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Development of Essential Oils Based New EC Formulation for the Management of Post Harvest Anthracnose of Pomegranate Caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.

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New EC formulations (10, 20, 30 and 40 EC) were developed using combination of lemongrass oil, cinnamon oil and thyme oil for the management of post harvest anthracnose of pomegranate caused by *Colletotrichum gloeosporioides* and tested at 0.1% concentration *in vitro*. Among them, essential oils based new 30 EC formulation, completely (100%) inhibited the pathogen growth at 0.1% concentration. The formulation was named as THYCILEM 30 EC and tested for its physical stability. The formulation was stable in all physical stability tests and was not inhibitory to antagonistic micro organisms. The *in vivo* results revealed that pre harvest spraying with new THYCILEM 30 EC (0.1%) followed by post harvest dipping with new THYCILEM 30 EC (0.1%) for 5 min was more effective in reducing pomegranate anthracnose which had the minimum PDI of 13.43 after 24 days of treatment under post harvest conditions, compared to 87.50 PDI in untreated control. The application of new THYCILEM 30 EC as pre harvest spray and post harvest dipping did not show any drying, scorching of leaves and browning of fruits which was noticed by visual observation.

Key words: Essential oil, THYCILEM 30 EC, Pomegranate, *Colletotrichum gloeosporioides*

Pomegranate (*Punica granatum* L.) known as "Fruit of paradise" and "Vital cash crop" is cultivated in an area of 510 ha with an annual production of 13.96 thousand MT with an average productivity of 27.43 MT.ha^{-1} in Tamil Nadu (Indiastat, 2017). Pomegranate cultivation is severely affected by the anthracnose disease which cause 30-40% losses to pomegranate fruits and renders the fruits unfit for consumption and marketting (Jagtap *et al*., 2015). As pomegranate is consumed directly, and development of awareness on organic farming was aimed towards the production of residue free fruits, botanical pesticides are considered as an alternate method to synthetic pesticides (Orlikowski and Skrzypczak, 2001). Though essential oils are effective in controlling the plant pathogens, an additional important problem in practical application of essential oils in agriculture is a lack of persistent efficacy in the field (Chen *et al.,* 2013). This problem could also be resolved by improved formulation. The EC formulated products are more effective than crude essential oils for its easy applicability and requirement in small quantity (Vanitha, 2010). Hence, attempts were made to prepare EC formulation using combination of essential oils and test their physical stability and antifungal activity *in vitro*. The efficacy of the EC formulated product was also tested under *in vivo* conditions.

Material and Methods

Fungal culture

Pomegranate variety Bhagwa was used for the isolation of post harvest anthracnose pathogen, *C. gloeosporioides* from Kavundanpalayam whole sale market, Coimbatore, Tamil Nadu. Phenotypic confirmation *viz.,* colour and zonation was studied as described by (Prashantha *et al*., 2013). Molecular confirmation was done by comparing the partially sequenced data sequence with the available resources using BLAST and the sequence was submitted to NCBI for acquiring accession number.

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Development of new EC formulations

The crude essential oils such as lemongrass oil, cinnamon oil and thyme oil with 100% inhibition of *C. gloeosporioides* under *in vitro* were combined and developed as various EC formulations *viz.,* 10, 20, 30 and 40 EC. The EC formulations were developed by mixing recommended quantities of essential oils, emulsifying agent, stabilizing agent and solvent (Vanitha, 2010).

In vitro efficacy of new EC formulations against C. gloeosporioides

Different EC formulations *viz.,* 10, 20, 30 and 40 EC at 0.1% concentration were screened under laboratory conditions by mixing 0.1 mL of each EC formulation with 100 mL of sterilized PDA medium separately. 20 ml of each of the poisoned medium was poured into sterilized petri plates separately. Each petri dish was seeded with an actively growing culture disc (8 mm diameter) of pathogen by placing at the centre of each petri dish and PDA plates without formulation served as control. The experiment was carried out by following Completely Randomized Design (CRD) with four replications. The plates were incubated at room temperature (28±2^{o}C) for ten days

and the radial mycelial growth (mm) of the pathogen was recorded. The per cent inhibition of the mycelial growth was calculated as per the formula mentioned below (Pandey *et al.*, 1981).

