractions of *H. triquetrifolium* against two pathogenic fungi *Fusarium oxysporum* and *Ascochyta rabiei* and found that Hypericin gave 87.50% and 88.23% MGI while f2B gave 81.62, 86.08 % MGI% against the two pathogenic fungi respectively.

The aims of this study is to investigate the effect of some local plant crude extracts and their derivatives on the inhibition of the well-known dangerous fungus (*P. expansum*) on pear fruits in Sulaimani province – Kurdistan/Iraq.

II. Materials and Methods:

A. Plant collection and identification

Samples of *Hypericum triquetrifolium* and *Vitus agnus-castus* were collected at full bloom from Dukan and Mawat during July 2010 [8], while *N. Sativa* and *Zingiber officinale* were taken as seeds and rhizomes alternatively from Sulaimani governorate. Plant samples were formerly identified at Herbarium section, Plant protection Department, College of Agriculture, University of Baghdad. *H.triquetrifolium* was dried in shade under ambient temperature; the dried samples were ground to powder by electric grinder, and kept in plastic containers under dried condition for the purpose of extraction and analysis.

B. Preparation of plant extract

100 gm sample powders from each plant (*H.triquetrifolium*) put in a thimble inside a soxhlet for continuous extraction

using 500 ml ethanol 70-80% for 4-6 hours at 45-50^{o}C depending on the species. The extract was concentrated by Rotary Vacuum Evaporator at 45^{o}C. The gummy residue of the total plant extracts were kept in a refrigerator until it is used in the experiment [9]. Rhizomes of *Z. officinale* were cut into small pieces; 100gm of these pieces were put in a thimble inside a Soxhlet using 500 ml ethanol 96% for 4 hrs. at 45^{o} C, the extraction was evaporated at low temperature 50-60 ^{o} C then put in a refrigerator until it is used [10].

C. Fruit collection and treatment

Pear fruits were collected from trees in Shanaxse, Sulaimani at a commercial maturity in November 2009. Uniform fruits were then washed by distilled water, dried up and kept in refrigerator at 4^{o}C until used in the study.

D. Isolation and purification of the pathogen(s) associated with fruits under study

Small pieces from surface of decayed pear fruit were placed on Potato Dextrose Agar (PDA), then the plate were incubated at 25 ± 2^{o} C for 7 day .The incubated fungus on PDA were purified by hyphal-tip method to obtain a pure culture of the fungus and conserved in a refrigerator at 4^{O} C for further studies. The fungus was formerly identified by using two methods: Morphological characters of the fungus according to [11], and by using Polymerase Chain Reaction (PCR) technique [12].

Six healthy pear fruits were taken and injured at different sites on the surface, then inoculated with 5 mm of isolated and purified *P.expansum* mycelia growth and incubated at 25±2^{o} C .Other pear fruits were maintained without inoculating, and sprayed only with distilled water as control treatment.

E. Effect of fixed oil of N. sativa seeds on the Inhibition zone area of the fungus

The seeds of *N. sativa* were ground into a fine powder in an electric grinder and oils were extracted with hexane by soxholet's extraction apparatus at 30 ^{o}C mixed with PDA to test seeds oil for the antifungal properties [13]. Required amount of oil extracts were dissolved in pure acetone and thoroughly mixed with PDA to provide 0.5, 0.1 and 0.15 % ml with 100 ml medium, some plate media kept untreated as control. 5mm fungal culture was placed in the centre of each petri plate and incubated at 25±2^{o} C, after that data inhibition zone area were recorded after 7 days of incubation. [14].

F. Effect of fraction1 and fraction2 of H.triquetrifolium on the growth of the isolated fungus

40000 µg ml^{-1} from fr1 and fr2 were put into P D A medium each petri dish was inoculated with 0.5cm of the fungal inoculums pieces, and then incubated at 25 ± 2^{o} C. The inhibition zone area was calculated [15].

G. Fractionation of the polar materials in fraction 2

Fraction 2 was diluted with 500 ml of dist water which stored for 10 days in a refrigerator the filtrate was concentrated in vacuum named fraction 2A which was kept in a fridge, the precipitate named fraction 2B which kept in a fridge [16].

H. Effect of Fr2A and Fr2B on the growth of the fungus

40000 µg ml^{-1} from fr2A and fr2B were prepared, and added to P D A

medium, the petri dish was inoculated with the fungus then incubated at 25 ± 2^{o} C, the inhibition zone area were calculated [17].

