

Antifungal Activity of Eco-friendly 10% Microemulsion Formulation of *Trans*-methyl Cinnamate against Some Important Phytopathogenic Fungi

Mohamed, A. A. Shaymaa

Pesticides Formulation Research Department, Central Agricultural Pesticides Laboratory, Agricultural Research Center

shimoabdewahab_2004@yahoo.com

Abstract: This study was undertaken to investigate the antifungal potential of *trans* – methyl cinnamate against four fungal strains economically important phytopathogenic fungi in the Egyptian environment named *S. rolfisii*, *R. solani*, *A. solani* and *F. solani*; preparation of microemulsion formulation and determinate its physical properties. Moreover, investigated the antifungal activity of the prepared formulation. *Trans*-methyl cinnamate prepared by Phase Inversion method. The prepared microemulsion presented polydispersity Index 0.296 and mean droplet size 55 nm. The LC₅₀'s of the tested compound *trans*-methyl cinnamate and the prepared 10% micro emulsion formulation against the tested fungi were determined according to the relationship drawn between the logarithm of concentration and the percent of growth inhibition (1dp lines), and the toxicity indices and relative potencies were calculated. The prepared formulation was more effective than the corresponding active material regarding the value of LC₅₀ in the case of all examined fungi. The LC₅₀ values of *trans*-methyl cinnamate on the tested phytopathogenic fungi *S. rolfisii*, *R. solani*, *A. solani* and *F. solani* were 115.56, 69.07, 165.21 and 422.77, respectively. Whereas, the LC₅₀ values of prepared *trans*-methyl cinnamate 10% ME formulation on the same examined fungi were 58.62, 60.86, 50.56 and 209.30 ppm, respectively. The toxicity indices were 50.72, 88.12, 30.6 and 49.51% for *trans*-methyl cinnamate 99% against *S. rolfisii*, *R. solani*, *A. solani* and *F. solani*, respectively when compared with for *trans*-methyl cinnamate 10% ME, which had the 100% toxicity index. Finally, the relative potencies of *trans*-methyl cinnamate 10% ME on *S. rolfisii*, *R. solani*, *A. solani* and *F. solani*, were 1.97, 1.14, 3.27 and 1.58 folds, respectively than *trans*-methyl cinnamate 99%.

[Mohamed, A. A. Shaymaa. **Antifungal Activity of Eco-friendly 10% Microemulsion Formulation of *Trans*-methyl Cinnamate against Some Important Phytopathogenic Fungi.** *N Y Sci J* 2018;11(7):99-107]. ISSN 1554-0200 (print); ISSN 2375-723X (online). <http://www.sciencepub.net/newyork>. 13. doi:[10.7537/marsnys110718.13](https://doi.org/10.7537/marsnys110718.13).

Keywords: *Trans*-methyl cinnamate – microemulsion formulation – ME – antifungal activity – phase inversion esterification method.

1. Introduction

Aromatic plants and essential oils (EOs) have been used since ancient times and are still widely used for the irbiological properties ^(1,2) and their applications in various industries; food, cosmetics, perfumery and pharmacy ⁽³⁾. Nowadays, EOs are attracting substantial interest from scientists because of their use in the treatment of certain infectious diseases for which synthetic antibiotics are becoming less and less active, or for preserving food against oxidation as alternatives to synthetic chemicals.

Cinnamates found in nature as secondary metabolites widely distributed in the plant kingdom. They synthesized by plants and actually represented components of essential oils. Numerous studies have demonstrated that essential oils with methyl cinnamate as one of the main components, exhibit different kind of biological activity, such as antibacterial, antifungal, antithrombotic, anti-inflammatory and antioxidative activity ⁽⁴⁻⁸⁾. According to *in vivo* observations, methyl cinnamate is not irritating to skin and mucous membrane ⁽⁹⁾. Cinnamates have wide use in different areas of industry, especially in food industry.