$$\text{Per cent inhibition over control} = \frac{C - T}{C} \times 100$$

where, C- Mycelial growth of pathogen in control

T- Mycelial growth of pathogen in treated plates

Evaluation of physical stability of new 30 EC formulation

Stability tests *viz.,* spontaneity test, test for stability of emulsion, heat stability test, cold stability test, centrifugation test, mechanical vibration test, light test and microbial stability test were performed to determine the physical stability of the formulation (Abo-El Seoud *et al.*, 2005; Bouranen, 2017; Vanitha, 2010).

In spontaneity test, a test tube containing 20 mL of water (Hard water and Soft water separately) was taken and the formulation was added drop by drop slowly into it to determine the spontaneity while mixing. In the test for stability of emulsion, 2 mL of the formulation was added to 100 mL of hard water and stirred continuously. The emulsion was kept undisturbed for 1 h by transferring it into a 100 mL measuring cylinder. The volume of the cream at the top and sediments at the bottom were noticed. Indian Standard Institute (ISI) criteria for the stability is that the creamy matter at the top and sediments at the bottom should not exceed 2 mL. In the heat and cold stability tests, 5 mL of the formulation was taken in a 50 mL test tube containing 10 mL of soft and hard water separately and it was placed in the water bath and refrigerator maintained at 95ºC and 4ºC for 1 h, respectively.

To perform the centrifugation test, 10 mL of the formulation was subjected to a cycle of 3000 rpm for 30 min at room temperature. Later, the formulation was examined for phase separation to assess the formulation instability. The formulation was evaluated by submitting it into mechanical vibration movement, which may cause instability detected as phase separation. Briefly, 10 mL of the formulation was subjected to vibration on a vortex shaker for 10 sec. In the Light test, the formulation was placed in transparent glass containers and subsequently exposed to extreme light source for 15 days, using a daylight bulb with photoperiodicity system (16 h light and 8 h dark). At the end of the exposure period, the formulation was examined for any changes in physical properties, such as appearance, clarity or colour and liquefaction. Any phase separation or change in colour observed was considered as indicator of product instability. The microbial stability of the formulation was evaluated through the microbial contamination test. 0.1 mL of the formulation was mixed with 100 mL of PDA and NA medium separately to give 0.1 % concentration to test fungal and bacterial contamination in the formulation, respectively. The plates were checked for microbial growth after inhibition which is an indication of contamination. The inhibitory activity of the formulation on beneficial micro organisms was evaluated. The biocontrol agents *viz., Trichoderma asperellum* (KX533985), *Bacillus amyloliquefaciens* (MH470473) and *Pseudomonas fluorescens* (BE0005) were received from Department of Plant Pathology, TNAU, Coimbatore. PDA and NA medium were mixed with the EC formulation proportionately and the antagonistic micro organisms were inoculated on the respective medium. Inhibitory activity of EC formulation on antagonistic micro organisms was observed.

Evaluation of pre harvest spray and post harvest dipping with new 30 EC formulation against anthracnose of pomegranate

Field trial was conducted in the farmer field at Theethipalayam, Coimbatore district, Tamil Nadu (Latitude: 10º 57' N and Longitude: 76º 53' E) during February 2018 to April 2018 in pomegranate variety Bhagwa to test the efficacy of the essential oil based new 30 EC formulation under field conditions against pomegranate anthracnose. Foliar application of the formulation was initiated after fruit set at ten days interval for three times. The fruits were collected from treated trees and dipped in same concentration as post harvest dip for 5 min. The spore suspension of *C. gloeosporioides* (6.0×10^6 conidia /ml) was artificially inoculated on the treated fruits by pin prick method and the fruits were incubated under room temperature (28±2°C). Five replications was maintained for each treatment with each replication having five fruits. Post harvest fungicide Thiophanate methyl (0.1%) was used as pre harvest spray and post harvest dip for comparision. The observation was calculated as PDI after 24 days of treatment at 4 days interval under room temperature storage (28±2°C). The disease incidence was estimated by using the score chart given below and per cent disease index was worked out for each treatment, using the formula (Rose 1974).

$$\text{Percent Disease Index (PDI)} = \frac{\text{Sum of individual ratings}}{\text{Total number of leaves observed}} \times \frac{100}{\text{Maximum grade}}$$

(No infection - 0 grade; 25% fruit surface infected - 1; 26 - 50% fruit surface infected - 2; 51- 75% fruit surface infected - 3; More than 75% fruit surface infected - 4).