I. Separation of Hypericin from fr2B

Hypericin was separated from fraction 2B, by Thin Layer Chromatography (TLC) using pre-coated silica gel plate (Aluminium sheets 20x22 cm,25/pk) from(A.G.fluka)as the stationary phase and using a solvent system mixture of to luene: formic acid: ethyl formate in a ratio: of 5:1:4 altreativey as mobilc phase. A sample of fr2B was dissolved in 1ml acetone, and was spotted on the above mentioned plate which developed by the above solvent system in a (TLC) tank. After drying up the plates were observed a spots as indication of Hypericin with a R*f* value of 0.45-0.50 [calculated by applying the formula: R*f*= Distance spot moved(cm)/Distance solvent moved(cm)] , with a deep reddish colour by a naked eye and red fluorescent and long wave U.V. lamp[7], [18] and [19].

Several plates were prepared as above, using all the solution of fraction 2B, the spots containing Hypericin were taken off from all the plates and put in a flask while the rest of the silica above the spots of Hypericin were taken off and kept in a separate flask, to both flasks, enough acetone was added to cover the Silica powder, the flasks were shaken by hand and filtered, this process was repeated three times to extract all the possible compound adsorbed to the silica, the combined acetone extracts of each fraction were concentrated in vacuum [16].

J. Effect of isolated Hypericin on the growth of the fungus

Acetonic extract of the spots containing Hypericin after drying was tested on the growth of the fungi Hypericin containing spots were scraped off the plates together with silica gel above the spots a sample of 2 gm of Hypricin containing silica and 2 gm of silica above Hypericin containing spots were spread over the surface of each of the four replication of petri dishes containing PDA, inoculums with the fungus. The Inhibition zone area was calculated [20].

K. Detection of active ingredient of H triquetrifolium by HPLC technique

3 µg particle size column (50 x 4.6 mm.ID) C-18DB ,mobile phase 0.01M ammonium phosphate butter A:acetonitrile B .eluted by linear gradient from 0-100% B in 10 min, detection Uv set at 254 nm, flow rate 1.2 ml/min ,temperature 30^{o}C. Standard 50 µg ml^{-1} of each standard.

L. Calculations of H.triquitrifolium derivatives

The amount of each active ingredient can be found by applying the following formula [21]:

Concentration of sample (µg ml^{-1}) =Area of sample / Area of standard x conc. of standard x dilution factor

The potential efficacy percentage was calculated by using the following formula [22].

P_e = Potential Efficacy, G_c= Growth (for control) and G_t = Inhibition Growth (for treatment)

M. Statistical Analysis:

The measured parameters were analysed by using computerized statistical analysis of X-LSAT package for analysis of variance (ANOVA) and means of treatments were compared using L.S.D. at 0.05 level.

III. Results and discussions:

A. Effect of different concentrations of essential oil and essential oil free of V. agnus-castus on the growth of P.expansum

Results of essential oi1 experiment effect of *V. agnas-castus* on MGI% , revealed that there were significant differences among the treatments themselves and with control treatment at different conc. The highest mycelial growth inhibition for the marc (the essential oil free component of the plant) reached to 80.23%, meanwhile remarkably and scientifically MGI% was in detected the first conc. (50 µl ml^{-1}) which was equal to 55.76%.(Table I).

*Pe (%) = Gc – Gt / Gc * 100*

Table.I: Effect of essential oil and essential oil free of *V.agnus* (Marc) on growth of *P. expansum*

Concentration (µl ml^{-1})	Mycelial growth (cm)	Growth inhibition (%)
Control	8.5	0.00
5	3.76	55.76
10	3.73	56.11
20	2.39	71.88
Marc	1.68	80.23
LSD (0.05)	0.936	

[17] proved that the inhibition percentage of the total crude extract of this plant after separating essential oil were 72.94 against *Helmenthosporium* spp. for the same conc. while **[7]** proved that MGI% of the essential oil of this plant was 74.59, 70.80 and 53.42 against *Fusarium oxysporum* . [23] reached 26.50% mycelia growth inhibition for *R.stolonifer* in using the essential oils of the flowers of *V. agnas-castus* and 49.36% inhibition for *A. niger.*

These results indicate that there were significant and crucial effect of the chemical component and essential oil extracted from the plant on the Inhibition of *P. expansum*. A thought was established about controlling of thin to xicogenic and dangerous fungus which infects pear fruits in our country, it was confirmed that there was alternative method despite of using chemicals because of great risks of them on the consumers which using fungicide coated pear fruit.