Besides naturally occurring, the cinnamates can be synthesized using different methods: esterification of corresponding cinnamic acids with alcohols ⁽¹⁰⁾, Wittig reaction ⁽¹¹⁾, Reformatsky reaction ⁽¹²⁾, Heck reaction ⁽¹³⁾, as well as other methods ⁽¹⁴⁾. Due to the characteristic flavor and fragrance, as well as high boiling point and stability, synthetic methyl, ethyl- and butyl cinnamate are widely used in food industry, especially for beverages and baked goods. Council of Europe ⁽⁹⁾ included methyl cinnamate in the group of substances safe for use in foodstuffs. Application of cinnamic acid esters in cosmetic and pharmaceutical industry is also significant.

Methyl cinnamate (MC) is a phenylpropanoid derivative that is abundant in the essential oil of several plants, such as *Ocimummicranthum* Willd., ⁽¹⁵⁾ *Alpina malaccensis* var. *Nobilis*, ⁽¹⁶⁾ *Alpiniazerumbet* ⁽¹⁷⁾ and *Kaempferia galanga* L. (Zingiberaceae) ⁽¹⁸⁾. In addition, a pleasant and strongly aromatic constituent of fruits (e.g. strawberries) and culinary spices (e.g. basil) is used in the flavorindustry. Because of its *in vitro* antifungal activity, ⁽¹⁹⁾ MC vapors released from

the edible film of strawberry puree extend the shelf life of strawberries⁽²⁰⁾.

Vegetables are important components of the human diet since they provide essential nutrients that are required for most of the reactions occurring in the body. Like other crops, vegetables attacked by pests and diseases during production and storage leading to damages that reduce the quality and the yield⁽²¹⁾. In order to reduce the loss and maintain the quality of vegetables harvest, pesticides used together with other pest management techniques to the presence of pesticide residues in vegetables after harvest⁽²³⁾.

Pesticides in developing countries in Asia and Pacific region are mainly available as dust, wet table powder, emulsifiable concentrates, solutions, etc. for vegetable pest management. These types of formulations are regarded now as in conventional, "old technology", "classical", or "traditional" because of their increased dose rate or repeated applications to get desired bio efficacy. These higher doses and repeated applications lead to accumulate pesticide residues in vegetable commodities along with environmental pollution and increase the resistance of insects towards the pesticides⁽²⁴⁾.

Microemulsions are very attractive alternatives to O/W emulsions for agrochemical formulations^(25, 26). The solutions can be prepared by simply mixing the components without resource to high energy and expensive equipment. The formulations do not separate in storage, and easily pour and disperse on dilution. Another important evidence of microemulsion formulations is their synergistic effect on the biological efficacy of pesticides by solubilizing the active ingredient (the chemical in pesticide known as the active ingredient in the terminology) in the microemulsion droplets.

There is a growing interest in replacing petroleum-based ingredients with natural materials, such as long and medium chain triglycerides and alkyl esters because of their many advantages⁽²⁷⁻³⁰⁾. Natural oils and their derivatives are renewable, biodegradable, harmless to the environment, and less of an irritant to the users^(31, 32).

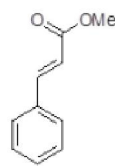
According to the consensus – building, in the present study we prepare microemulsion formulation (ME 10%) of *trans*-methyl cinnamate and determine its physical properties while the fungicidal potency were evaluated for both active substance and the local prepared formulation.

2. Materials and Methods:

I. Materials:

1. Chemical Compound:

Trans-methyl cinnamate 99%; (M. formula C₁₀H₁₀O₂), MP. 34 – 37 °C purchased from Alfa Aesar, Germany.



Trans-methyl cinnamate

2. Tested Fungi:

Five fungal isolates *Scortium rolfsii*, *Rhizoctonia solani*, *Alternaria solani* and *Fusarium solani* obtained from plant protection department, Faculty of Agriculture, Al-Azhar University.

II. Methods:

1. Preparation of the Microemulsion Base and Microemulsion Systems (Phase Inversion Method):

The method carried out at room temperature. 10 grams *Trans*-methyl cinnamate 99% dissolved in 15% wt. of solvents so 100 and 22.5% wt. Surfactants (Tween 20 and Rhodical BR/60) poured together in a beaker. The surfactant concentration optimized in order to obtain long-term stability. Water then added drop by drop until a concentration of 50 - 95% wt. reached. The vessel was agitated with a magnetic stirrer at 250 rpm that is the minimum speed required to ensure emulsion stability (low energy mixing)⁽³³⁾.

