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THESIS

INTEGRATED CONTROL OF *Penicilium digitatum* (Pers.:Fr.) Sacc., A GREEN MOLD CITRUS DISEASE, USING YEASTS AND MEDICINAL PLANTS

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A Thesis Submitted in Partial Fulfillment of
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Green mold caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. is responsible for 90% of losses during post-harvest handling. Currently, control of post harvest diseases is mainly dependent on the use of chemical fungicides, that is becoming increasingly restricted because of environment and health concern.

Biological control and biofungicide are investigated and use as an alternative to chemical. Seven yeasts and seven crude extracts of medicinal plants were tested against *P. digitatum*. *In vitro* and *in vivo* test was done for screening of yeasts and plant crude extracts.

A combination of *Candida utilis* TISTR 5001 and *Eugenia caryophyllata* crude extract was the best combination to attain a reduction to the disease incidence and the disease severity. Combination of *E. caryophylata* crude extract and *C. utilis* TISTR 5001 significantly reduced the natural development of green mold of citrus fruits. The combination had no effect to fruits quality. Based on HPLC and NMR (^{1}H and ^{13}C) confirmed that the active compound of *E. caryophylata* was eugenol. Hence, the results indicate a combination of the plant extracts and yeasts posses antifungal activity that can be exploited as an ideal treatment for future plant disease management.

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INTEGRATED CONTROL OF P*enicillium digitatum* (Pers.:Fr.) Sacc., A GREEN MOLD CITRUS DISEASE, USING YEASTS AND MEDICINAL PLANTS

INTRODUCTION

Citrus is a major world horticultural commodity, in terms of exports and imports (Storey and Walker, 1999). World production of citrus fruit continuous increase in the last decades of the twenty century, total annual citrus production was estimated at over 85, 357 million tons in the period 2012. Oranges share more than half of global citrus production (51.1 million tons). The rise in citrus production is mainly due to an increasing of cultivation areas and the preferences towards more health and convenience food consumption and the rising incomes (FAO, 2012) .

Green mould, caused by *Penicillium digitatum* is the most devastating pathogen of citrus fruit, being responsible for about 90% of losses during post-harvest handling (Macarisin *et al.*, 2007; Kinaya *et al*., 2007). The fungus infects fruit through injuries where moisture and nutrients are available to stimulate spore germination and infection (Brown *et al*., 2000).

Currently, control of post harvest diseases is mainly dependent on the use of chemical fungicides, the use of chemicals is increasingly restricted because of concerns for the environment and health, as well as the cost of developing new pesticides to cope with pathogens resistance (Huanga *et al*., 1995), then, to find an effective and safe non-fungicide are needed (Sholberg and Conway, 2004; Conway *et al*., 2005; Droby, 2006; Yu *et al*., 2007).

Biological control is an alternative to reduce chemical for controlling postharvest diseases that caused severe loss worldwide (Huanga et al., 1995). The biological control by using antagonistic yeasts was one of the most promising non-

fungicidal especially to control the wound invading pathogens (Janisiewicz and Korsten, 2002).

The mechanism of this biological control is based on ecological interactions, such as competition for space and nutrients, mycoparasitism, antibiosis, predation or induction of plant defenses. Traditionally, yeasts are the organism mostly used as biocontrol agents due to their fast colonization of plant surfaces and production of extracellular polysaccharides that enhance their survival and restrain both colonization sites and inhibit germination of pathogen propagules (Pimenta e*t al.*, 2010).

Currently, two commercial products, Aspire (based on *Candida oleophila*) and Yield Plus (based on *Cryptococcus albidus*) have been registered in the United States or South Africa (Janisiewicz and Korsten, 2002; Fravel, 2005), BioSave, and Shemer etc., have also been developed and registered (Sharma *et al*., 2009).

Effective control of fruit rot of citrus with yeasts such as *Pichia guilliermondii*, *Candida oleophila*, *Candida sake*, *Candida formata*, *Candida saitona*, *Debaryomyces hansenii*, *Aureobasidium pullulans*, *Pantoea agglomerans*, *Saccharomyces cerevisiae*, *Metschnikowia fructicola* had been reported by El-Ghaouth *et al*. (1998); Wilson and Chalutz (1989); Chalutz and Wilson (1990); Droby *et al*. (1991); Arras (1996); Ippolito *et al*. (2000); Nunes *et al*. (2001); Teixido *et al*. (2001); Karabulut *et al*. (2003); Spadaro *et al*. (2004); Droby (2006); Morales *et al*. (2008).

The result of control of *Penicillium digitatum* by the predacious yeast S*accharomycopsis crataegensis*, the yeast showed a high potential (39.9% disease severity reduction) of this fungus (Pimenta *et al*., 2010).

Mixed cultures of the microbial antagonists appear to provide better control of postharvest diseases over individual cultures or strains. Different microbial

antagonists such as *Debaryomyces hansenii*, *Cryptococcus laurentii*, *Bacillus subtilis*, and *Trichoderma harzianum* are also being used (Sharma *et al*., 2009).

Many plant extracts have been reported to have activity against a wide range of fungi and those with fungitoxic properties may include volatile constituents. When two or more substances are present in the extracts, the fungitoxic potential might be enhanced (Kumar and Tripathi, 1991; Doubrava *et al*., 1998). Possible synergism among compounds could be beneficial for postharvest treatment because the pathogens would be less development of resistance against mixed components (Tripathi and Dubey, 2004).

Cinnamon bark, cinnamon leaf, and clove oil have fungistatic and fungicidal activity against the anthracnose and crown rot pathogens (Ranasinghe *et al*., 2002). Cinnamon extract completely inhibited conidial germination and mycelial growth of *Colletotrichum musae*, *Fusarium* spp. and *Lasiodiplodia theobromae* at 5.0 g /L (Win *et al*., 2007).

In vitro, the crude extracts of nightshade fruits and cinnamon bark have completely inhibited growth of *Penicillium digitatum* isolates at IC50 = 57.5 μg/ mL (Kanan and Al-Najar, 2008). Control of *Fusarium oxysporum*, *Aspergilus niger*, and *A. flavus* with leaf extracts of *Xylopia aethiopica* and *Zingiber officinale* showed that *X. aethiopica* inhibited *F. oxysporum* (52.2%), *A. niger* (28.3%), and *A. flavus* (27.3%), whereas *Z. officinale* inhibited *F. oxysporum* (31.5%), *A. niger* (33.3%), and *A. flavus* (18.2%). The impact of lemongrass oil-enrichment on fungal sporulation in PDA revealed spore production was reduced by 70% of *Botrytis. cinerea*, 58% of *Colletotrichum coccodes*, 41% of *A. niger*, 40% of *C. herbarum*, and 35% of *Rizhopus stolonifer* at 25 mg/L . Moreover, spore production was completely inhibited at the highest oil concentration (500 mg/L) for all of the pathogens (Okigbo and Nmeka, 2008).

On the other hand, many studies of the use of plant extracts such as essential oils and pure compound against plant pathogenic fungi have been conducted.

A number of antifungal compounds have been found in plants. These compounds belong mainly to six chemical groups such as phenolics and phenolic acids, coumarins and pyrones, flavonoids, isoflavonoids, steroids and steroidal alkaloids, and miscellaneous compounds (Mitra *et al*., 1984). Based on biosynthetic origins, plant natural product can be divided into three major groups namely the terpenoids, the alkaloids, and the phenylpropanoids and allied phenolic compounds (Croteau *et al*., 2000). Treatments with mixed aqueous leaf extract of *Solonum virginionum* and *Trichoderma viridae* were found to effectively control anthracnose disease of tomato at 70 % (Mogle, 2011). A treatment comprising *Bacillus amyloliquefaciens* HF-01 combined with 50 µg /mL tea saponin was revealed to be as effective as the fungicide treatment and resulted in excess of 90% control of green and blue molds and sour rot (Hao *et al*., 2011). Plants extracts are an options that have recently received attention. However, use of these extracts to control post-harvest pathogens of fruits and citrus pathogens is still limited (Obagwu and Korsten, 2003; Ghassan, 2008).

OBJECTIVES

The objectives of this research were:

1. Select potential plant extracts and determine their efficacy in controlling of *P.digitatum* both *in vitro* and *in vivo*.

2. Select potential yeast and determine their efficacy in controlling of *P.digitatum* both *in vitro* and *in vivo*.

3. Evaluate microbial antagonist integrated treatment with plant extracts to control *P.digitatum in vitro*.

4. Evaluate microbial antagonist integrated treatment with plant extracts to control *P.digitatum in vivo.*

5. Determine the mode of action of plant extracts and yeast as bio-pesticide

LITERATURE REVIEW

Citrus fruits, belonging to the genus of the family Rutaceae, are well known for their refreshing fragrance, thirst-quenching ability, and providing adequate vitamin C as per recommended dietary allowance (RDA). In addition to ascorbic acid, these fruits contain several phytochemicals which play the role of neutraceuticals, such as carotenoids (licopene and β-carotene), limonoids, flavanones (narigin and rutinoside), and vitamin-B complex and related nutrients (thiamine, riboflavin, nicotinic acid/niacin, pathothenic acid, pyridoxine, folic acid, biotin, chlorine, and inositol). The flavanoids from citrus juices, particularly those from oranges and grapefruit, are effective in improving blood circulation and possess antialergic, anticarcinogenic, and anti viral properties (Filatova and Kolesnova, 1999).

1. Citrus world production and consumption

Citrus is the most widely produced fruit, as group of several species, and it is grown in more than 100 countries (Chang, 1992). Citrus is the first among fruit crops in the world with respect to production leaving behind banana, grapes, apples, and pears (FAO, 2007 and 2009). World citrus production increased at the rate of 0.75% per year during 2007 to 2011, and decreased 7% in 2012, because small crops in Brazil, European Union, and Mexico, which resulted in an output of 85, 357 million tons (FAO, 2012). Production trends indicate that orange constitute about 60% of citrus the total citrus output, followed by madarins, elementins, satsumas, and tangerins, which compared about 26% of the output. The group of lemons and limes constitute 7.27%, and grapefruit and pammelos comprise roughly 6.35% (USDA, 2012)

World population is nearly 10 billion by 2050, therefore, citrus production has to increase at a suitable growth rate to meet this increasing demand. The 2012 orange production was 51.1 million metric tons and 28.498 million metric tons was expected to be consumed fresh. Consumption of fresh citrus is relatively high in develop countries. Citrus consumption per person in 2009 totaled 84.1 pounds. of the total,

20.7 pounds was consumption of fresh citrus and 63.4 pounds was consumption of processed citrus (Boriss, 2011), while top ten total citrus fruits producers 2007 (tonnes) as shown in Table 1.

Tangerine fruit is the most economically important citrus crop in Chiang Mai (Thailand). The most widely grown cultivar is Sai Num Pung (SARDI Citrus Information, 2007). As with other fruits, citrus is attacked by several pre and / or post harvest pathogens that affect on fruit quality. One of the most pathogen is *Penicillium digitatum* cause of green mold disease (Droby *et al*., 1989).

2. Green mold

Green mold caused by *Penicillium digitatum* is ubiquitous to all citrus growing regions. Spores of this organism are airborne and produced a large number on the infected fruit surface. These spores will contaminate the packinghouse and its equipment, storage rooms, transit containers, and an the retail market. Spores will also accumulate in water used for drenching and cooling (Brown and Chambers, 1996).

P. digitatum is commonly known as green-mold, grows on the surface of postharvested citrus fruits producing a characteristic powdery olive-colored conidia. This pathogen is main concern as it is responsible for 90% of citrus losses due to diseases occurring during the storage period, and it causes serious damages in commerce (Canamas *et al*., 2008).

P. digitatum classification: Kingdom: Fungi, Phylum: Ascomycota, Class: Eurotiomycetes, Subclass: Eurotiomycetidae , Order: Eurotiales, Family: Trichocomaceae, Genus: *Penicillium*, Species: *P. digitatum*, Binomial name: *Penicillium digitatum*, (Pers.) Sacc., (1883), Synonyms: *Monilia digitata* Pers., (1801), *Mucor digitata* (Pers.) Mérat, (1821).

Table 1 Top ten total citrus fruits producer in 2007 (tonnes)

Country	Grapefruit	Lemons and limes	Oranges	Tangerines, etc.	Other	Total
Brazil	72	1,060,000	18,279,309	1,271,000	-	20,682,309
China	547	745,1	2,865,000	14,152,000	1,308,000	19,617,100
United States	1,580,000	722	7,357,000	328	30	10,017,000
Mexico	390	1,880,000	4,160,000	355	66	6,851,000
India	178	2,060,000	3,900,000	-	148	6,286,000
Spain	35	880	2,691,400	2,080,700	16,5	5,703,600
Iran	54	615	2,300,000	702	68	3,739,000
Italy	7	546,584	2,293,466	702,732	30	3,579,782
Nigeria	-	-	-	-	3,325,000	3,325,000
Turkey	181,923	706,652	1,472,454	738,786	2,599	3,102,414
World	5,061,023	13,032,388	63,906,064	26,513,986	7,137,084	115,650,545

Source: FAO (2007)

Initial symptoms of the small decayed area appears as a soft watery spot, moist and light than comparable stages of sour rot, a softening of the exocarp. It is visible within 48 h (Droby *et al*., 2008). The rot eventually extend to the peel and into the edible fruit segments. Under high RH conditions, the fruit collapses into a soft, decomposing mass. If the relative humidity low, the fruit shrivels and dries up. If the relative humidity is high, other rot pathogen can invade and become soft (Droby *et al*., 1997)

White mycelium is produced on the lesion surface, and when the lesion enlarges to approximately one inch in diameter, olive green spores are produced in the center (Figure 1). A broad zone of white mycelium surrounds the sporulating area, and the outer region of the lesion is composed of softened rind. The entire fruit is soon encompassed by a mass of olive green spores which are easily dispersed by any physical motion or air currents (Florida Department of Citrus, Scientific Research Department, 2010).