Statistical analysis

The data obtained were statistically analyzed (Gomez and Gomez, 1984) and the treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was IRRISTAT version 92 developed by the IRRI, Biometrics Unit, The Philippines.

Results and Discussion

Fungal culture

Phenotypic characterization of *C. gloeosporioides* revealed that the colony colour was blackish grey with grey pigmentation. Molecular confirmation was done by

comparing the partially sequenced data sequence with the available resources using BLAST which showed 99% homology and the sequence was submitted to NCBI and the assigned accession number was MH480385.

Table 1. *In vitro* efficacy of different new EC formulations against *C. gloeosporioides*

Different EC formulations	Growth (mm)	Per cent inhibition over control
10 EC (0.1%)	27.00[c] (5.24)	70.00
20 EC (0.1%)	25.00[b] (5.05)	72.22
30 EC (0.1%)	0.00[a] (0.71)	100.00
40 EC (0.1%)	0.00[a] (0.71)	100.00
Control	90.00[d] (9.51)	0.00

*Mean of four replications.

*Figures in parentheses are square root transformed values.

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

In vitro efficacy of new EC formulations against C. gloeosporioides

Among the four different EC formulations of essential oils *viz.,* 10, 20, 30 and 40 EC, the formulation of 30 and 40 EC at 0.1% concentration completely inhibited the mycelial growth (100%) of *C. gloeosporioides*. The EC formulations of 10 and 20 EC at 0.1% concentration were also effective and recorded 70.00 and 72.22% inhibition of the pathogen, respectively (Table 1, Fig. 1). Hence, the effective 30 EC formulation was used for further studies, packed in autoclavable, white, HDPE container 500 mL capacity with inner cap and outer screw cap with 12 mm gauge thickness. The formulation was named as THYCILEM 30 EC (Fig. 2).

Evaluation of physical stability of new THYCILEM 30 EC

In the spontaneity test, the new THYCILEM 30 EC formulation of essential oils combination (lemongrass oil, cinnamon oil and thyme oil) immediately formed milky white emulsion when mixed with both hard and soft water. In the test for stability of emulsion, the volume of cream at the top and sediments at the bottom did not exceed 2 mL in the new THYCILEM 30 EC formulation. In the heat and cold stability test, the new THYCILEM 30 EC formulation did not show turbidity, solid or oil matter when kept in waterbath at 95ºC and refrigerator at 4ºC, respectively. At the end of centrifugation test, the new THYCILEM 30 EC formulation did not show any phase separation which indicated that the formulation was stable. The new THYCILEM 30 EC formulation was stable when subjected to vibratory movement because it did not show any phase separation. The new THYCILEM 30 EC formulation remained unaltered in its physical properties by the end of light exposure period. PDA and NA medium amended with the new THYCILEM 30 EC formulation did not show any fungal and bacterial contamination, respectively throughout the incubation time which indicated that the formulation was free from contamination. The new THYCILEM 30 EC was not inhibitory to the fungal (*Trichoderma asperellum*) and bacterial (*Pseudomonas fluorescens* and *Bacillus amyloliquefaciens*) antagonists at 0.1% concentration.