B. Effect of different concentrations of fixed oils of N. Sativa seeds on the growth of P. expansum

Table II shows that the minimum inhibition of *P. expansum* growth by *N.sative* seed oil started from 10 µl ml^{-1} which gave 59.63% mycelia growth inhibition while the highest MGI% was for 50 µl ml^{-1} which was 82.77% in this experiment result showed that there was a significant effect between the three concentration that were used to inhibit the mycelia growth of the fungus *P. expansum* and an excellent inhibition was obtained for the last concentration which was 50 µl ml^{-1} . Many studies are available about the bioactivity of *N. sative* fixed oil. [14]and [24] referred to the fact that the fixed oil of *N.sativa* seeds possess a remarkable

antifungal activity against *Aspergillus niger*, *A.flavus*, *F.moniliforme*, *F.oxysporum*, *F. nivale*, *F. semitectum*, *Alternari alternata* that showed strong fungicidal activity at the 0.5%.

In general fixed oil which was natural products from black seeds, have great potential as novel fungicide sources for controlling pathogenic fungi because it contain non-phytotoxic compounds and potentially effective against plant pathogenic fungi and its safer antifungal agents to control phytopathogenic in agriculture. That's because these components affect not only the permeability but also other function of the cell membranes, and might also the cell membrains. Thus penetrating into the interior of the cell and intersecting with critical intracellular sites. In this experiment, the present results of antifungal screening indicated that fixed oils derived from *N. sativa* seeds markedly inhibited the mycelial growth of the tested fungi at a concentration of 50 µl ml^{-1}. fixed oils components would act on the hyphae of the mycelium, provoking exit of components from the cytoplasm, the loss of rigidity and integrity of the hypha cell wall, resulting in its collapse and death of the fungus [25].

Table.II: Effect of *N.sativa* seeds oil on the growth of *P. expansum*

Concentration (µl ml^{-1})	Mycelial growth(cm)	Growth inhibition (%)
Control	8.3	0.00
10	3.35	59.63
15	2.70	67.46
50	1.43	82.77
LSD (0.05)	0.453	

C. Effect of fractions of H. triquetrifolium and Hypericin on mycelia growth inhibition of P. expansum

Table III shows that hypericin is superior significantly superior to other component fractions with respect to MGI % followed by F2B and so on little relative effect of F1 component may be due to its content of lipophilic compound, which affect on the fungus activity [26]. While F2 was more effective on the inhibition because of their contents of hydrophilic compounds. In this result we indicated that the highest mycelia growth inhibition against the fungus was given by hypericin which was 92.67%. The result agrees with [15] who found that extracts of hypericin was 100 % effective in the inhibition of *Pythium aphanidermatum.* and [17] found that hypericin extracted from *H. triquetrifolium* gave 88.82, 88.23, 94.12 MGI% against three pathogenic fungi *Pyricularia oryzae* , *Helmenthosporium oryzae* , *Fusarium accuminatum* respectively. *H. triquetrifolium* contains different compounds and secondary metabolites, these components are of phenolic nature (Diathrone derivetives). Such as (Hypericin and Pseudohypericin).There were really considered as active ingredients used for different medicinal purpose, as a antidepressant therapy and other psychological human disorders [27]. Although counted as a excellent inhibitor for many plant pathogenic fungi [15]. Several researches attributed the mechanism of action of the secondary metabolites of plants to the phenolic compounds the amphipathicity of these compounds can explain their interactions with bio membrane and thus the antifungal activity [28].

D. HPLC analysis of bioactive ingredient of H . triquetrifolium

H. triquetrifolium contains a variety of structurally diverse natural products which possess a wide array of biological properties [29]. In HPLC polyphenol analysis 3 groups of secondary metabolites were shown as a result of analysis, which are:

1- Diathrone derivatives; which include: Pseudohypericin, Hypericin.
2- Flavanoids; including; Leucocyanidin.
3- Coumarin; including; Scopoletin and Umbelliferone (Table IV, Fig 1 the peaks 1, 2, 3, 4, 5).

H.triquetrifolium is traditional medicinal containing a broad spectrum of secondary metabolites [30]. Hypericin gave a highest secondary metabolites with (225.073 µg/ml) and second Pseudohypericin gave (148.538 µg/ml).while Leucocyanidin gave the lowest concentration of (53.023 µg/ml). [31] assume that the discrepancy of the secondary metabolites contents of *H. triquetrifolium* could be attributed to macro- and micro-environmental factors as well as seasonal variations, harvesting time and drying and storage conditions. There are several worldwide studies which have shown such variations in the concentration of the bioactive constituents. For example *H. triquetrifolium* growing in different locations of the world during plant growth showed variation of their secondary metabolite contents. Habitat altitude and developmental stage were also found to influence hypericins levels of *H. triquetrifolium.*Hypericin and pseudohypericin are photodynamic pigments [32]. Photodynamic hypericin activities displayed under the influence of light are used for therapy in various diseases. The highest hypericin content was obtainedfrom the full flowering stage of this plant. This finding is parallel to previous published results [31] and [33]. Achieved from the fractionation of *H. triquetrifolium* showed that hypericin was able to inhibit the growth of *P. expansum* mycelia when its concentration reach 93.05 %. (Table IV) because it have many Ecologic functions include defense against microbial pathogens and herbivorous animals. The synthesis of phenolic compounds in plants can be modulated by the application of herbicides and, to a lesser extent, insecticides and fungicides.