Lifecycle, ecology, and biology. *Penicillium digitatum* survives in gardens from season to seasons (Droby *et al*., 1997). The fungus survives in the field on plant debris and produces spores that infect split and injured fruit in the tree and on the ground. At cooler fall and winter temperatures that favor fungal development, large numbers of spores are produced and carried by wind currents to surfaces of fruit in the tree canopy. The infection is initiated by spores in the air, entering the peel through the wounds, even the oil glands. The fungi do not spread by contact between adjacent fallen fruit, healthy, and intact. Dormant *Penicillium* spores present on the fruit surface become active if the peel is injured. The spores germinate rapidly and colonize the injured tissue (Droby *et al*., 2008).

Infection and sporulation cycle can be repeated many times during the season and inoculum pressure increases in the site if no precautions are taken. The green conidia develops more rapidly at temperatures near 24 °C and more slowly above 30°C and below 10 °C. The rot is almost inhibited at temperatures of 1 °C (Droby *et al*., 2008).

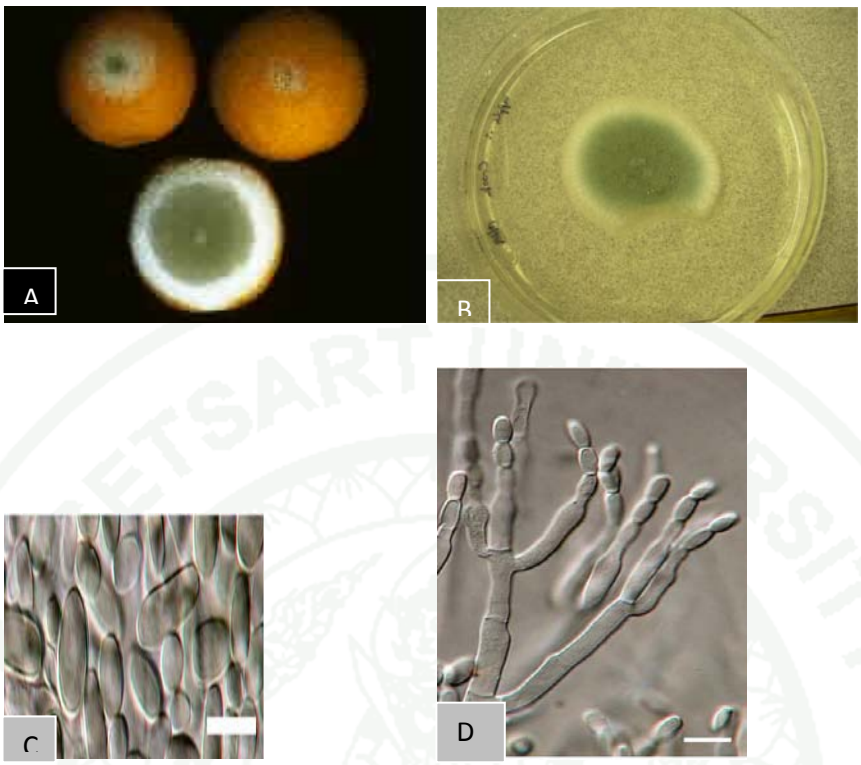


Figure 1 A. Symptom of green mold disease on citrus fruit, B. *P. digitatum* colony on PDA medium (Wilkipedia, 2010), C. Conidia, D. Conidiophores. White bar = 10 µm (Samson and Frisvad, 2004)

The spores germinate on the fruit when nutrients and moisture are released at injuries that occurred during harvesting and handling. Even injuries that involve only a few oil glands are sufficient to induce infection. The fungus can also invade fruit through certain physiologically injuries, such as injuries associated with chilling injury and stem-end rind breakdown. Fruit decaying with green mold produce relatively large amounts of ethylene gas which is a natural plant hormone that promotes respiration, senescence, and premature color development. *P. digitatum* does not usually spread from infected to healthy fruit in packed cartons but it can cause a condition known as soilage when masses of spores produced on infected fruit are disseminated to surfaces of healthy fruit. So, soiled fruit must be cleaned before retail sale. The infection and sporulation cycle can be repeated many times in a packinghouse and in storage rooms during extended storage *P. digitatum* enables to

develop strains with resistance to chemical fungicide treatments (Florida Department of Citrus, Scientific Research Department, 2010).

The accumulation of phenolic and lignin like materials in cell walls of injured exocarp cells appeared to be the major factor involved in resistance of healed tissue to penetration by *P. digitatum.* These materials may inhibit fungal growth, interfere with enzymatic degradation and/or lignify the tissue (Brown, 1983).

Conidia of *P. digitatum* placed on water agar plugs on the surface of intact oranges did not germinate, but if plugs were placed over puncture wounds in the peel germination were >80%. Peel oils from several species of citrus and volatiles emitted from injured oranges were reported to be responsible for stimulating germination of *P. digitatum* conidia. Ten *Penicillium* species tested, only *P. digitatum* and *P. italicum* spores germination was enhanced on water agar when exposed to volatiles from injured oranges (Eckert *et al*., 1992).

In studies using amended water agar, germination of *P. italicum* was stimulated by citral, nonanal, citronella, and citronellal, but *P. digitatum* was stimulated only by citral (500 µL/L), this stimulation was further enhanced if nonanal (500 µL/L) was added. Combinations of limonene, acetaldehyde, ethanol and carbon dioxide stimulated spore germination of *P. digitatum*, although individual components alone were inactive (Eckert and Ratnayake, 1984). Octanal was the most potent, with an IC-50 of 60 µL/L. Citral has been shown to reduce germ tube elongation of *P. digitatum*, with an IC-50 of 100 µL/L (Droby *et al*., 2008).

3. Citrus post harvest disease control

Harvested citrus fruit is very susceptible to wound infection by *Penicillium digitatum*, which causes green mold of citrus (Sommer *et al*., 2002). Minimizing wounds on fruit, proper temperature management, and postharvest fungicide treatments are the main methods of reducing losses by this pathogen (Eckert and Eaks, 1989).

Continuous use of fungicides such as imazalil, thiabendazole, and *o*-phenylphenol in citrus packing facilities in California resulted in the appearance of *P. digitatum* isolates with multiple fungicide resistances within natural populations (Holmes and Eckert, 1999). In addition, maximum residue limit for imazalil, the most important fungicide in use is lower in most citrus importing countries than in the United States (United States Department of Agriculture-Foreign Agricultural Service, 2004).

An efforts to develop methods to control fungal decay in citrus that are considered to be safer to consumers, workers, and the environment than the fungicides are developed. These include biological control (Janisiewicz and Korsten, 2002), low toxicity chemicals (Smilanick *et al*., 1999), and physical treatments such as heat and UV-C, which can enhance disease resistance (D'hallewin *et al*., 2000; Porat *et al*., 2000). Currently, the biofungicides Aspire and Bio- Save are registered for postharvest use on citrus in the United States (Janisiewicz and Korsten, 2002), although both have shown limitations in efficacy as individual treatment for citrus fruit (Brown and Chambers, 1996; Brown *et al*., 2000; Droby *et al*., 1998). Most alternative treatments need further effort in research and development to be implemented, there is still a need in the citrus industry for effective and environmentally friendly means of postharvest decay control.

4. Introduction of microbial antagonists

Many factors are involved for the introduction of a microbial antagonist for effective control of postharvest diseases of fruits and vegetables. Various studies have indicated that microbial agent should be applied at wound site before the arrival of the pathogen (Smilanick, 1994; Barkai-Golan, 2001; El-Ghaouth *et al*., 2004; Droby, 2006; Singh and Sharma, 2007). For instance, *Trichoderma viride* antagonist was only effective in controlling *Lasiodiplodia theobromae* in banana (*Musa* spp.) fruit when it was introduced 4 h prior to the inoculation of the pathogen (Mortuza and Ilag, 1999), otherwise it was not effective at all. Another factor, which is equally important for the effectiveness of a microbial antagonist is the presence of moisture in the wound sites.

For example, antagonistic yeast *Candida oleophila* was effective in controlling *Botrytis cinerea* in apple when it was applied to fresh wounds but when moisture was not available, it became a limiting factor for yeast growth (Mercier and Wilson, 1995). In addition, a microbial antagonist should have certain desirable characteristics to meet the basic requirements of the biological control.

5. Criteria for an ideal antagonist

A potential microbial antagonist should have certain desirable characteristics to make it an ideal bioagent (Wilson and Wisniewski, 1989; Barkai and Golan, 2001). The antagonist should be: (a) genetically stable; (b) effective at low concentrations; (c) not fastidious in its nutritional requirements; (d) capable of surviving under adverse environmental conditions; (e) effective against a wide range of the pathogens and different harvested commodities; (f) resistant to pesticides; (g) a non-producer of metabolites harmful to human; (h) non-pathogenic to the host; (i) preparable in a form that can be effectively stored and dispensed; and (j) compatible with other chemical and physical treatments. In addition, a microbial antagonist should have an adaptive advantage over specific pathogen (Wilson and Wisniewski, 1989).

For example, *Rhizopus stolonifer* is more sensitive to low temperature than many other pathogens. Thus, for its effective control, a microbial antagonist should have the ability to grow, multiply, and suppress the pathogen at low temperature. Similarly, *Candida oleophila* was effective along with dicloran to reduce the incidence of *Penicillium expansum* and Rhizopus rot in nectarine even under controlled atmosphere storage conditions (Lurie *et al*., 1995). Most of the pome fruits are stored in cold storage. Thus, for controlling their postharvest diseases to a satisfactory level, a microbial antagonist should have the ability to survive under cold stored conditions as well. Considering these factors, research work on the use of microbial antagonists for the control of postharvest diseases of fruits and vegetables has been re-oriented in many countries.

6. Mechanism of antagonist yeast on *P. digitatum*

Several modes of action have been suggested to explain the biocontrol activity of microbial antagonists. Still, competition for nutrient and space between the pathogen and the antagonist is considered as the major modes of action by which microbial agents control pathogens causing postharvest decay (Droby *et al*., 1992; Wilson *et al*., 1993; Filonow, 1998; Ippolito *et al*., 2000; Jijakli *et al*., 2001).

At the wound site, the microbial antagonist should be better adapted to various environmental and nutritional conditions than the pathogen (Barkai and Golan, 2001; El-Ghaouth *et al*., 2004). Rapid colonization of fruit wound by the antagonist is critical for decay control and manipulations leading to improved colonization enhance biocontrol (Mercier and Wilson, 1995). Thus, microbial antagonists should have the ability to grow more rapidly than the pathogen. Similarly, it should have the ability to survive even under conditions that are unfavorable to the pathogen (Droby *et al*., 1992).

Research work conducted on this mode of action of microbial antagonists supports the hypothesis that competition for nutrients plays a major role in the mode of action of *Pichia guilliermondii* against *Penicillium digitatum* in citrus (Droby *et al*., 1992; Arras *et al*., 1998), *Enterobacter cloacae* against *Rhizopus stolonifer* on peach (*Prunus persica*) (Wisniewski *et al*., 1989), *Cryptococcus laurentii* against *Botrytis cinerea* on apple (Roberts, 1990), and *Rhodotorula glutinis* and *Cryptococcus laurentii* against *Penicillium expansum* and *Botrytis cinerea*, respectively (Castoria *et al*., 1997) and *Metschnikowia pulcherrima* on apples (Kim *et al*., 1997). *M. pulcherrima* competes pathogens *Botrytis cinerea* and *Penicillium expansum* in apple through iron depletion (Saravanakumar *et al*., 2008).

As a result of its ability for suppressing postharvest diseases, Kurtzman and Droby (2001) and Grebenisan *et al*. (2008) have recommended it as potential yeast for controlling fruit rots. Biocontrol of grey mold (*Botrytis cinerea*) on apple by *Metschnikowia pulcherrima* was reduced or totally suppressed by the addition of

several nutrients suggesting that competition for nutrients plays a role in the biocontrol capability of *Metschnikowia pulcherrima* against *Botrytis cinerea* (Piano *et al*., 1997).

Non-pathogenic species of *Erwinia*, such as, *E. cypripedii* showed antagonistic activity against various isolates of *Erwinia caratovora* sub sp. *caratovora*, the causal agent of soft rot of many vegetables such as carrot, tomatoes (*Lycopersicon esculentum*) and pepper (*Capsicum annuum*), primarily by competing for nutrients (Moline, 1991; Moline *et al*., 1999). It has been demonstrated through *in vitro* studies that microbial antagonists take up nutrients more rapidly than pathogens, spore germination of the pathogens was reduced at the wound site (Wisniewski *et al*., 1989; Droby and Chalutz, 1994; Droby *et al*., 1998).

Attachment by microbial antagonist to the pathogen hyphae appears to be an important factor necessary for competition for nutrients as shown by the interactions of *Enterobacter cloacae* and *Rhizopus stolonifer* (Wisniewski *et al*., 1989), and *Pichia guilliermondii* and *Penicillium italicum* (Arras *et al*., 1998).