2. Physical Parameters:

a. Mean droplet size and polydispersity index:

The mean droplet size and polydispersity index (PDI) of *trans*-methyl cinnamate 10% ME formulation was performed by a dynamic light scattering method using Zetasizer Nano ZS (Malvern Instruments, UK) at room temperature. Formulated emulsion diluted with deionized water using Millipore Corporation (milli-Q) to avoid multiple scattering effects⁽³⁴⁾.

b. Physical Properties of Formulation:

The physical properties of *trans*-methyl cinnamate 10% ME was carried out at initial, after storage at 0 °C for 7 days and after storage at 54 ± 2 ° for 14 days to detect storage stability at elevated temperature⁽³⁵⁾. In addition, the physical properties of the formulation done as follow:

c. Physical Properties of Spray Solutions:

The physical properties of 5% spray solutions of prepared *Trans*-methyl cinnamate 10% ME in both soft and hard water; persistent foam, emulsion stability and re-emulsification, conductivity⁽³⁵⁾, pH⁽³⁶⁾ and surface tension⁽³⁸⁾ recorded.

3. Antifungal Assay:

The *trans*-methyl cinnamate prepared in dimethyl sulfoxide (DMSO) and tested for mycelial growth inhibition activity against four fungal isolates *Scortium rolfsii*, *Rhizoctonia solani*, *Fusarium solani* and *Alternaria solani* using the food poison technique

in Potato dextrose agar medium ⁽⁴²⁾. To test the antifungal activities of the selected compounds, sterile Petri dishes containing the compound dissolved in DMSO/Tween 80 emulsifier (80/20 v/v) diluted in PDA medium prepared. The prepared 10% microemulsion formulation of *trans*-methyl cinnamate applied directly to the PDA media. Tween 80 alone as

a control (0.025% by volume) added to PDA medium as an emulsifier control. Plates containing media mixed with DMSO (0.1% by volume) included as a solvent control. Also, DMSO and Tween 80 (4/1 v/v) added to PDA medium as a solvent/emulsifier control. Finally, PDA plates treated with distilled water served as a negative control.

Test	Instrument	Test Method
Acidity /or alkalinity	Automatic Titrator, (Hanna model HI 901)	CIPAC (MT 191) ⁽³⁶⁾
pH Measurement	pH Meter (Jenway model pH 3510)	CIPAC (MT 75.3) ⁽³⁶⁾
Viscosity	Brookfield Viscometer DV-II + Pro, USA	ASTM (D 2196-15) ⁽³⁷⁾
Surface Tension	“Sigma 700” by du Noüy Ring, a platinum/iridium ring	ASTM (D1331 – 14) ⁽³⁸⁾
Refractive Index	ABBE Refractometer, ATAGO	ASTM (D 1218-02) ⁽³⁹⁾
Density and Specific Gravity	Rodulph Densitometer (DDM 2910, USA)	ASTM (D 4052 – 11) ⁽⁴⁰⁾
Flash point	Koehler instrument (tag closed-cup)	ASTM (D 3828 – 12a) ⁽⁴¹⁾

Agar disks (5 mm in diameter) of the tested fungi cut from completely grown cultures and placed at the center of the plates containing antifungal substances of the used concentration in ppm ($\mu\text{g/ml}$).