Table.III: Effect of fractions of *H. triquetrifolium* and Hypericin on mycelial growth of *P. expansum*:

Concentrations (40000 µg ml^{-1})	Mycelial growth (cm)	Growth inhibition (%)
Control	8.05	0.00
Fraction 1	3.91	51.45
Fraction 2A	3.17	60.62
Fraction 2	2.57	68.07
Fraction 2B	2.10	73.91
Hypericin	0.59	92.67
Spots above Hypericin	5.52	35.05
LSD (0.05)	0.821	

Table 4: Amounts and retention times of the metabolites of *H. triquetrifolium* from HPLC analysis.

Sq.	Metabolites	Retention time (Minute)	Area	Concentration µg ml^{-1}
1	Leucocyanidin	1.017	11887	53.023
2	Pseudohypericin	2.827	32844	148.538
3	Hypericin	3.217	41212	225.073
4	Scopoletin	4.000	11277	58.6488
5	Umbelliferone	4.838	13383	55.723

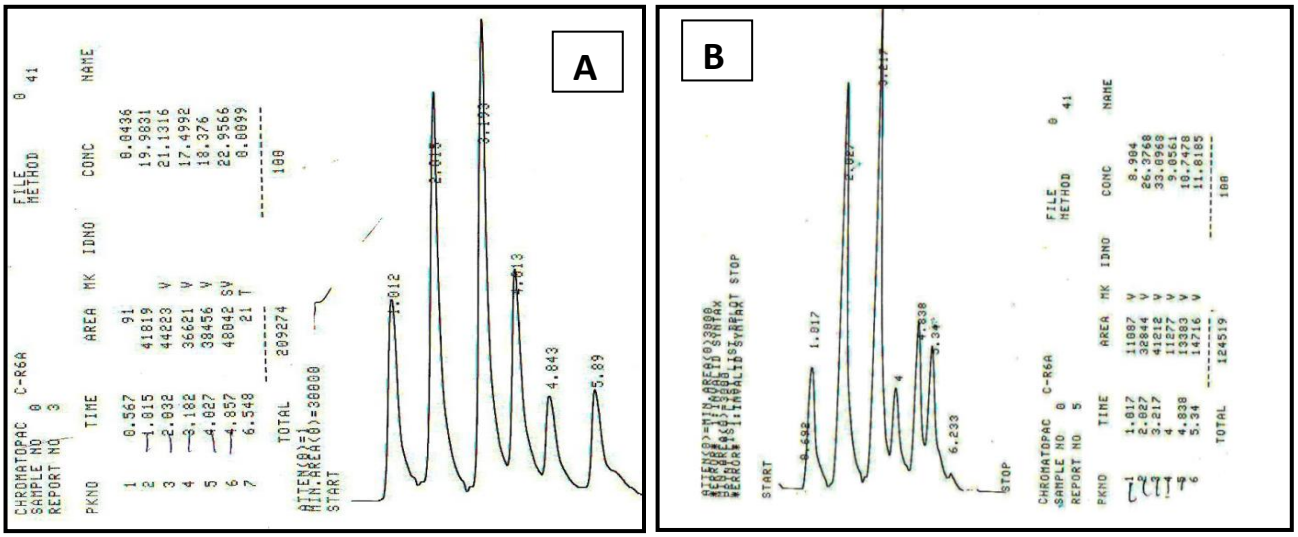

Fig. 1: HPLC analysis of five secondary metabolites found in *H . triquetrifolium* .

1- Leucocyanidin 2- Pseudohypericin 3- Hypericin 4- Scopoletin 5- Umbelliferone (**A**) are the peaks of Reference compounds , while the peaks in (**B**) are the compounds of the sample of plant extract. Separation of active ingredients by FLC (fast liquid chromatographic) , 3 µg particle size (50 x 4.6 mm. ID) C – 18 DB , mobile phase 0.01M ammonium phosphate buffer A: acetonitrile B eluted by linear gradient from 0 – 100 % in 10 min. detection UV set at 254 nm , flow rate 1.2 ml/min. , temperature 30°C Standard (Reference) 50 µg ml^{-1} of each standard.