In vitro studies conducted on such interactions revealed that due to direct attachment, antagonistic yeasts and bacteria take nutrients more rapidly than target pathogens and thereby prevent spore germination and growth of the pathogens (Droby *et al*., 1989, 1998; Wisniewski *et al*., 1989).

In contrast, direct physical interaction did not appear to be required for the antagonistic activity of *Aureobasidium pullulans* against *Botrytis cinerea, Penicillium expansum, Rhizopus stolonifer*, and *Aspergillus niger* infecting table grapes (*Vitis vinifera*) and *Botrytis cinerea* and *Penicillium expansum* on apple (*Malus domestica*) fruit (Castoria *et al*., 2001). In these examples, antagonism was not the result of direct attachment of the microbial antagonist(s) with hyphae of the pathogens, but other mechanisms like antibiosis might have played a significant role for antagonism.

The level of control provided by the microbial antagonists is also highly dependent on the initial concentration of the antagonists applied on the wound site and the ability of the antagonist to colonize the wound site (Janisiewicz and Roitman, 1988; Wisniewski *et al*., 1989; McLaughlin *et al.*, 1990). In general, microbial antagonists are the most effective in controlling postharvest decay on fruits and vegetables when applied at a concentration of 10^7–10^8 cfu/mL (McLaughlin *et al*., 1990; El-Ghaouth *et al*., 2004), and rarely, higher concentrations are required.

The biocontrol activity of microbial antagonists with most harvested commodities increased with the increasing concentrations of antagonists and decreasing concentrations of pathogen. For example, *Candida saitona* was effective at a concentration of 10^7 cfu/mL for controlling *Penicillium expansum* on apples (Mc.Laughlin *et al*., 1990).

In an another study, El-Ghaouth *et al*. (1998) reported that *Candida saitona*, a concentration of 10^8 cfu/mL was better in controlling blue mold (*Penicillium expansum*) on apples. This qualitative relationship, however, was highly dependent on the ability of the antagonists to multiply and grow at the wound site. This was demonstrated by using a mutant of *Pichia guilliermondii*, which lost its biocontrol activity against *Penicillium digitatum* on grapefruit and against *Botrytis cinerea* on apples, when applied to the wounds at concentrations as high as 10^{10} cfu/mL (Droby *et al*., 1991). The population of this mutant remained constant at the wound sites during incubation period, while that of the wild type increased 10- to 20-folds, within 24 h.

In addition, production of antibiotics (antibiosis), direct parasitism, and possibly induced resistance are other modes of action of the microbial antagonists by which they suppress the activity of postharvest pathogens on fruits and vegetables (Janisiewicz *et al*., 2000; Barkai and Golan, 2001; El-Ghaouth *et al*., 2004).

Production of antibiotics is the second important mechanism by which microbial antagonists suppress the pathogens of harvested fruits and vegetables. For

instance, bacterial antagonists like *Bacillus subtilis* and *Pseudomonas cepacia* are known to kill pathogens by producing the antibiotic iturin (Gueldner *et al*., 1988; Pusey, 1989).

The antibiotic produced by *Bacillus subtilis* was effective in controlling fungal rot in citrus (Singh and Deverall, 1984) and *Monilinia fructicola* in peaches and cherries (Pusey and Wilson, 1984; Utkhede and Sholberg, 1986). Further, *Pseudomonas cepacia* inhibited the growth of postharvest pathogens *Botrytis cinerea* and *Penicillium expansum* in apple by producing an antibiotic, pyrrolnitrin (Janisiewicz and Roitman, 1988; Janisiewicz *et al*., 2000). *Pseudomonas cepacia* was also effective in controlling green mold (*Penicillium digitatum*) in lemon *(Citrus limon* L.) by producing antibiotics (Smilanick and Denis-Arrue, 1992).

Although, antibiosis might be an effective tool for controlling postharvest diseases in fruits and vegetables, at present emphasis is being given for the development of non-antibiotic producing antagonists for the control of postharvest diseases of fruits and vegetables (El-Ghaouth *et al*., 2004; Singh and Sharma, 2007). Researchers are aiming to isolate, evaluate or to develop those antagonistic microorganisms that control postharvest diseases of harvested commodities by the mechanism of competition for space and nutrient, direct parasitism or induced resistance (Droby, 2006).

Wisniewski *et al*. (1991) observed that *Pichia guilliermondii* cells had the ability to attach to the hyphae of *Botrytis cinerea* and *Penicillium*. After yeast cells were dislodged from the hyphae, the hyphal surface appeared to be concave and was partial degradation of the cell wall of *Botrytis cinerea* at the attachment sites. In contrast, co-culturing *Botrytis cinerea* with non-antagonistic yeast elicited only a loose attachment to the fungus with no pitting in the hyphae (Wisniewski *et al*., 1991). Similarly, *Candida saitona* attached strongly to the hyphae of *Botrytis cinerea* and caused swelling (El-Ghaouth *et al*., 1998).

Microbial antagonists also produce lytic enzymes such as gluconase, chitinase, and proteinases that make lysis the cell wall degradation of the pathogenic fungi (Lorito *et al*., 1993; Castoria *et al*., 1997, 2001; Jijakli and Lepoivre, 1998; Kapat *et al*., 1998; Mortuza and Ilag, 1999; Chernin and Chet, 2002). Bonaterra *et al*. (2003) reported that direct parasitism was a major factor that permitted *Pantoea agglomerans* to control *Monilinia laxa* or *Rhizopus stolonifer* decay on stone fruits.

Induction of defense responses in the harvested fruits and vegetables by the microbial antagonists has been suggested and shown as another mode of action of microbial antagonists for controlling postharvest decay (Arras, 1996; El-Ghaouth *et al*., 1998; Ippolito *et al*., 2000). *Cryptococcus saitona* induced chitinase activity and formed structural barrier (papillae) on host cell walls in apple against *Penicillium expansum* (El-Ghaouth *et al*., 1998).

Similarly, *Aureobasidium pullulans* caused a transient increase in the activity of 1,3-gluconase, peroxidase, and chitinase enzymes in apple wounds which stimulated wound healing processes and induced defense mechanisms against *Penicillium expansum* (Ippolito *et al*., 2000). Microbial antagonists induced disease resistance in the harvested commodities by the production of antifungal compounds, as in avocado (*Persea americana*) fruit (Prusky *et al*., 1994; Yakoby *et al*., 2001), and accumulation of phytoalexins, like scoparone and scopoletin in citrus fruit (Rodov *et al*., 1994; Arras, 1996).

Production of antifungal compounds by microbial antagonists in the host cells help in inducing defense mechanism and hence provide biocontrol on the harvested commodities. A large amount of extra-cellular mucilage is to be implicated in cell adhesion and may well contain active chemical elicitor that provides signals for recognition and subsequent responses, providing defense mechanism (Wisniewski *et al*., 1991; Castoria *et al*., 1997; El-Ghaouth *et al*., 1998). Further, oligosaccharide fragments of yeast cell wall polysaccharides are known to be active elicitors of host defense responses (Base *et al*., 1992).

7. Application of mixtures of microbial antagonists

Biological control of postharvest diseases with microbial antagonists have much progressed during the last two decades. However, it has been difficult to select an individual microbial strain with a broad spectrum of activity against major postharvest pathogens. Hence, compatible strains are needed to provide the necessary spectrum of activity for effective control of postharvest diseases of fruits and vegetables (Janisiewicz, 1988; Barkai-Golan, 2001; El-Ghaouth *et al*., 2004). Mixtures of microbial antagonists should have wide spectrum of microbial activity resulting in the control of two or more postharvest diseases, increasing the effectiveness under different situations such as cultivars, maturity stages, and locations. Moreover, it is enhancing the efficiency and reliability of biocontrol as the components of the mixtures act through different mechanisms like antagonism, parasitism, and induction of resistance in the host, and the combination of different biocontrol traits without the transfer of alien genes through genetic transformation (Singh and Sharma, 2007).

Use of mixed strains of microbial antagonists is a challenging work, as microorganisms have different growth habits, and requirements for nutrition and cultural conditions. However, some success has been achieved in this area as well. For instance, a combination of the bacteria *Pseudomonas syringae* and the yeast *Sporobolomyces roseus* proved to have a marked advantage over each of the antagonists in controlling *Penicillium expansum* in apple, both in reducing the incidence of wound infections and in limiting rot diameter (Janisiewicz and Bors, 1995).

The advantage of antagonistic pairs over a single antagonist was described by Schisler *et al*. (1997) in the control of Fusarium dry rot (*Gibberella pulcaris*) in stored potatoes. The black rot of pineapple (*Ananas comosus*), caused by *Ceratomyces paradoxa* could be controlled by the yeast *Pichia guilliermondii*, its combination with five yeast isolates was still more effective and the level of control was comparable to

current industry practice of holding fruit at a low temperature (8–10 ^{o}C) (Reyes *et al*., 2004).

Mixture of *Aureobasidium pullulans* (10^6 cfu/mL) and *Bacillus subtilis* (10^8 cfu/mL) obtaining a level of control equivalent to fungicides for *Penicillium expansum* and *Botrytis cinerea* (Leibinger *et al*., 1997). The antagonistic mixture consisting of *Candida sake* CPA-1 (10^7 cfu/mL) and *Pantoea agglomerans* (10^7 cfu/mL) controlled blue mold rot in 'Blanquilla' pear and brought the maximum control in blue mold rot on 'Golden Delicious' apples (Nunes *et al*., 2002a). Under natural infection conditions, dipping grapes in a cell suspension culture of Kloeckera and Candida was effective in controlling Rhizopus decay but had no effect on Aspergillus decay caused by *Aspergillus niger* in storage (McLaughlin *et al*., 1992).

The efficiency of an antagonist is affected both by the concentration of the yeast cells in the wound and by the number of pathogen spores used for inoculation. For example, when wound inoculation was done with a higher concentration of *Botrytis spores* (10^6 spores/mL), a reduced percentage of infection was achieved only by the highest yeast concentration (10^9 cells/mL) and vice versa (Chalutz *et al*., 1991).

Similarly, the best activity of *Trichoderma* spores was achieved at higher concentrations of the antagonist and at lower inoculation levels of pathogen (Elad *et al*., 1982; Mortuza and Ilag, 1999). In potato, antagonist pairs effectively controlled Fusarium dry rot over asingle use (Schisler *et al*., 1997). Mixed cultures of *Candida sake* and *Pantoea agglomerans* gave better control on blue and gray mold both in apple and pears than their individual use (Nunes *et al*., 2002b). Janisiewicz *et al*. (2008) reported that mixed cultures of *Metschnikowia pulcherrima* and *Cryptococcus laurentii* exhibited greater biocontrol activity on blue mold (*P. expansum*) than either yeast applied alone or in combination with sodium carbonate or bicarbonate in a pilot test conducted on citrus in controlled atmosphere. Although the use of antagonistic mixtures offers more effective control, the economic viability of this approach appears to be a major obstacle for its adoption, as registration of two microbial antagonists will cause additional burden for the industry.

8. Medicinal plant as anti fungal compound

The activity of natural plant products on the host tissue may involve direct interaction with the pathogen or induction of host resistance. Host resistance induction, on the other hand, may involve several complex mechanisms including hypersensitive responses, build up of cell wall barriers, increased production of phytoalexins, accumulation of pathogenesis-related (PR) proteins, and fungal cell wall hydrolases (Porat *et al.*, 2002)

Eckert and Ratnayake (1994) in which different mixtures of variety of volatiles were suggested to be responsible for stimulation of spore germination. Recently, many studies on the use of plant extracts essential oils, and pure compound against plant pathogenic fungi have been conducted. Wilson *et al*. (1997) found that species of allium and capsicum and essential oils of palmarosa, red thyme, cinnamon leaf, and clove buds were highly active against *Botrytis cinerea*.

A number of antifungal compounds have been found in plants. These compounds belong mainly to six chemical groups, such as phenolics and phenolic acids, coumarins and pyrones, flavonoids, isoflavonoids, steroids and steroidal alkaloids, and miscellaneous compounds (Mitra *et al*., 1984). However, a few commercial products from plant are used in practical plant protection.

Methanolic extracts of *Withania somnifera* and *Acacia seyal,* two plants from Ethiopia, were tested *in vivo* on citrus fruit for their efficacy to control *Penicillium digitatum*, could control the pathogen by 70% and 75% of wounded fruits after storage at 25 ^{o}C and >85% RH (Mekib *et al*., 2007). Plant aqueous extracts obtained from *Chuquiraga atacamensis*, *Parastrephia phyliciformis* and *Parastrephia lepidophylla*, were able to inhibit *in vitro P. digitatum* growth, while *P. lepidophylla* extract was active against *G. citri-aurantii* cultures. *P. lepidophylla* aqueous formulations showed MIC100 values of 300 mg/L against both *P. digitatum* and *G. citri-aurantii* strains. *In vivo* tests showed that the *P. lepidophylla* aqueous

formulations (600 mg/L) exert curative and protective effects on the fruit against infection by a *P. digitatum* wild strain (Jorge, 2012).

Combining biocontrol agents with other approaches such as heat treatment increased interest (Obagwu and Korsten, 2003), application of chemical components generally regarded as safe (Nunes *et al.*, 2002a; Droby *et al*., 2003). *P. membranefaciens* or *C. laurentii* combined with NH4-Mo or NaBi showed less decay incidence of brown rot than fruit treated with antagonists or chemicals alone at 20 ^{o}C. The treatment comprising *Bacillus amyloliquefaciens* HF-01HF-01 combined with 50 g/mL tea saponin was as effective as the fungicide treatment, which gave more than 90% control of green and blue mold and sour rot (Hao *et al.*, 2011).