Table 2. Evaluation of pre harvest spray and post harvest dipping with new THYCILEM 30 EC against anthracnose of pomegranate

Treatments	PDI					
	Days after treatment					
	4	8	12	16	20	24
T1 - Pre harvest spray with THYCILEM 30 EC (0.1%)	1.33c (6.62)	4.23c (11.87)	8.50d (16.95)	11.33c (19.67)	14.63d (22.49)	18.50c (25.48)
T2 - Pre harvest spray with Thiophanate methyl (0.1%)	0.96b (5.62)	3.50b (10.78)	6.33c (14.57)	10.97c (19.34)	13.50c (21.56)	16.33b (23.83)
T3 - Pre harvest spray and Post harvest dipping with THYCILEM 30 EC (0.1%)	0.00a (1.28)	0.00a (1.28)	0.00a (1.28)	3.50a (10.78)	8.77a (17.22)	13.43a (20.82)
T4 - Pre harvest spray and Postharvest dipping with Thiophanate methyl (0.1%)	0.00a (1.28)	0.00a (1.28)	2.26b (8.65)	5.21b (13.19)	9.19b (17.64)	12.63a (18.47)
T5 - Control	5.00d (12.93)	20.00d (26.56)	48.33e (44.04)	58.90d (50.13)	76.67e (61.12)	87.50d (69.34)

*Mean of five replications.
Figures in parentheses are arc sine transformed values.

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Evaluation of pre harvest spray and post harvest dip with new THYCILEM 30 EC against anthracnose of pomegranate

To assess the bioefficacy of THYCILEM 30 EC, a field experiment followed by post harvest storage trials were carried out against pomegranate anthracnose. The percent disease index varied from 12.63 to 18.50 after 24 days of treatment with THYCILEM 30 EC and Thiophanate methyl. But the PDI in untreated control was 87.50. Among the treatments, pre harvest spraying with new THYCILEM 30 EC (0.1%) followed by post harvest dipping with new THYCILEM 30 EC (0.1%) was more effective in reducing pomegranate anthracnose which had a

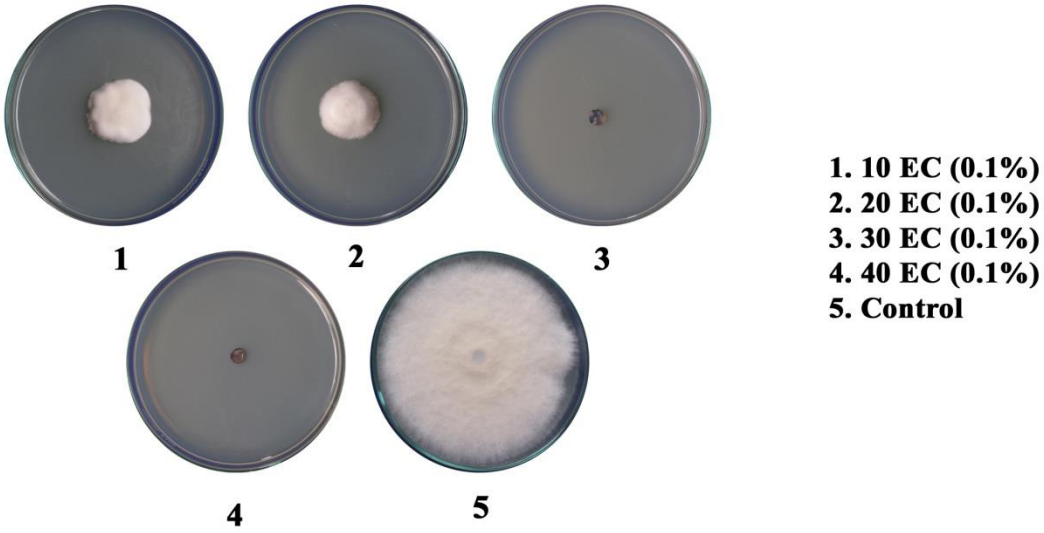


Fig.1. *In vitro* efficacy of different EC formulations against *C. glocosporioides*

minimum incidence of 13.43 PDI after 24 days of treatment. It was equally effective as that of fungicide Thiophanate methyl (0.1%) (pre harvest spray + post harvest dip) treated which had PDI 12.63. The next best treatments were pre harvest spray with Thiophanate methyl (0.1%) and pre harvest spray with new THYCILEM 30 EC (0.1%) which recorded PDI of 16.83 and 18.50, respectively. The highest PDI of 87.50 was recorded in control treatment (Table 2, Fig. 3).