E. Effect of plant extracts alone and in combina-
tions with them and /or with fludioxonil on the
blue mold fungus

Table V shows the effect of plant extracts alone or in combination with them and with the postharvest fungicide fludioxonil (Scholar). The high percentage of growth zone of the fungus was in (Z) treatment extract, with less potential efficacy (49.23%) . The zero growth zone started at (N+F) treatment and the other ones (H + F) , (H + N +Z) and (H + N + Z + F) , potential efficacy percentage

was 100%. In this experiment, we conclude that the combinations of plant extracts in with high benefit to reduce the % of fungal growth zone, also as a result of this experiment the treatment that completely eliminated *P. expansum*-incited decay was the combination of (H + N + Z) without any fungicides treatment. The proper combination of alternative control measures can provide commercially acceptable and it is excellent for applying and protecting the pear fruits from blue mold fungus attacking suitable. Other researchers found that mixtures of plant extracts inhibition the fungul growth of the treatment sets, for instance , the essential oils of *Z. officinale* and *N. sativa* with stand high inoculation density of the test fungus[34]. Potentials of oils and other components are exploited to be used as botanical fungistatic (fungicide) such plant fungicides have long life shelf and remained naturally at different temperatures (5 – 50 ^{o} C) [35]. Due to the fore mentioned concerns about their effects on human health and the continuing development of resistance in postharvest pathogens to the commonly used fungicides makes it of paramount importance to find alternatives to the use of fungicides to reduce losses from postharvest decays. Alternative control methods alone do not have as wide a spectrum of activity under various conditions as fungicides and most of them cannot achieve the effectiveness of fungicides even under optimal conditions. Therefore, a combination of promising alternatives must be used to develop a control strategy suitable for commercial application. Biological control is an alternative to chemical control that shows effectiveness in controlling postharvest diseases [35]. The goals of the experiment was reduced the rate of extracts to a third or a half ratio to avoid the unfavorable toxicity in the plant material, also for synergetic plant extracts reduce mold growth on pear fruit surface, since the selected plants, are well known as folk medicinal for their high values in health as they contain phenolic like substances, for example *H.friquetrifolium* contains the active ingredient Hypericin [36], which is used as antidepressant. *Z.officinale* active ingredient “gengerol” used as antitumor and antioxidant [37]. Finally, active ingredient “Nigellone” in *N.sativa* used against various diseases and disorders [38]. Furthermore, these plants are usually known as antibacterial and antifungal potent. Furthermore, combination of plant extracts alone or with fungicides to reduce mixture ratios, though the overall purpose is to remove the fungal growth on the fruit surface and thus obtaining better quality for human being. The increased global chemo phobia and reduced efficiency of chemicals due to pathogen resistant strains have forced producers to evaluate the contents of sustainable agriculture (organic cultivation.

Table.V: Effect of dominant plant extracts alone and in combinations with them and /or with fludioxonil on the blue mould fungus of pear fruits.

Treatment sets*	Concentration (µg ml^{-1})	Growth zone (cm) after 14 days storage	Potential Efficacy Control (%)
Control		2.167	
Z	40000	1.100	49.23
N	40000	0.967	55.37
H	40000	0.100	95.38
H+Z	20000+20000	0.067	96.90
H+N	20000+20000	0.05	97.67
F	2500	0.017	98.98
Z+F	20000+1250	0.015	99.30
N+F	20000+1250	0.00	100.00
H+F	20000+1250	0.00	100.00
H+Z+N	13333+13333+13333	0.00	100.00
H+Z+N+F	10000+10000+10000+625	0.00	100.00
LSD (0.05)		0.212	

*Z=*Zingibar officinale* extract, N= *Nigella sativa* extract, *Hypericum triquetrifolium* extract, F=fludioxonil fungicide.

V. Conclusions:

Penicillium expansum is the main causal agent of blue mold disease of pear fruits (Herme Naske cv.) in Sulaimani governorate. Ethanol extract of the tested plants showed high potentional in mycelial growth of this fungus, fixed oil of *N. sativa* seeds exhibited high mycelial growth inhibition percentage. TLC-isolated Hypericin from *H.triquetrifolium* achieved high MGI% of the fungus, and a large of it have been obtained (225.073 µg ml^{-1}) by using HPLC technique . Combination of differ plant exreacts (Hypericum + Nigella + Zingeber).

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