Seven plants are used in this research, namely *Cymbopogon citrates, Zingiber officinale, Momordica charantia, Curcuma longa, Eugenia caryophyllata, Cinnamomum cassia* and *Tinospora crispa.*

Fresh *C. citratus* grass contains approximately 0.4% volatile oil. The oil contains 65 - 85% citral, a mixture of two geometric isomers, geraniol and neral. Related compounds geraniol (Figure 2), geranic acid, and nerolic acid have also been identified. Other compounds found in the oil include myrcene (12 - 25%), diterpenes, methylheptenone, citronellol, linalol, farnesol, other alcohols, aldehydes, linalool, terpineol, and more than a dozen other minor fragrant components. Geographical variations in the chemical constituents have been noted. Nonvolatile components of *C. citratus* consist of luteolins, homo-orientin, chlorogenic acid, caffeic acid, *p* - coumaric acid, fructose, sucrose, octacosanol, and others. Flavonoids luteolin and 6-C-glucoside have also been isolated. *C. flexuosus* volatile oil typically contains up to 85% citral. However, many strains have a higher concentration of geraniol (50%), with citral (10 - 20%) and methyl eugenol as minor components. Another type of East Indian lemongrass is reported to contain no citral but up to 30% borneol (http://www.drugs.com/npp/citronella-oil.html)

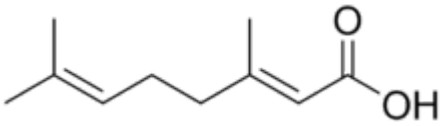


Figure 2 3,7-Dimethyl-2,6-octadienoic acid, Geranic acid

Zingiber officinale rhizome. [6]-gingerol, is the active constituent of fresh ginger. Chemically, gingerol (Figure 3) is a relative of capsaicin and piperine, the compounds which give chilli peppers and black pepper their respective spicyness. It is normally found as a pungent yellow oil, but also can form a low-melting crystalline solid (http://www.drugs.com/npp/galangal.html)

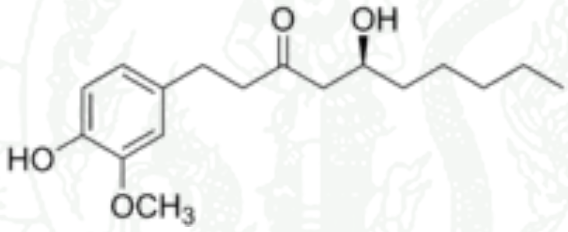


Figure 3 (S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone (Gingerol)

Momordica charantia fruit. Momordicin I, or 3,7,23-trihydroxycucurbitan-5,24-dien-19-al (Figure 4) is a chemical compound found in the leaves of the bitter melon vine (*Momordica charantia*), possibly responsible for its reputed medicinal properties. The compound was isolated and characterized. It is a white crystalline solid with formula $C_{30}H_{48}O_4$, that melts at 125–128 °C. The compound can be extracted from ground dry leaves by dichloromethane (Singh *et al.*, 2011).

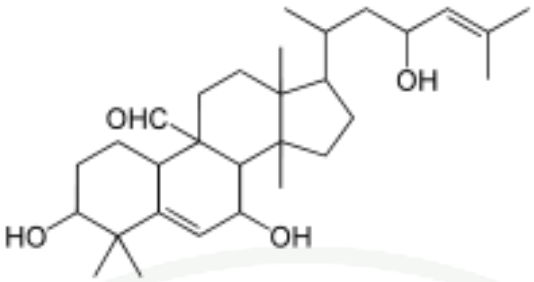


Figure 4 Momordicin

Curcuma longa rhizome. It can exist at least in two tautomeric forms, keto and enol (Figure 5A and B). The keto form is preferred in solid phase and the enol form in solution. Curcumin is a pH indicator. In acidic solutions (pH < 7.4) it turns yellow, whereas in basic (pH > 8.6) solutions it turns bright red (Awasthi and Dixit, 2009)

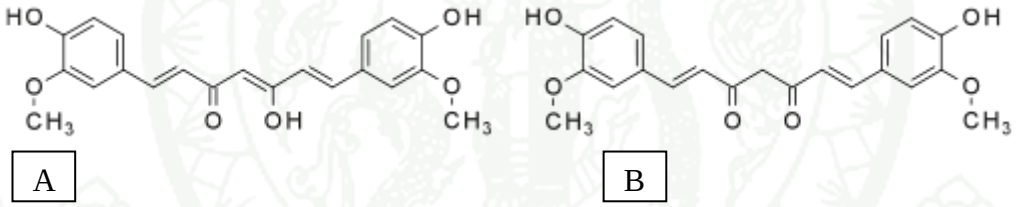


Figure 5 A. Enol form Curcumin B. keto form Curcumin

Dried *Eugenia caryophyllata* bud, comprises 72-90% of the essential oil extracted from cloves, and is the compound most responsible for the cloves' aroma. Other important essential oil constituents of clove oil include acetyl eugenol, beta-caryophyllene and vanillin; crategolic acid; tannins, gallotannic acid, methyl salicylate (painkiller); the flavonoidseugenin, kaempferol, rhamnetin, and eugenitin; triterpenoids like oleanolic acid, stigmasterol and campesterol; and several sesquiterpenes. Eugenol (Figure 6) has pronounced antiseptic and anaesthetic properties. Of the dried buds, 15 - 20 % is essential oils, and the majority of this is eugenol. A kilogram (2.2 lbs) of dried buds yields approximately 150 mL (1/4 of pint) of eugenol (Xing *et al*., 2011).

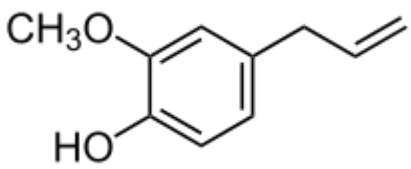


Figure 6 Eugenol

Cinnamomum cassia bark. The essential oil of cinnamon bark is about 90% cinnamaldehyde. Cinnamaldehyde (Figure 7) is the organic compound that gives cinnamon its flavor and odor. This pale yellow viscous liquid occurs naturally in the bark of cinnamon trees and other species of the genus Cinnamomum. The natural product is *trans*-cinnamaldehyde. The molecule consists of a phenyl group attached to an unsaturated aldehyde. As such, the molecule can be viewed as a derivative of acrolein. Its color is due to the $\pi \rightarrow \pi^*$ transition: increased conjugation in comparison with acrolein shifts this band towards the visible (http://en.wikipedia.org/wiki/Cinnamaldehyde)

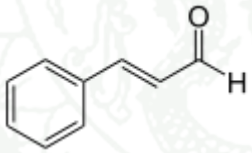


Figure 7 Cinnamaldehyde

Tinospora crispa. The whole plant contains a bitter principle, colombine, 2.22%; traces of an alkaloid and a glucoside. It also contains an amorphous bitter principle, picroretine, and traces of berberine. *Tinospora crispa* stem contains flavone O-glycosides (apigenin, Figure 8), picroretoside, berberine, palmatine, picroretine, and resin (Cavin *et al*., 1998)

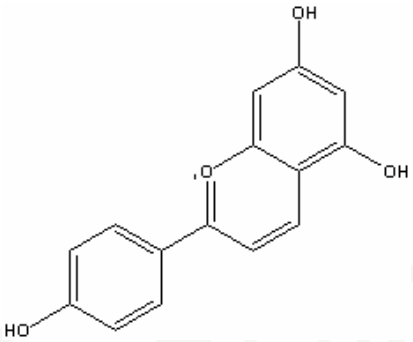


Figure 8 Apigenin

MATERIALS AND METHODS

Materials

1. Plant material: Fresh *Cymbopogon citratus* stem, *Zingiber officinale* rhizome, *Momordica charantia* fruit, *Curcuma longa* rhizome, dried *Eugenia caryophyllata* flower bud, *Cinnamomum cassia* Bark, *Tinospora* crispa bark (Figure 9)

2. Yeasts : *Candida tropicalis* TISTR 5010, *Pichia membranefaciens* TISTR 5093, *Candida utilis* TSITR 5001, *Aureobasidium pullulans* TISTR 3384, *Candida guillermondii* BCC 5389, *Candida sake* TISTR 5143, and *Debaryomyces hansenii* TISTR 5155

3. A highly virulent strain of *P. digitatum*
4. Potato dextrose agar medium
5. Yeast malt extract medium
6. "Sai Num Pung" tangerine fruits
7. Sodium hypochlorite
8. CH_2Cl_2
9. Sterile water
10. Imazalil
11. Sodium hydroxide (NaOH)
12. Phenolphthalein
13. Sodium bicarbonate ($NaHCO_3$)
14. Methaphosporic acid (HPO_3)

Figure 9 A. Fresh *Cymbopogon citratus* stem, B. *Zingiber officinale* rhizome, C. *Momordica charantia* fruit, D. *Curcuma longa* rhizome, F. dried *Eugenia caryophyllata* flower bud, G. *Cinnamomum cassia* Bark, H. *Tinospora* crispa bark

Methods

1. Experimental preparation

1.1 Plant crude extracts material

Preparation of the plant crude extracts for the antifungal screening against pathogenic fungi was conducted as previously described by Samsam-Shariat (1992) with modification. Fresh *Cymbopogon citratus* stem, *Zingiber officinale* rhizome, *Momordica charantia* fruit, *Curcuma longa* rhizome were bought from Amornpan traditional market, Chatuchak, Bangkok while dried *Eugenia caryophyllata* flower bud, *Cinnamomum cassia* Bark, *Tinospora* crispa bark were bought from China town market, Bangkok. The fresh plants were dried under shade and blended to powder. The powder samples were stored at ambient temperature in glass bottle in the dark until further used. Powdered plant with a mass of 300 g was soaked in 400 mL of 96% ethanol for 3 days with frequent agitation. The mixture was filtered through Whatman filter paper no 1 and the crude extract subsequently collected. The crude extract was evaporated at 40ºC with a rotavapor at 200 mbar. The dried extract was partitioned with dichloromethane and water. Only the dichloromethane phase was further separated and subjected to qualitative antifungal bioassay. The phase was subsequently added with 20% ethanol and kept in -20^{o}C until used.

1.2 Yeast preparation

Seven antagonist yeasts were used in this research were *Candida tropicalis* TISTR 5010/ATCC 13803 (http://www.biotec.or.th/tncc/dbstore/detail.asp?DB= TISTR*) and used for production of xylitol (Choi *et al*., 2000); *Pichia membranefaciens* TISTR 5093 (source isolate from coconut toddy and used for ester production (http:// www.tistr.or.th/tistr_culture/list_en.php?type=y&key=P*);

*accessed at 18 December 2012

Candida utilis TSITR 5001, used for production single cell protein from cassava (Trien *et al*., 2000); *Aureobasidium pullulans* TISTR 3384 (http://www.straininfo.net/strains/ 505092*) and used for fructo-oligosaccharides (FOS) production for prebiotic purpose (Fungsin *et al*., 2012); *Candida guillermondii* BCC 5389 (http://www.biotec.or.th/tncc/dbstore/ detail.asp?pg=113&wr=&DB=BCC*); *Candida sake* TISTR 5143 substrate is decayed fruit (http://www.biotec.or.th/tncc/ dbstore/Strain Details.asp? Genus=Candida &Species = sake&id=376&DB=TISTR*) and *Debaryomyces hansenii* TISTR 5155 (http:// www.tistr.or.th/ tistr_culture/ list_en.php?type=y&key=D*) and used for economical kefiran production (Cheirsilp *et al*., 2011), the yeasts were acquired from the National Science and Technology Development Agency (NSTDA) and the Thailand Institute of Scientist and Technological Research (TISTR). The yeast cells were cultured on yeast malt extract agar (YMA) and incubated at 28 °C for 48 h.

Aqueous antagonist suspension prepared by culturing the yeast cells on yeast malt extract agar (YMA) and incubated at 28 °C for 24 h. Then the cells suspension was prepared and added with 10 mL of sterile distilled water and counted with a haemocytometer. The cell suspension was adjusted to 1 x 10^8 cells/mL (Nunes *et al*., 2001; Yu *et al*., 2007).

1.3 *P. digitatum* preparation

P. digitatum was isolated from decayed citrus “Sai Num Pung” tangerine fruit by culturing on potato dextrose agar (PDA contained 200 g potato, 20 g glucose, and 20 g agar in 1 L of distilled water). To get a highly virulent strain of *P. digitatum*, the pathogen was inoculated on citrus fruits, and *P. digitatum* which showed fast symptom was chosen. The pathogen was grown on PDA c at 25 ^{o}C for 7 days. The conidial suspension was then prepared and added with 10 mL of sterile distilled water with 0.01% Tween 80 to the surface of the cultures.

*accessed at 18 December 2012

Conidia were scraped from the PDA with a sterile loop and the suspension was subsequently transferred to a test tube. Conidia concentration was determined with a haemacytometer and conidia suspension was adjusted to 1 x 10^5 conidia/mL with sterile water.

1.4 Fruit preparation

"Sai Num Pung" tangerine fruits (*Citrus reticulata*) were obtained from a commercial orchard in Fang, Chiang Mai province, Thailand, and fruits free of defect were chosen. The fruits surfaces were disinfected with immersed in 1% sodium hypochlorite for 3 minutes, rinsed with sterile water, and dried in a sterile chamber, exclude for semi commercial test did not need immersed in 1% sodium hypochlorite.