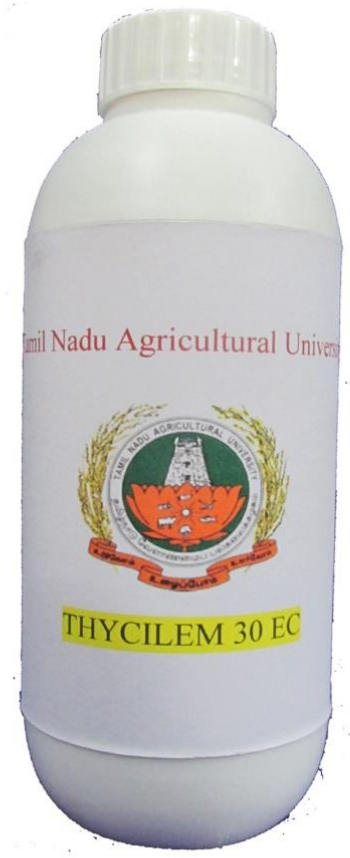
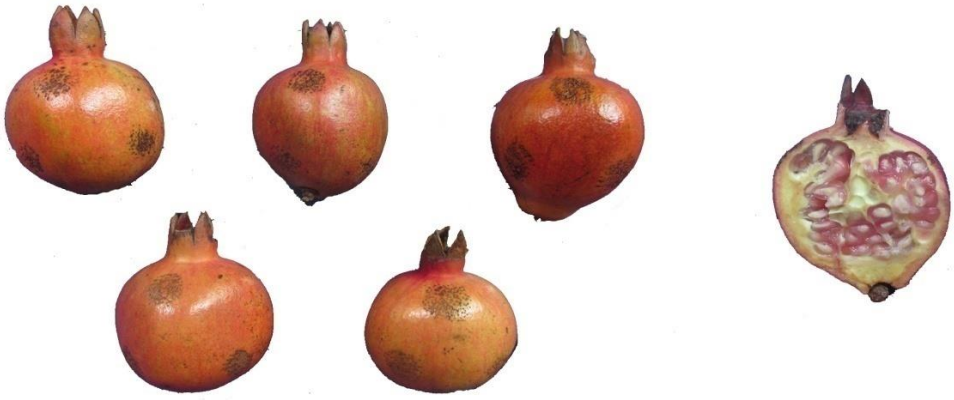


Fig.2. TNAU Essential oil formulation - New THYCILEM 30 EC against post harvest anthracnose of pomegranate

The EC formulated essential oil sprayed on pomegranate plants and pomegranate fruits dipped with the formulated product did not show any adverse effect *viz.,* drying, scorching of leaves and browning of fruits which was recorded by visual observation. Under field conditions, flower set was increased without any symptoms. Fruits under storage remained without symptom development upto 12 days after inoculation and after that the symptom development was very less when compared to control.

The present findings about new EC formulated essential oil are in agreement with the findings of many workers are discussed below. Neem oil and pungam oil formulations *viz.,* NO 60 EC (citric acid), NO EC (acetic acid) and NO + PO 60 EC (citric acid) were developed and evaluated against *Saracladium oryzae* (Narasimhan *et al.,* 1998) and *Helminthosporium oryzae, Pyricularia oryzae* (Rajappan *et al.,* 2001). Rajappan *et al.* (1999) developed Neem oil based EC formulations (NO 60EC, (A)) and showed its efficacy against green gram powdery mildew by spraying 30 mL/L of formulation and the disease incidence was reduced upto 21.67%. Vanitha (2010) evaluated different EC formulation (10, 20, 30 and 40 EC) of lemongrass oil, wintergreen oil and combination of both oils against leaf blight of *Solanum nigrum*. The crude plant oils of wintergreen, lemongrass oil and their combinations inhibited the pathogen *in vitro* at 0.025% of 30 and 40 EC formulations. The foliar spray of 30 EC formulation of wintergreen, lemongrass oil and their combinations (0.1%) were effective against *Alternaria chlamydospora* however, the combination treatment recorded the least PDI of 62.27 on 60 days after planting in pot culture experiment. The field experimental results showed that the 30EC