Four replicates of each concentration 25, 50, 100, 200, 300 and 400 $\mu\text{g/ml}$ for fungus incubated at 28°C for all tested fungi. Radial growth measured from the centers of the dishes sides after the fungal growth of the control of each fungus completed and the mean calculated of two perpendicular colony diameters in each replicate. Inhibition of growth calculated in relation to the growth in the control, according to the equation ⁽⁴³⁾:

$$\% \text{ of inhibition} = 1 - \left(\frac{\text{Diameter of treated colony}}{\text{Diameter of control colony}} \right) \times 100$$

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$$\text{Toxicity Index} = \frac{\text{LC}_{50} \text{ of the most effective compound}}{\text{LC}_{50} \text{ of the other compound}} \times 100 = \dots\%$$

$$\text{Relative Potency} = \frac{\text{LC}_{50} \text{ of the least effective compound}}{\text{LC}_{50} \text{ of the other compound}} = \dots \text{Fold}$$

3. Results and Discussion

1. Formulation characteristics:

Table (1): Mean droplet size and polydispersity index of *trans*-methyl cinnamate 10% microemulsion

	Size (d. nm)	% Number	Width (d.nm)	PdI
Peak 1	55.21	100	19.68	0.296

Micro-emulsion is clear, thermodynamically stable, isotropic liquid mixture. It is prepared by using oil, water, surfactant and a co-surfactant. It incorporates very small size particles up to nano size as compared to conventional emulsion. IUPAC defines micro-emulsion as dispersion made of water, oil, and surfactant (s) that is an isotropic and thermodynamically stable system with dispersed domain diameter varying approximately from 1 to 100 nm ⁽⁴⁶⁻⁴⁸⁾. The polydispersity index (PDI) is an important characteristic of the microemulsion. PDI is a measure of homogeneity and stability of the droplet size in the microemulsion system. PDI of the particle

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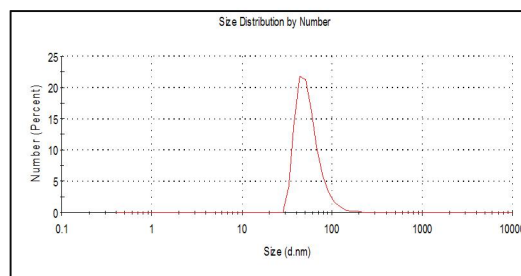


Fig. 1. Mean droplet size of *trans*-methyl cinnamate microemulsion

a. Physical Properties of Formulation:

The most important parts of chemical stability are performances on accelerated testing and kinetics of pH profiles⁽⁴⁹⁾. The formulation exhibited acidic pH value. The pH values of the prepared formulation were in range (4.24 -4.36), and the free acidity values were in range of (0.1233 – 0.1764 % as H₂SO₄) indicating that the formulation having acidic character implying that it will have good biological activity⁽⁵⁰⁾. The prepared formulation having the surface tension range (27.025-27.289 mN/m). Lower surface tension is a desirable characteristic for most agricultural sprays because it facilitates the spreading of droplets upon impaction on leaves or other target surfaces, to

increase the surface-active area and improves penetration and uptake of the product into the plant⁽⁵¹⁾. The prepared formulation in the all storage condition having high value of flash point more than 60°C and this is quite safe. The refractive indices of the prepared formulation were ranged (1.4253 – 1.4267), the densities were ranged from 0.9877 to 0.9902 and specific gravities range were 0.9907 – 0.9932 that showed no valuable change according to different storage conditions. Viscosities were in range (18.75 – 19.71 cP.). Increasing viscosity of spray solution caused reduction drift and an increase in retention sticking and pesticidal efficiency⁽⁵²⁾.

Table (2): Physical Properties of *trans*-methyl cinnamate 10% ME at initial, after storage at 0 °C for 7 days and after accelerated hot storage at 54 ± 2 °C for 14 days

Test	Initial	After storage at 0 °C for 7 days	After accelerated hot storage at 54 ± 2 °C for 14 days
pH	4.24 ± 0.03	4.42 ± 0.02	4.36 ± 0.1
Acidity (% as H ₂ SO ₄)	0.1233 ± 0.0032	0.1666 ± 0.0012	0.1764 ± 0.0011
Surface Tension (mN/m)	27.025 ± 0.004	27.289 ± 0.014	27.006 ± 0.090
Density (gm/cm ³)	0.9877± 0.0000	0.9886 ± 0.0000	0.9902 ± 0.0000
Specific Gravity	0.9907 ± 0.0000	0.9916 ± 0.0000	0.9932 ± 0.0000
Flash Point (°C)	Over 60	Over 60	Over 60
Refractive Index	1.4253 ± 0.0001	1.4254 ± 0.0001	1.4267 ± 0.0001
Viscosity (cP.)	18.75 ± 0.023	19.71 ± 0.069	19.18 ± 0.1