2 Part I: Screening ethanol concentration for plant crude extracts solvent

This method was used to screen ethanol concentration for plant crude extracts solvent. Ethanol percentages of plant solvent were 0, 20, 40, 60, 80, and 100%. The concentration of ethanol in each percentage was at 5,000, 10,000, 15,000, and 20,000 mg/L. *P. digitatum* conidia were used in this research mixed with 0.5% WA (100 mL water and 0.5 g agar), one inoculating loop of *P. digitaum* mixture added on petridish in the centre, incubated in 25 ^{o}C for 7 days, and colony diameter of *P. digitatum* hyphal growth was measured. Inhibition (%) of fungal radial growth was calculated using the formula ((radius of control-radius of treatment)/radius of control)) x 100% (Skidmore and Dickinson, 1976).

3 Part II. *In vitro* plant crude extracts screening to control *P.digitatum*

3.1 *In vitro* plant crude extracts screening in wide concentration

Poisonous food technique was used to screen plant crude extracts for their efficacy against *P. digitatum.* Potato dextrose agar was mixed with plant crude extracts at a concentration of 0 (no plant crude extracts added), 5,000, 10,000,

15,000, and 20,000 mg/L. A small block of the medium (2 cm x 2 cm) was aseptically cut and transferred to a sterile slide prior to inoculation with the *P. digitatum* on the 4 sides of a media block using sterile needle, and then incubated in a moist Petri dish for 48 h (Figure 10). *P. digitatum* growth was investigated, added with lactophenol cotton blue on the media, covered with the cover slip, and observed under a microscope. The experiment was performed in 5 replications. Inhibition (%) of fungal radial growth was calculated using the formula ((radius of control-radius of treatment)/radius of control)) x 100% (Skidmore and Dickinson, 1976).

3.2 *In vitro* plant crude extracts mix with citrus juice screening in wide concentrations

Poisonous food technique was used to screen plant crude extracts for their efficacy against the fungal pathogen *P. digitatum* was conducted with. Potato dextrose agar was mixed with 2% citrus juice and plant crude extracts at a concentration of 0 (no plant crude extracts added), 5,000, 10,000, 15,000, and 20,000 mg/L. A small block of the medium (2 cm x 2 cm) was aseptically cut and transferred to a sterile slide prior to inoculation with the *P. digitatum* on the 4 sides of a media block using sterile needle, and then incubated in a moist Petri dish for 48 h (Figure 10). *P. digitatum* growth was investigated by adding with lactophenol cotton blue on the media, covered with the cover slip, and observed under a microscope. The experiment was performed in 5 replications. Inhibition (%) of fungal radial growth was calculated using the formula ((radius of control-radius of treatment)/radius of control)) x 100% (Skidmore and Dickinson, 1976).

4 Part III: *In vivo* plant crude extracts screening on citrus fruits

Four promising plant crude extracts which were used in this part are from the result of 3.1 and 3.2. The plant crude extracts were tested on citrus fruit at different concentration comparing with imazalil 500 mg/L, 20% ethanol, and water. A sterile needle was used to make wound on 3 mm depth. Ten micro liter of conidial suspension of *P. digitatum* ($1x10^5$ conidia/mL) or 10 μL of sterile water (control -)

was added using a sterile pipette on the citrus wound and allowed to dry under aseptic conditions. Then 10 µL of plant crude extracts, 10 µL of sterile water (control

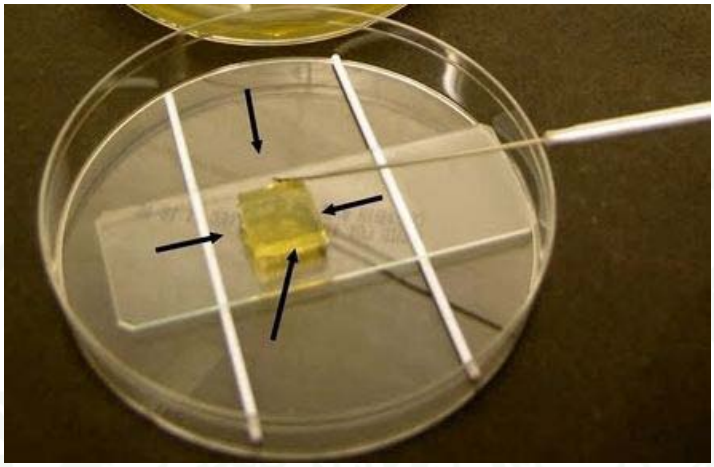


Figure 10 Slide culture method for screening plant crude extracts, arrows were inoculation sites

treatment +), imazalil 150 mg/L, or 20% ethanol was also applied on the same wound. The inoculated fruits were incubated in a 100% RH chamber at 25ºC for 7 days.Each treatment contained 45 fruits in 3 replications and the experiment was arranged as randomized complete design. Disease incidence and disease severity were observed after 7 days of incubation. To calculate disease incidence, the number of infested citrus fruits was observed. The disease incidence calculated using this formula: Disease incidence = (number of infected fruits/total number of fruit assessed) x 100. The disease severity on citrus was determined according to the portion of the infected area of citrus fruit. The disease severity calculated using this formula: Disease severity = (infected tissue area/total tissue area) x 100% (Masood *et al*., 2010). The reduction of disease incidence was calculated using the formula: ((disease incidence in control – disease incidence in treatment)/ disease incidence in control)) x 100%, while the reduction of disease severity was calculated using the formula: ((disease severity in control – disease severity in treatment)/ disease incidence in control)) x 100%.

Three promising plant crude extracts were selected from previous step, after that would be selected 2 promising plant crude extracts with same method. The plant crude extracts were tested on citrus fruit at different concentration comparing with 20% ethanol. A sterile needle was used to make wound on 3 mm depth. Ten micro liter of conidial suspension of *P. digitatum* ($1x10^5$ conidia/mL) was added using a sterile pipette on the citrus wound and allowed to dry under aseptic conditions. Then 10 µL of plant crude extracts or 10 µL of 20% ethanol (control) was also applied on the same wound. The inoculated fruits were incubated in a 100% RH chamber at 25ºC for 7 days. Each treatment contained 45 fruits in 3 replications and the experiment was arranged as randomized complete design. Disease incidence and disease severity were observed after 7 days of incubation. To calculate disease incidence, the number of infested citrus fruits was observed. The disease incidence calculated using this formula: Disease incidence = (number of infected fruits/total number of fruit assessed) x 100. The disease severity on citrus was determined according to the portion of the infected area of citrus fruit. The disease severity calculated using this formula: Disease severity = (infected tissue area/total tissue area) x 100% (Masood *et al*., 2010). The reduction of disease incidence was calculated using the formula: ((disease incidence in control – disease incidence in treatment)/ disease incidence in control)) x 100%, while the reduction of disease severity was calculated using the formula: ((disease severity in control – disease severity in treatment)/ disease incidence in control)) x 100%.

5 Part IV: *In vitro* antagonist screening for controlling *P. Digitatum*

5.1 *In vitro* antagonist-*P.digitatum* screening for controlling *P. Digitatum*

The screening was performed to select 4 promising yeasts. *In vitro* of antagonist screening was done according to the method describe by Spadaro *et al*. (2002). Interaction between yeasts and *P.digitatum* hyphae was assessed for direct parasitism in 90 mm diameter Petri dish which contained PDA medium with dual culture technique. Antagonist yeast was streaked on the medium, 30 mm from the border and one inoculating loop of *P.digitatum* conidia mix with 0.5% water agar was

added on 30 mm from the border in the opposite side of the yeast. The radial growth of the mycelium toward the yeast strip was measured after storage at room temperature for7 days. The effect of antagonist on mycelium of pathogen was observed under a compound microscope. Each treatment was 50 Petri dishes in 5 replications and the experiment was arranged as randomized complete design. Inhibition (%) of fungal radial growth was calculated using the formula ((radius of control-radius of treatment)/ radius of control)) x 100% (Skidmore and Dickinson, 1976).

5.2 *In vitro* of synergism effect of two antagonist yeasts on pathogen

In section 5.1, four promising yeasts, namely A,B,C, and D were selected, so combinations for this study were AB,AC,AD, BC, BD, and CD. *In vitro* of antagonist screening was done according to the method describe by Spadaro *et al*. (2002) with slightly modifications. Interaction between yeast(s) and *P. digitatum* hyphae was assessed for direct parasitism in 90 mm diameter Petri dish which contained the PDA medium with dual culture technique. Mixture of antagonist cells was streaked on the medium, 30 mm from the border and 1 inoculating loop of mixture of *P.digitatum* conidia with 0.5% water agar was added on 30 mm from the border in the opposite side of yeast. The radial growth of the mycelium toward the yeast strip was measured after storage at room temperature for 7 days. The effect of antagonist on mycelium of pathogen was observed under a compound microscope. Each treatment was 30 Petri dishes in 3 replications and the experiment was arranged as randomized complete design. Inhibition (%) of fungal radial growth was calculated using the formula ((radius of control-radius of treatment)/ radius of control)) x 100% (Skidmore and Dickinson, 1976).

5.3 *In vitro* test effect of plant crude extracts in two promising antagonist yeasts

Two promising yeasts as a result from section 5.2 were tested in PDA medium plus plant crude extracts. The aim of this step was to know whether plant

crude extracts can affect to the yeasts growth or not affected. Two promising yeasts streak on the PDA medium plus plant crude extracts in different concentration. All treatment kept at 25^{o}C, 95% RH for 5 days. Each treatment was 30 plates in 3 replications. Randomized complete design was used in this research.

6 Part V: *In vivo* antagonist - plant crude extracts to control *P.digitatum* on citrus fruit

6.1 Effect of antagonist-plant crude extracts on disease of citrus fruit

Two promising plant crude extracts and two yeasts as a result of part III and IV were tested on citrus fruit both individually and in their combination. A sterile needle was used to make wound on citrus fruits 3 mm depth. Ten micro liter of conidial suspension of *P. digitatum* (1x10^{5} conidia/mL) was added with a sterile pipette on the citrus wound and allowed to dry under aseptic conditions. Then, 10 µL of cells suspension of yeast at 1 x 10^{8} cells/mL were also added on the same wound. After drying, 10 µL of the plant crude extracts were added on the wound site, and then incubated in a 100% RH, at 25 ^{o}C, for 7 days. Disease incidence and disease severity were observed after 7 days of incubation. Each treatment was 45 fruits in 3 replications and the experiment were arranged as randomized block design.

6.2 Efficacy of yeast and plant crude extracts for reducing natural decay development on citrus fruit

The best combination of plant crude extracts and yeast as a result of section 6.1 was tested for reducing natural development on citrus fruits. The effectiveness of yeast and plant crude extracts to control naturally infected citrus fruits determined in semi-commercial experiment. For the purpose, the best combination of plant crude extracts and yeast was tested compare with imazalil 150 mg/L, water and 20% ethanol. Each treatment containing 200 fruits were dipped in the suspension of yeast and plant crude extracts, imazalil, water, or 20% ethanol for 2 minutes, then dried and packed into plastic box and cover with plastic bag. After storage at 25 ^{o}C,

95% RH for 2 weeks, disease incidence and disease severity were recorded. Each treatment was four replications, 50 fruits per replication, and the experiment was arranged as randomized complete design. Disease incidence and disease severity were observed after 14 days of incubation. The disease incidence calculated using this formula: Disease incidence = (number of infected fruits/total number of fruit assessed) x 100. The disease severity on citrus was determined according to the portion of the infected area of citrus fruit. The disease severity calculated using this formula: Disease severity = (infected tissue area/total tissue area) x 100% (Masood *et al*., 2010).

6.3 Effect of antagonist-plant crude extracts on *P.digitatum* colonization on citrus fruit

The colonization ability of *P.digitatum* on the citrus wounds was observed in the treated fruits at 1, 2, 3 day(s) after treatment, each fruit had 5 wounds and each wound represent 1 treatment (total 5 treatments: plant crude extracts + yeast+ *P.digitatum*; yeast + *P.digitatum*; *P.digitatum*; plant crude extracts + *P.digitatum*; yeast, Figure 11). A sterile needle was used to make wound in citrus fruits 3 mm depth, added with 10 µL of conidial suspension of *P.digitatum* at with 1x 10^5 conidia/mL by using sterile pippete, then 10 µL of cell suspension of yeast at 1x 10^8cells/mL, and allowed to dry under sterile condition, then 10 µL of plant crude extracts added to the same wound. Fifteen fruits per treatment in 3 replication, randomized block design was used in this research. Treated fruits were kept at 100% RH, at 25 ºC. *P.digitatum* colonization was determined at 1, 2, and 3 day(s) after treatment, 1.0 cm^2 of citrus peel in around wounded was cut, removed using sterile scalpel, transferred to tubes contained 10 mL of sterile water, diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, and then followed by plating 100 µL of the suspension in triplicate on PDA supplemented with 200 mg/l of streptomycin. The colonies of *P.digitatum* were counted after incubated at 25ºC for 5 days.