a. Pre harvest spray with new THYCILEM 30 EC (0.1%) + Post harvest dipping with new THYCILEM 30 EC (0.1%)



b. Pre harvest spray with Thiophanate methyl (0.1%) + Postharvest dipping with Thiophanate methyl (0.1%)

c. Untreated control

Fig.3. Evaluation of pre harvest spray and post harvest dipping with new THYCILEM 30 EC against anthracnose of pomegranate

formulation of combination of wintergreen and lemongrass oil (0.1%) sprayed plots significantly proved its superiority (7.10 PDI) over the control.

Conclusion

The perusal of literatures showed that there is no literature pertaining on EC formulation of essential oil combination (lemongrass oil, cinnamon oil and

thyme oil) and its efficacy on pomegranate fruits. Hence, it seems to be a new result on usage of EC formulated essential oil on pomegranate fruits under storage conditions.

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References

Abo-El Seoud, M., Sarhan, M., Omar, A., and M. Helal. 2005. Biocides formulation of essential oils having antimicrobial activity. *Archives of Phytopathology and Plant Protection,* **38(3)**: 175-184.

Bouranen, A. 2017. Determination of the stability of cosmetic formulations with incorporation of natural products. http://hdl.handle.net/10198/14416.

Chen, K.N., Chen, C.Y., Lin, Y.C., and M. J. Chen. 2013. Formulation of a Novel Antagonistic Bacterium Based Biopesticide for Fungal Disease Control Using Microencapsulation Techniques. *Journal of Agricultural Science,* **5(3)**: 153.

Gomez, K. A. and A. A. Gomez. 1984. *Statistical Procedure for Agricultural Research.* John Wiley Sons, New York.

Indiastat. 2017. Statistical Data on India. https://www.indiastat.com/

Jagtap, N. M., Ambadkar, C. V., and G. A. Bhalerao. 2015. Morphological and cultural characters of *Colletotrichum gloeosporioides* causing anthracnose of pomegranate. *Advance Research Journal of Crop Improvement,* **6(1)**: 56-58.

Narasimhan, V., Rajappan, K., Ushamalini, C., and A. A. Kareem. 1998. Efficacy of new EC formulations of neem oil and pungam oil for the management of sheath rot disease of rice. *Phytoparasitica,* **26(4)**: 301-306.

Orlikowski, L. B., and C. Skrzypczak. 2001. Biopreparations from grapefruit progress in the biological control of plant diseases. *Horticultura,* **23:** 261-269.

Pandey, D. K., Tripathi, R. N., Tripathi, N.N., and R. D. Tripathi. 1981. Antifungal activity in some seed extracts. *Environment India,* **4(I, II)**: 83.

Prashantha, A., Sataraddi, A. R., Patil, S. V., Lokesh, M.S., Chandan, K., and S.B. Gurumurthy. 2013. Effect of chemicals inducing systemic resistance and efficacy of bioagents and botanicals against pomegranate (*Punica granatum* L.) anthracnose (*Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc.). *International Journal of Plant Protection,* **6(2)**: 289-292.

Rajappan, K., Mariappan, V., and A. A. Kareem. 1999. Development and evaluation of EC and dust formulation of plant derivatives against green gram powdery mildew. *Annals of Plant Protection Sciences,* **7(1)**: 110-112.

Rajappan, K., Ushamalini, C., Subramanian, N., Narasimhan, V., and A. A. Kareem. 2001. Management of grain discoloration of rice with solvent-free EC formulations of neem and pungam oils. *Phytoparasitica,* **29(2)**: 171-174.

Rose, D.H. 1974. Diseases of apple fruits in the market. Bull US Department of Agriculture. **1253**: 24.

Vanitha, S. 2010. Developing new botanical formulation using plant oils and testing their physical stability and antifungal activity against *Alternaria chlamydospora* causing leaf blight in *Solanum nigrum*. *Research Journal of Agricultural Science,* **1(4)**: 385-390.

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