* Results ± SD

b. Physical Properties of Spray Solutions:

Table (3) showed the physico-chemical properties of spray solutions of the prepared 10% ME formulation in both soft and hard water at different storage conditions. The prepared formulation passed successfully emulsion stability test also there is no oil separation, precipitation or creamy layer. In addition, there were no valuable changes in the physico-chemical properties of the prepared local microemulsion formulation before and after heat and cold storage test.

The conductivity of spray solution tests, showed the highest value 705 µMHOS in hard water after hot storage. The highest values of surface tension and pH were 29.18mN/m and 4.24 in soft water of cold stored

sample, respectively. Increased electric conductivity of the spray solution coupled with increased mortality rate due to increased deposition and penetration of the formulated extracted particles⁽⁵³⁾. It also shown that the decrease in surface tension of pesticidal spray solution gives a prediction of increasing wettability and spreading over tested surface with increasing pesticidal efficiency⁽⁵⁴⁾. The decrease pH value of spray solution would lead to the deionization of the content which increase in its deposit's and penetration in the tested surface with a consequence increase in their pesticidal efficiency⁽⁵⁵⁾.

It is clear that cold and hot storage has no valuable effect on all tested physical properties of the prepared formulation.

Table (3): Physical Properties spray solutions (soft and hard water) of *trans*-methyl cinnamate 10% ME at initial, after storage at 0 °C for 7 days and after accelerated hot storage at 54 ± 2 °C for 14 days

Method	Storage Conditions		After storage at 0 °C for 7 days		After accelerated hot storage at 54 ± 2 °C for 14 days	
	Soft Water	Hard Water	Soft Water	Hard Water	Soft Water	Hard Water
Foaming (ml)	2	2	3	2	-	2
Emulsification	Pass	Pass	Pass	Pass	Pass	Pass
pH	4.18	4.02	4.24	4.13	4.14	3.95
Surface Tension (mN/m)	27.34	28.82	29.18	29.14	28.23	28.23
Conductivity (µMHOS)	148.3	694	145.8	691	205	705

2. Antifungal Assay:

Cinnamic acid esters and their derivatives are widely distributed in plants including cereals, legumes, oilseeds, fruits, vegetables and tea or coffee beverages⁽⁵⁶⁾. Due to the common occurrence in plants and the low toxicity for humans, animals and environment^(57, 58), cinnamic acid derivatives have attracted much attention of many pharmacologists. In the past decades, cinnamic acid derivatives including natural and non-natural compounds had proved to possess diverse pharmacological actions such as antimicrobial^(59, 60). Although cinnamic acid itself and its derivative cinnamaldehyde had found to have inhibition activity against some plant pathogenic fungi^(61, 62), few reports have found on systematic investigation on the activity of cinnamic acid esters against plant pathogenic fungi. The purpose of the present research is to explore the bioactivity of a *trans*-methyl cinnamate against phyto-pathogenic fungi, and meanwhile discover new potent antifungal compounds.