Figure 11 A methods for testing colonization of *P. digitatum* and yeast on citrus fruits. Number 1 was plant crude extracts + yeast+ *P.digitatum* treatment; 2 was yeast + *P.digitatum* treatment; 3 was *P.digitatum* treatment; 4 was plant crude extracts + *P.digitatum* treatment; 5 was yeast treatment

For deeply observation of the colonization ability of *P.digitatum* was observed at 2, 4, 6, and 8 hours after treatment to know ignition time of colonization ability. The colonization ability of *P.digitatum* on the citrus wounds was observed in the treated fruits, each fruit had 4 wounds and each wound represent 1 treatment (total 4 treatments: plant crude extracts + yeast + *P.digitatum*; yeast + *P.digitatum*; *P.digitatum*; plant crude extracts + *P.digitatum*; Figure 12). A sterile needle was used to make wound in citrus fruits 3 mm depth, added with 10 µL of conidial suspension of *P.digitatum* at with 1x 10^5 conidia/mL, then 10 µL of cell suspension of yeast at 1x 10^8cells/mL, and allowed to dry under sterile condition, then 10 µL of plant crude extracts added to the same wound. *P.digitatum* colonization was determined by using the method described by Pimenta (2010); 1cm^2 of citrus peel in around pricked area was cut into small pieces (0.5 mm x 3 mm) and then they were subsequently cultured in both PDA and YMA media (Figure 12). The numbers of *P. digitatum* colonized pieces were observed during 7 days after incubation. Fifteen fruits per treatment in 3 replication, randomized complete design was used in this research. Colonization of *P. digitatum* can be determined with formula which was defined as: A number of colonized of citrus peel / Total pieces of citrus peel x 100%

Figure 12 A methods for testing colonization of *P. digitatum* and yeast on citrus fruits. Number 1 was plant crude extracts + yeast+ *P.digitatum* treatment; 2 was yeast + *P.digitatum* treatment; 3 was *P.digitatum* treatment; 4 was plant crude extracts + *P.digitatum* treatment

6.4 Effect of yeast, plant crude extracts, and pathogen on yeast colonization on citrus fruit

The colonization ability of yeast on the citrus wounds was observed in the treated fruits at 1, 2, 3 day(s), each fruit had 5 wounds and each wound represent 1 treatment (total 5 treatments: plant crude extracts + yeast+ *P.digitatum*; yeast + *P.digitatum*; *P.digitatum*; plant crude extracts + *P.digitatum*; yeast, Figure 11). A sterile needle was used to make wound in citrus fruits 3 mm depth, added with 10 µL of conidial suspension of *P.digitatum* at 1x 10^5 conidia/mL by using sterile pippete, then 10 µL of cell suspension of yeast at 1x 10^8cells/mL, and allowed to dry under sterile condition, then 10 µL of plant crude extracts added to the same wound. Fifteen fruits per treatment in 3 replication, randomized block design was used in this research. Treated fruits were kept at 100% RH, at 25 ºC. Yeast colonization was determined at 1, 2, and 3 day(s) after treatment, 1.0 cm^2 of citrus peel in around wounded was cut, removed using sterile scalpel, transferred to tubes contained 10 mL of sterile water, diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} , and 10^{-5}, and then followed by plating 100 µL of the suspension in triplicate on YMA supplemented with 200 mg/l of streptomycin. The colonies of yeast were counted after incubated at 25ºC for 5 days.

For deeply observation of the colonization ability of yeast was observed at 2, 4, 6, and 8 hours after treatment to know ignition time of colonization ability. The colonization ability of yeast on the citrus wounds was observed in the treated fruits, each fruit had 4 wounds and each wound represent 1 treatment (total 4 treatments: plant crude extracts + yeast + *P.digitatum*; yeast + *P.digitatum*; *P.digitatum*; plant crude extracts + *P.digitatum*; Figure 12). A sterile needle was used to make wound in citrus fruits 3 mm depth, added with 10 µL of conidial suspension of *P.digitatum* at with 1x 10^5 conidia/mL, then 10 µL of cell suspension of yeast at 1x 10^8cells/mL, and allow to dry under sterile condition, then 10 µL of plant crude extracts added to the same wound. Yeast colonization was determined by using the method described by Pimenta (2010); 1cm^2 of citrus peel in around pricked area was cut into small pieces (0.5 mm x 3 mm) and then they were subsequently cultured in both PDA and YMA media (Figure 12). The numbers of yeast colonized pieces were observed during 7 days after incubation. Fifteen fruits per treatment in 3 replication, randomized complete design was used in this research. Colonization of yeast can be determined with formula which was defined as: A number of colonized of citrus peel / Total pieces of citrus peel x 100%

7 Part VI: Effect plant crude extracts to yeast conidia and pathogen spore growth

To know the effect of yeast and plant crude extracts to *P. digitatum* and plant crude extracts on *C. utilis* growth were investigated. Both microorganisms were grown on the PDA, PDA plus 5,000 mgL^{-1} of *E. caryophyllata*, and PDA plus 5,000 mgL^{-1} of *E. caryophyllata* and citrus juice 2% as origin substrate. Effect of *E. caryophyllata* and *C. utilis* to *P. digitatum* was observed under fluorescent microscope Carl Zeis GmbH, Germany. Isolates were transferred from petri dish then smeared on a glass slide and stained with one drop of Nile red solution (1 mg mL^{-1} of 100% ethanol), waited for 3 minutes, afterward observed under the microscopes with normal and fluorescent light source.

8 Part VII: Determination of active compounds of plant crude extracts

A thin layer chromatography (TLC) method was used to separate compound which was contained in plant crude extracts. The following solvent were combination of toluene, dichloromethane, and acetone to obtain the best separation of phenolic compounds. The best crude extracts was spotted on the surface of TLC plates (20X20 cm, coated with silica gel, F254 (Merck, Darmstadt, Germany), and then developed in glass tank containing running solvent as a mobile phase. After drying separated fractions were visualized with a UV lamp at 254 and 366 nm, retention factor (Rf) was recorded.

In parallel with bioassay test, conidial suspension of *Cladosporium cladosporioides* and *P. digitatum* were prepared in potato dextrose broth (PDB), and those were sprayed on to the surface of TLC developed plate. The plate was incubated in a moist chamber at room temperature for 36 h. Inhibition zone that indicated of active ingredient and retention factor (rf) were recorded, where Rf was distance of inhibition zone (cm) / distance from beginning to solvent front (cm).

The purity control of the isolated compound of plant crude extracts was done by high performance liquid chromatography (HPLC) and the structure was elucidated with nuclear magnetic resonance spectroscopy (NMRs). The HPLC measurement was carried out by Agilent 1100 series with UV-DAD detection at 230 nm with a reference wavelength at 600 nm. The separation was accomplished by applying of a Hypersil BDS C-18 column with the dimensions of 250 x 4.6 mm and 5µm particle size. The mobile phase was an aqueous buffer containing 15 mM ortho-phosphoric acid and 1.5 mM tetrabutylammoniumhydroxide (A) and methanol (B) with a linear gradient 20-90% B for 0-15 min followed by 100 % B for 5 min. This was kept for further 8 min. The flow rate was set to 0.8 mL/min. and the injection volume was 10 µL.

To identify the chemical formula of the compound was used nuclear magnetic resonance (NMR) was performed using a Bruker DRX-400 AVANCE spectrometer

(Bruker, Rheinstetten, Germany) equipped with a 400.13 MHz (^{1}H) or 100.61 MHz (^{13}C), and the Topspin 1.2 software was used.

9 Part VIII: Effect of yeast and plant crude extracts on postharvest quality of citrus fruit

The effect of yeast and plant crude extracts on postharvest quality of citrus fruit was examined. The weight loss (%), fruit firmness (kg), total juice percentage (%), total soluble solid (%), titratable acidity (%), and ascorbic acid content (mg/100g) of fruits were determined from 20 symptomless fruits were randomly selected from each replication after storage at 25 ^{o}C for 2 weeks. For the weight loss, fruits measured before treatment (A) and after storage (B), and the weight loss was calculated as (A/B)/A. Fruit firmness was measured at four points of the equatorial region by using a firmness tester (N.O.W., FHR-5) with a 5mm probe. Total juice percentage was calculated by using the formula: (juice weight/fruits weight) x 100%. The total soluble solid in juice was determined with a digital refractometer (N1-E, Atago Co. Tokyo, Japan) (Lacey *et al*., 2009). Determination of total titratable acidity was taken for triplicate measurements. About 10 mL of citrus juice or 10 mL of sterile distillated water (control), add with 2 drops of phenolphthalein as indicator, and then titrate with 0.1N sodium hydroxide (NaOH) solution. Total acidity was calculated as percentage of citric acid on fresh weight basis using the standard formula, Percent acid= (0.1 x 0.064 x mL 0.1 M NaOH used)/mL of sample taken (Khan *et al*., 2010). Ascorbic acid (vitamin C) was determined by titrating 2 mL of citrus juice or 2 mL sterile distillate water (as a control), add with 5 mL acid stabilization solution 3% which content 15g of methaphosporic acid (HPO_3) and 40 mL of glacial acetic acid, dissolved in 500 mL of distilled water. Titrated with standard dye solution 0.5% (0.042 g of sodium bicarbonat ($NaHCO_3$), 0.05 g of sodium 2, 6 dichloroindophenol in 200 ml distilled water) drops of meta phosphoric acid to a light pink end point which should persist for at least 15 seconds (JBT Food Tech, 2011).

10 Statistical analysis

All data were analyzed by statistical analysis of variance (ANOVA) procedure using statistical analysis system (SAS) software. Statistical significant was assessed at $p < 0.05$ and Tukey'S HSD Multiple range test.

RESULTS AND DISCUSSION

Results

The experiments were done through *in vitro* and *in vivo.* In this research divided into seven parts which consists screening ethanol concentration for plant extracts solvent, *in vitro* plant crude extracts screening to control *P.digitatum, in vivo* plant extracts screening on citrus fruits, *in vitro* antagonist screening for controlling *P. digitatum, in vivo* antagonist - plant extracts to control *P.digitatum* on citrus fruit, determination of active compound of plant extracts, effect of yeast and plant extracts on postharvest quality of citrus fruit.

1 Screening of ethanol concentration for plant extracts solvent

Screening of ethanol as a solvent for plant extracts was started from concentration of 20 - 100 % of ethanol diluted in sterile purified water. The best solvent was ethanol 20%, which had the lowest inhibition to *P.digitatum* (Table 2).

2 *In vitro* plant extracts screening for controlling *P.digitatum*

The screening of plant extracts by using food poisoning technique (Table 3 and Figure 13). The result demonstrated that *Eugenia caryophyllata* crude extracts reduced *P. digitatum* hyphal growth by 100% at concentration of 5,000 - 20,000mg/L, while others the plant crude extracts, *Curcoma longa, Cymbopogon citratus,* and *Momordica charantia* crude extracts were effectively reduce *P. digitatum* hyphal growth up to 70%, at concentrations of 10,000 - 20,000 mg/L (Table 3). Mean while, screening of the plant crude extracts on PDA medium plus plant extracts and 2% citrus juice were effective reduction *P. digitatum* hyphal growth at concentration of 5,000 - 20,000 mg/L of *E. caryophyllata* crude extracts by 100%, and at concentrations 15,000 -20,000 mg/L of *C. longa* crude extracts by 47% to 79% (Table 4).

Table 2 *In vitro* screening of ethanol concentrations at different percentages on their inhibition of hyphal growth of *P.digitatum*

Treatment	Inhibition of *P.digitatum* growth (%) in several concentrations of ethanol (mg/L) on PDA			
	5,000	10,000	15,000	20,000
20% ethanol	4.75 a	13.20 b	30.10 e	35.46 fgh
40% ethanol	15.74 bc	24.43 d	33.34 f	38.31 hi
60% ethanol	17.96 c	28.40 e	34.79 fg	39.49 ij
80% ethanol	29.29 e	35.99 gh	44.94 k	45.07 k
100% ethanol	35.87 gh	41.07 j	49.58 l	53.93 m

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 5 replicates.