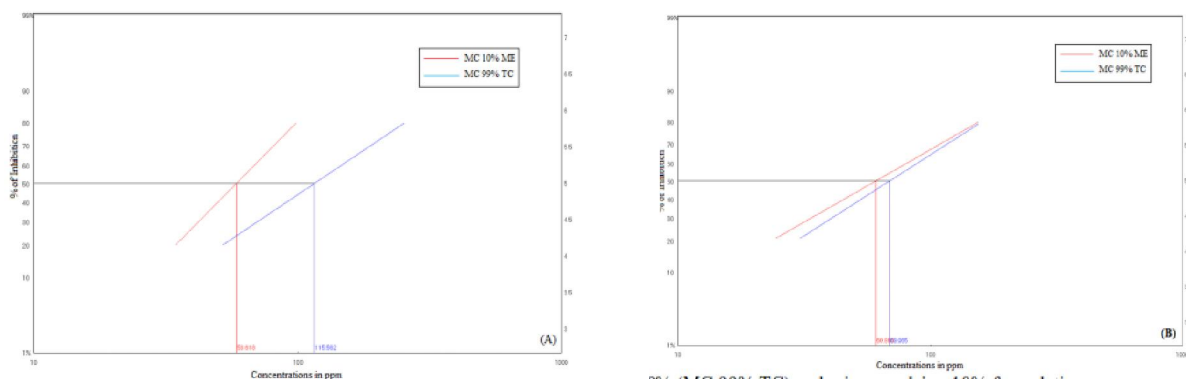
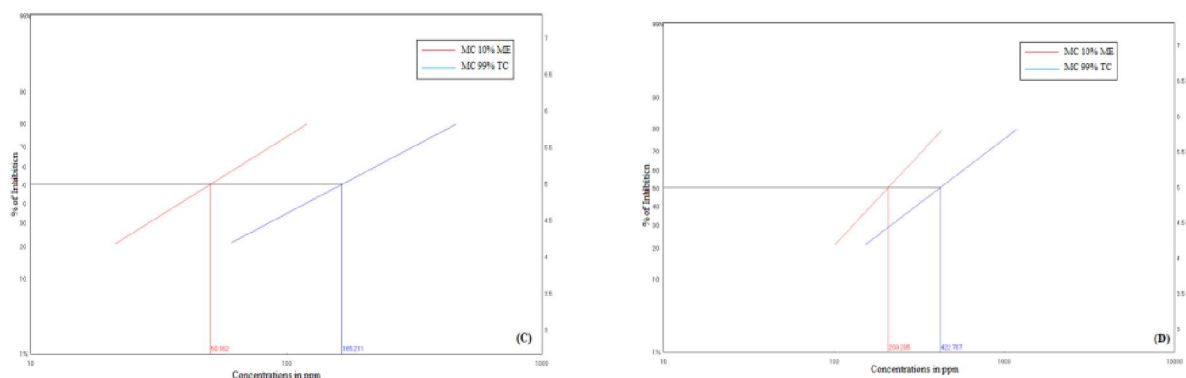
The *in vitro* antifungal activity of *trans*-methyl cinnamate 99% and its prepared 10% microemulsion formulation by food poison techniques reported in Table 4. The results indicated that as the concentration of the methyl cinnamate increases the percentage inhibition of mycelial growth increases suggesting that it inhibits the growth of all tested phytopathogenic fungi in a dose dependent manner. The prepared formulation demonstrated strong mycelial growth inhibition in all tested phytopathogenic fungi; the

prepared formulation more effective than the corresponding active material regarding the value of LC₅₀ in the case of all examined fungi. The lower the value of LC₅₀ is the higher the efficacy of the tested material in the test under consideration. The LC₅₀ values of *trans*-methyl cinnamate on the tested phytopathogenic fungi *S. rolfsii*, *R. solani*, *A. solani* and *F. solani* were 115.56, 69.07, 165.21 and 422.77, respectively. Whereas, the LC₅₀ values of prepared *trans*-methyl cinnamate 10% microemulsion formulation on the tested phytopathogenic fungi were 58.62, 60.86, 50.56 and 209.30 ppm, respectively.

The toxicity indices were 50.72, 88.12, 30.6 and 49.51% for *trans*-methyl cinnamate 99% against *S. rolfsii*, *R. solani*, *A. solani* and *F. solani*, respectively when compared with for *trans*-methyl cinnamate 10% microemulsion, which had the 100% toxicity index. While the relative potencies of *trans*-methyl cinnamate 10% microemulsion on *S. rolfsii*, *R. solani*, *A. solani* and *F. solani*, were 1.97, 1.14, 3.27 and 1.58 folds, respectively than *trans*-methyl cinnamate 99%. The Ldp-lines of the *trans*-methyl cinnamate 99% and *trans*-methyl cinnamate 10% microemulsion against *S. rolfsii*, *R. solani*, *A. solani* and *F. solani*, plotted in figure 2 and 3, respectively. The slope of each line calculated separately. The highest slope values were that of *trans*-methyl cinnamate 10% microemulsion. They were 3.68, 2.29, 2.19 and 2.57 for the prepared microemulsion formulation while they were 2.44, 2.05, 1.85 and 1.84 for the pure compound against *S. rolfsii*, *R. solani*, *A. solani* and *F. solani*, respectively.