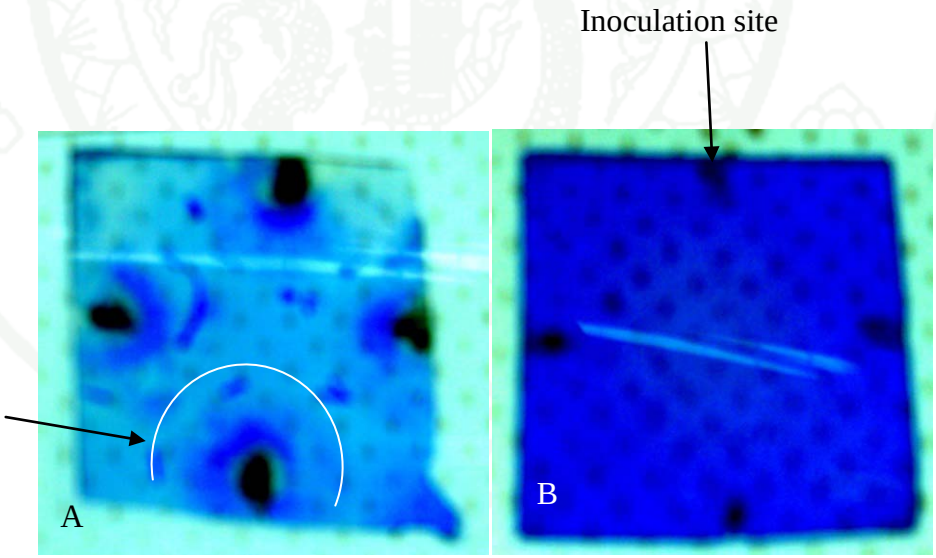


Figure 13 A. *P. digitatum* hyphal growth on PDA plus 20% ethanol as a control and B. PDA plus *E. caryophyllata* crude extracts 5,000 mg/L, stained with lactophenol blue. An arrow indicated the edge of mycelium

Table 3 *In vitro* screening of 7 plant crude extracts in wide concentration dissolved in 20% ethanol amended in PDA on their inhibition of hyphal growth of *P.digitatum* at 25°C for 24 h

Treatment	Inhibition *P.digitatum* hyphae growth (%) by plant crude extracts in several concentrations (mg/L)			
	5000	10000	15000	20000
Cymbopogon citratus	65.71 kl	67.86 kl	69.12i	77.00 o
Zingiber officinale	56.93 i	46.30 f	50.00 g	61.95 j
Momordica charantia	66.67 kl	53.17 hi	75.21 no	77.00 o
Curcoma longa	51.79 gh	78.32 op	79.21 p	73.60 mn
Eugenia caryophyllata	100.00 q	100.00 q	100.00 q	100.00 q
Cinnamomum	33.87 c	57.19 i	56.99 i	77.96 op
Tinospora crispa	54.66 hi	43.07 e	57.47 i	72.76 mn
20% ethanol (control)	37.16 d	13.74 a	24.37b	39.37d

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 5 replicates

3 *In viv*o plant extracts screening for controlling green mold rot disease

C. citratus, M. charantia, C. longa, and *E. caryophyllata* were selected as 4 promising plant crude extracts from the previous studied which were used for further experiment in citrus fruits. The concentration of *C. citratus* crude extracts and *M. charantia* crude extracts was 20,000 mg/L, while *E. caryophyllata* crude extracts of 15,000 mg/L (because in 20,000 mg/L affect to citrus peel, showed burning, Figure 14), The concentration of *C. longa* crude extracts was 30,000 mg/L. Laksanaphisut and Sangchote (2010) reported crude extracts of turmeric powder in 20% of ethanol at 30,000 ppm reduced disease incidences of green mold disease on treated fruit by 25%. Reduction of disease incidence of *E. caryophyllata* crude extracts was 36.85%, the others were 20.97%, 26.30%, and 20.97% for *C. citratus, M. charantia, C. longa* crude extracts respectively. While the disease severity, 3 of 4 plant crude extracts, namely *M. charantia, C. longa*, and *E.caryophyllata* crude extracts showed reduction

more than 30%, but those results lower than imazalil 500 mg/L treatment, which was 100% for reduction of disease incidence and disease severity (Table 5 and Figure 15).

Figure 14 Effect of high concentration (20,000 mg/L) of *Eugenia caryophyllata* crude extracts on citrus peel (an arrow)

Table 4 *In vitro* screening of 7 plant crude extracts dissolved in 20% ethanol at different concentrations amended in PDA plus 2% citrus juice on their inhibition of hyphal growth of *P.digitatum* at 25 ^{o}C for 24 h for 7 days

Treatment	Inhibition of hyphal growth of *P.digitatum* (%) on plant crude extracts + citrus juice in several concentrations (mg/L)			
	5000	10000	15000	20000
C. citratus	31.35 cd	40.08 e	49.21 g	53.97 h
Z. officinale	53.97 h	55.95 hi	55.30 hi	69.05 k
M. charantia	23.89 b	33.73 d	58.89 i	64.29 j
C.longa	46.67 f	64.68 j	73.41 l	78.97 m
E. caryophyllata	100.00 n	100.00 n	100.00 n	100.00 n
Cinnamomum	0.00 a	0.00 a	50.86 g	75.00 l
T. crispa	42.86 e	55.95 hi	56.75 hi	65.87 j
20% ethanol	0.00 a	0.0 a	0.00a	0.00a

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 5 replicates

Table 5 Disease severity (%), disease incidence (%), reduction of disease incidence (%), and reduction of disease severity (%) of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$conidia/ml, 10 µL), and Imazalil (500 mg/L), *E. caryophyllata* (crude extracts 15,000 mg/L,10 µL), *C. longa* crude extracts (30,000 mg/L,10 µL), *C. citratus* crude extracts and *M. charantia* crude extracts (20,000 mg/L, 10 µL) incubated at 25 ^oC for 7 days

Treatment	Disease Incidence (%)	Reduction of disease incidence (%)	Disease severity (%)	Reduction of disease severity (%)
Water (control -)	0.00 a		0.00 a	
Water (control +)	84.40 f		81.00 f	
Imazalil 500mg/L	0.00 a	100.00	0.00 a	100.00
P.digitatum	64.40 cd	23.69	74.30 e	8.27
Ethanol 20%	73.30 e	13.15	71.00 d	12.35
E.caryophyllata	53.30 b	36.85	47.20 b	41.73
C. citratus	66.70 d	20.97	76.90 e	5.06
M.charantia	62.20 c	26.30	54.80 c	32.35
C. longa	66.70 d	20.97	56.80 c	29.88

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 3 replicates. Water (control -) = 10 µL sterile water; Water (control +) = treated with *P. digitatum* ($1x10^5$conidia/ml, 10 µL) + 10 µL sterile water

Figure 15 A. Green mold rot development on citrus fruits which were treated with water (10 µL), B. *P. digitatum* ($1x10^5$conidia/mL, 10 µL), and C. water (10 µL) and *P. digitatum* ($1x10^5$conidia/mL, 10 µL); D. Imazalil (500 mg/L) and *P. digitatum* ($1x10^5$conidia/mL, 10 µL); E. 20% ethanol (10 µL) and *P. digitatum* ($1x10^5$conidia/mL,10 µL); F. *E.caryophyllata* crude extracts (15,000 mg/L,10 µL) and *P. digitatum* ($1x10^5$conidia/mL,10 µL); G. *C. longa* crude extracts (30,000 mg/L,10 µL) and *P. digitatum* ($1x10^5$conidia/mL,10 µL); H. *C.citratus* crude extracts (20,000 mg/L,10 µL) and *P. digitatum* ($1x10^5$conidia/mL, 10 µL); and I. *M. charantia* (20,000 mg/L,10 µL) and *P. digitatum* ($1x10^5$conidia /mL,10 µL) incubated at 25°C for 7 days

Three of promising plant extracts as a result from previous research were tested on citrus fruits for getting two propitious plant extracts. The result revealed that of *E. caryophyllata* crude extracts (15,000 mg/L,10 µL) and *C.longa* crude extracts (30,000 mg/L,10 µL) were two promising plant crude extracts that appeared

reduced the disease incidence by 37.5% and 26.7% respectively, and the disease severity by 10,12% and 7.54% respectively (Table 6, Figure 16).

Table 6 Disease severity (%), disease incidence (%), reduction of disease incidence (%), and reduction of disease severity (%) of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$conidia/mL, 10 µL), 20% ethanol, 10 µL, crude extracts of *E. caryophyllata* (15,000 mg/L,10 µL), crude extracts of *C. longa* (30,000 mg/L,10 µL), crude extracts of *M. charantia* (20,000 mg/L, 10 µL) incubated at 25°C for 7 days

Plant extracs	Disease Incidence (%)	Reduction of Disease Incidence (%)	Disease severity (%)	Reduction of Disease severity (%)
20% ethanol (control)	77.10 d		88.90 b	
E.caryophyllata	48.30 a	37.50	79.90 a	10.12
M. charantia	63.80 c	17.25	87.10 b	2.02
C. longa	57.00 b	26.07	82.20 a	7.54

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 3 replicates.

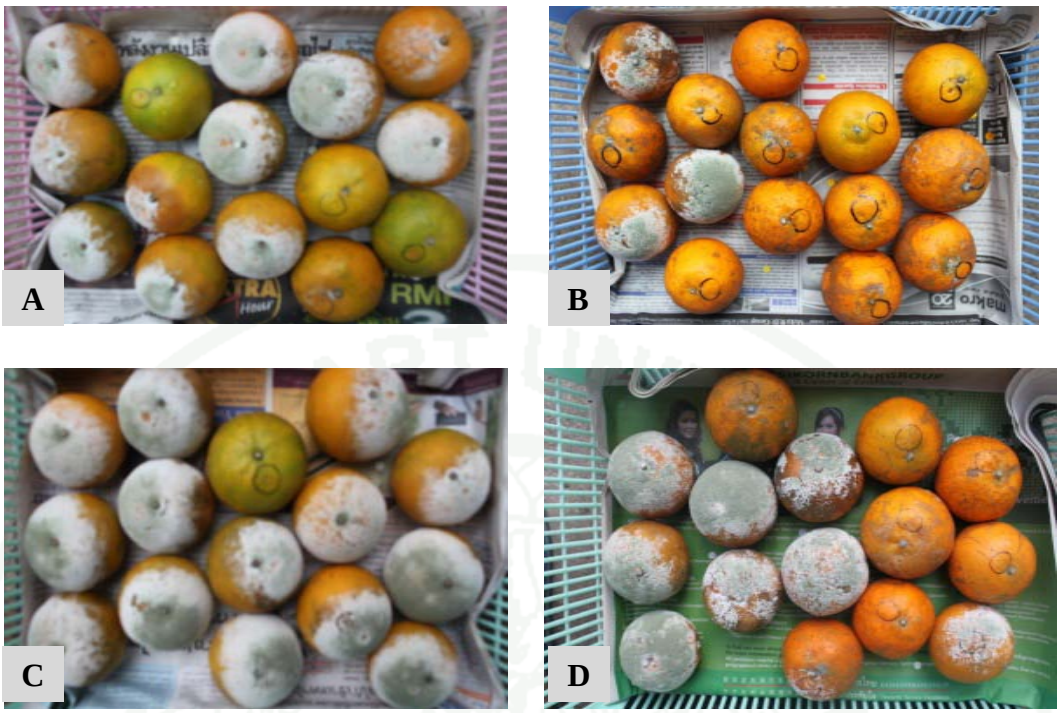


Figure 16 A. Green mold rot development on citrus fruits of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$conidia/ml, 10 µL), 20% ethanol, 10 µL, B. crude extracts of *E. caryophyllata* (15,000 mg/L, 10 µL), C. crude extracts of *C. longa* (30,000 mg/L,10 µL), and D. crude extracts of *M. charantia* (20,000 mg/L, 10 µL) incubated at 25°C for 7 days

Two of promising plant extracts as a result from previous studied was used for this research. Treatment of *E. caryophyllata* crude extracts (15,000 mg/L, 10 µL) reduced the disease incidence of green mold rot on citrus fruit by 26.29% and *C. longa* crude extracts (30,000 mg/L, 10 µL) by 14.43%, while the disease severity of *E.caryophyllata* crude extracts (15,000 mg/L, 10 µL) and *C. longa* crude extracts (30,000 mg/L, 10 µL) were 29.79% and 44.35% respectively. The disease incidence and disease severity of both crude extracts were lower than 20% ethanol (Table 7 and Figure 17).

Table 7 Disease severity (%), disease incidence (%), reduction of disease incidence (%), and reduction of disease severity (%) of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$conidia/ml, 10 µL), ethanol 20%, 10 µL, crude extracts of *E. caryophyllata* (15,000 mg/L, 10 µL), and crude extracts of *C.longa* (30,000 mg/L,10 µL), incubated at 25 ^oC for 7 days

Plant extracts	Disease incidence (%)	Reduction of disease incidence (%)	Disease severity (%)	Reduction of disease severity (%)
20% ethanol (control)	70.00 c		29.20 c	
E.caryophyllata	51.60 a	26.29	20.50 b	29.79
C. longa	59.90 b	14.43	16.25 a	44.35

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 3 replicates

4 *In vitro* yeasts screening for controlling *P. digitatum*

In this study, all of yeasts able to inhibit the hyphal growth of of *P. digitatum.* Five yeasts were selected to be promising yeast for controlling green mold rot disease. The yeasts were reduced of radial growth of *P. digitatum* to more than 48%, which *Candida tropicalis* TISTR 5010 was found to inhibit mycelial growth at 78.8%, and, *Candida guillermondii* BCC 5389, *Candida utilis* TISTR 5001, and *Candida sake* TISTR 5143 were found to inhibit mycelial growth at 69.3, 65, 50, and 48% respectively (Table 8 and Figure 18). Because of growth typical of *Pichia membranefaciens* TISTR 5010 inhibition on *P.digitatum* which can inhibit only in the short time and then *P.digitatum* can grow normaly (a red arrow, Figure 18A). For further research only four yeasts were used namely *Candida tropicalis* TISTR 5010, *Candida guillermondii* BCC 5389, *Candida utilis* TISTR 5001, and *Candida sake* TISTR 5143.

Figure 17 A. Green mold rot development on citrus fruits which were treated with *P. digitatum* ($1x10^5$conidia/ml, 10 µL), ethanol 20%, 10 µL, B. crude extracts of *E.caryophyllata* (15,000 mg/L,10 µL), C. crude extracts of *Curcuma longa* (30,000 mg/L,10 µL), incubated at 25°C for 7 days

Table 8 Effect of yeast on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated technique by 3 cm, incubated at 25°C for 7 days

Treatment	Radial growth inhibition (%)
Candida sake TISTR 5143	48.06b
Candida tropicalis TISTR 5010	78.05e
Pichia membranefaciens TISTR 5093	69.33d
Debaryomyces hansenii TISTR 5143	41.43a
Aureobasidium pullulans TISTR 3384	42.22a
Candida guillermondii BCC 5389	65.12c
Candida utilis TISTR 5001	50.49b

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of three replicates.