Table (4): Antifungal activities of the *trans*-methyl cinnamate 99% (T) and microemulsion 10% formulation (F) on *Sclerotium rolfisii*, *Rhizoctonia solani*, *Alternaria solani* and *Fusarium solani*

		Concentrations in ppm ($\mu\text{g/ml}$)						Slope	LC ₅₀	LC ₉₀	Toxicity Index	Relative Potency
		25	50	100	200	300	400					
<i>Sclerotium rolfisii</i>	T	5.28	18.77	43.92	71.91	84.35	90.54	2.44	115.56	388.07	50.72	1
	F	8.69	39.98	80.31	97.46	100	100	3.68	58.62	130.79	100	1.97
<i>Rhizoctonia solani</i>	T	15.62	37.41	64.35	85.47	92.78	95.96	2.05	69.07	136.12	88.12	1
	F	21.42	43.06	67.08	85.51	92.21	100	2.29	60.86	129.84	100	1.14
<i>Alternaria solani</i>	T	6.47	16.84	34.33	56.10	68.42	76.14	1.85	165.21	813.57	30.6	1
	F	25.18	49.58	74.14	90.41	100	100	2.19	50.56	194.95	100	3.27
<i>Fusarium solani</i>	T	1.27	4.47	12.54	27.55	39.23	48.25	1.84	422.77	2112.02	49.51	1
	F	0.9	5.50	20.46	47.97	65.63	76.54	2.57	209.30	658.89	100	1.58

**Fig. (2):** Ldp lines of the selected *trans*-methyl cinnamate 99% (MC 99% TC) and microemulsion 10% formulation (MC 10% ME) on (A) *Sclerotium rolfisii*, and (B) *Rhizoctonia solani***Fig. (3):** Ldp lines of the selected *trans*-methyl cinnamate 99% (MC 99% TC) and microemulsion 10% formulation (MC 10% ME) on (C) *Alternaria solani* and (D) *Fusarium solani*

The presence of high levels of methyl (E)-cinnamate in the bark and twig oils of *C. pubescens* (95.2% and 84.0%, respectively) and *C. impressicostatum* (85.9% and 67.6%, respectively) could explain their strong inhibition on a wide spectrum of fungal growth⁽⁶³⁾.

In general, the formulation in form of microemulsion (10% ME) increased the fungicidal efficiency of the active substance on all the fungi used. That comes from the aim that, microemulsion formulation is one of the most new generation types of

formulations which characterized by the nature of water that helps to penetrate inside fungus cells.

Conclusion

In this study, synthetic *trans*-methyl cinnamate and its prepared 10% microemulsion formulation, tested for their *in vitro* antifungal activity. This synthetic cinnamates could be potential antifungal agents against some important phytopathogenic fungi. Previous literatures found that in comparison of the activities and structures of the compounds in

substitution patterns of the benzene ring and the type of alkyl groups in the alcohol moiety significantly influence the activity of cinnamic esters. For cinnamate derivatives, introducing of substituents to the benzene ring leads to increase of the activity. For electron-donating groups like OH, OMe, and OAc, the order of the activity of various position isomers is *o*-substituted isomer > *p*-substituted isomer > *m*-substituted isomer, whereas for electron-withdrawing groups like halogen atoms and trifluoromethyl, *p*-substituted isomers generally have the highest activity⁽⁶⁴⁾.

Finally, *trans*-methyl cinnamate as eco-friendly microemulsion formulation might be used for controlling phytopathogenic fungi at the recommended dose.

Acknowledgments

I wish to express our thanks to Prof. Yasser Wahied Abdel Fattah El Sheikh and Dr. Mohamed Helal Ahmed Aly at Pesticides Formulation Research Department, Central Agricultural Pesticides Laboratory, Agricultural Research Center for their help.

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7/25/2018