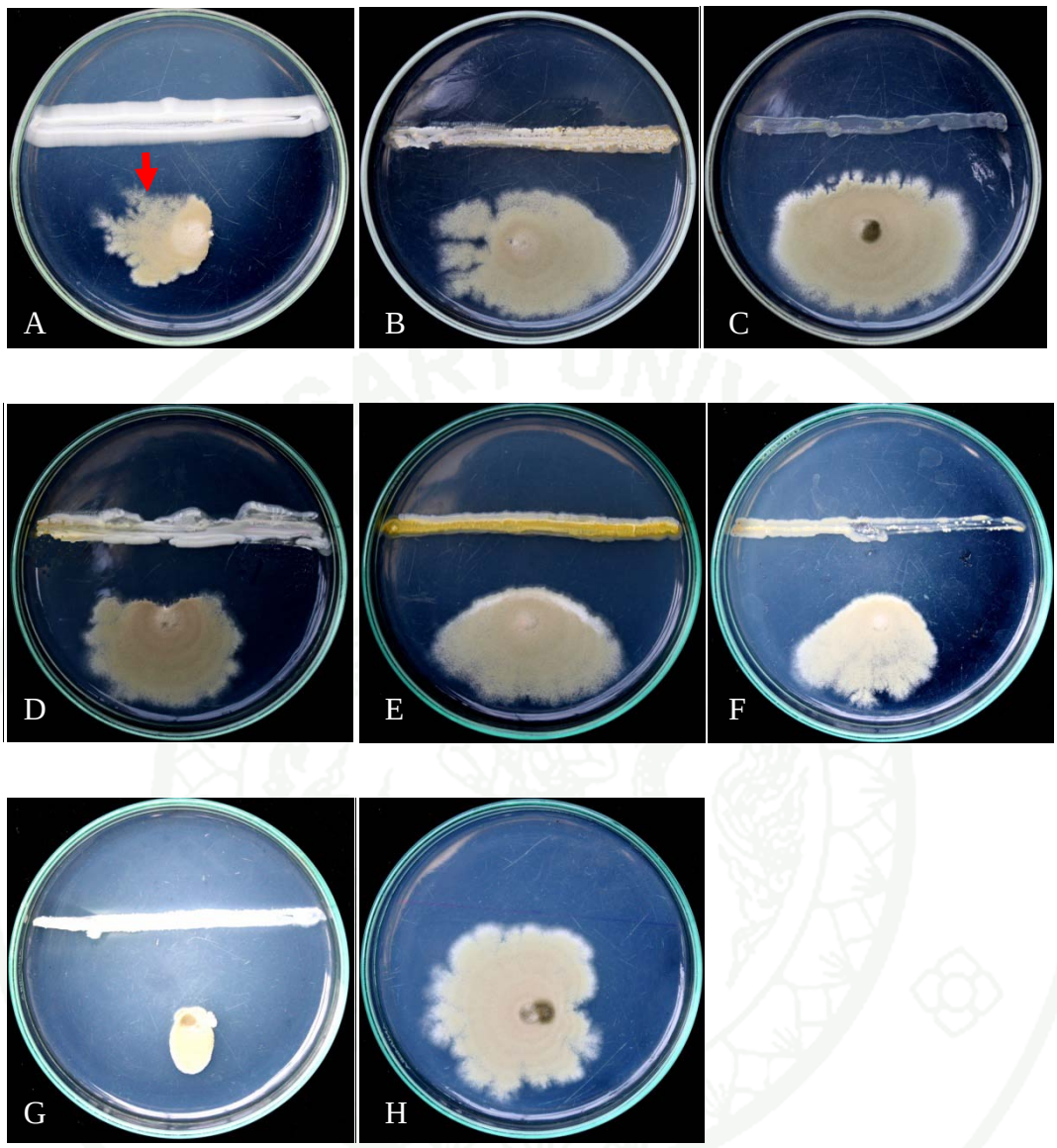


Figure 18 Effect of yeast on hyphal radial growth inhibition of *P. digitatum* with dual culture technique segregated by 3 cm, incubated at 25 ^{o}C for 7 days. A. *P. membranefaciens* TISTR 5093, B. *A. pullulans* TISTR 3384, C. *D. hansenii* TISTR 5155, D. *C. sake* TISTR 5143, E. *C. utilis* TISTR 5001, F. *C. guillermondii* BCC 5389, G. *C. tropicalis* TISTR 5010, and H. Control. An red arrow was *P. digitatum* which growth normally

To know whether yeasts had integrated effect, in this studied the2 yeasts mixed and streaked on medium along 3 cm to *P. digitatum.* The result revealed that mixing of 2 yeasts was not as effective as individual yeast. *C. tropicalis* TISTR 5010 and *Candida utilis* TISTR 5001 were two promising yeast which were 82% and 29% inhibition of *P. digitatum* respectively (Table 9, Figure 19).

Table 9 Effect of yeast(s) individual or in combination on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated by 3 cm, incubated at 25^{o}C for 7 days

Treatment	Inhibition growth of *P. digitatum* (%)
C. tropicalis TISTR 5010 (Ct)	82.42 f
C. utilis TISTR 5001 (Cu)	29.12c
C. sake TISTR 5143 (Cs)	26.59bc
C. guillermondii BCC 5389 (Cg)	17.58a
CtxCu	76.92e
CtxCs	75.82e
CtxCg	79.12e
CuxCs	25.27bc
CuxCg	45.05d
CsxCg	24.73b

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 3 replicates

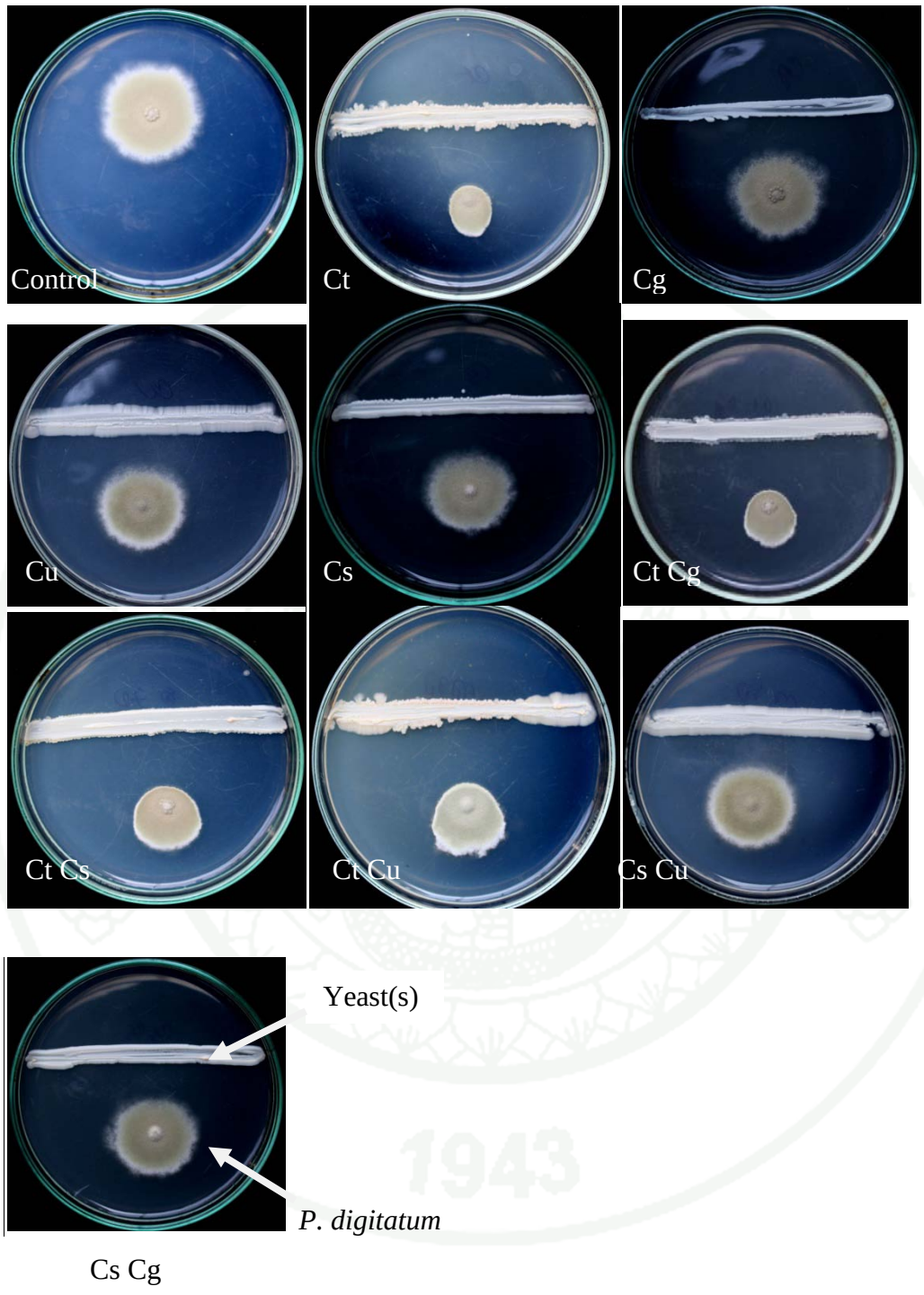
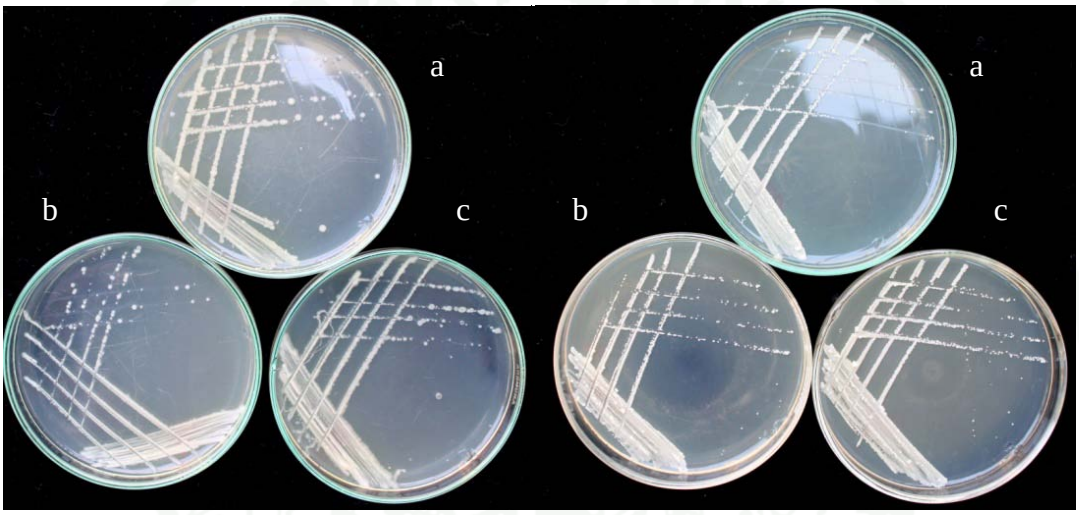


Figure 19 Effect of yeast(s) individual and in combination on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated by 3 cm, incubated at 25°C for 7 days . *C. tropicalis* TISTR 5010 (Ct), *C.utilis* TISTR 5001 (Cu), *C. sake* TISTR 5143 (Cs), *C.guillermondii* BCC 5389 (Cg)

Two promising yeasts as a result from previous studied were tested. To recognize whether *C.utilis* TISTR 5001 and *C. tropicalis* TISTR 5010 could growth at medium plus plant extracts, both yeasts were grown on PDA plus *C. longa* crude extracts 30,000 mg/L or *E. caryophyllata* crude extracts 15,000mg/L. The result showed that C. *utilis* TISTR 5001 and *C. tropicalis* TISTR 5010 could grow on those media (Figure 20).



Candida utilis TISTR 5001 *Candica tropicalis* TISTR 5010

Figure 20 Effect plant extracts on yeast growth, a. Control (PDA), b. *C. longa* crude extracts 30,000 mg/L on PDA, and c. *E. caryophyllata* crude extracts 15,000mg/L on PDA

5 *In vivo* antagonist - plant extracts for controlling *P.digitatum* on citrus fruit

All of the combinations of plant crude extracts and yeast could reduce the disease incidence in excess of 70%. The combination of *E. caryophylata* crude extracts at 15,000 mg/L, 10 µL, and *C. utilis* TISTR 5001 at $1x10^8$ cells/mL, 10 µL, was established to be the best combination to attain a reduction in disease incidence of 90.3%, while the combinations of *Curcuma longa* crude extracts at 30,000 mg/L, 10 µL, and *C. utilis* TISTR 5001 at $1x10^8$ cells/mL, 10 µL; *E. caryophylata* crude extracts at 15,000 mg/L, 10 µL, and *C.tropicalis* TISTR 5010 at $1x10^8$ cells/mL, 10 µL; *C. longa* crude extracts at 30,000 mg/L, 10 µL, and *C.tropicalis* TISTR 5010 at $1x10^8$ cells/mL, 10 µL , resulted in a reduction in the disease incidence at 80.7, 77.4, and 71%, respectively (Table 10 and Figure 21). Likewise, for the disease severity, the combination of *E.caryophylata* crude extracts at 15,000 mg/L, 10 µL, and *C. utilis* TISTR 5001 at $1x10^8$ cells/mL, 10 µL, was found to be the best combination to attain a reduction in disease severity by 96.26%, while the combinations of *C. longa* crude extracts at 30,000 mg/L, 10 µL, and *C. utilis* TISTR 5010 at $1x10^8$ cells/mL, 10 µL; *E. caryophylata* crude extracts at 15,000 mg/L, 10 µL, and *C.tropicalis* TISTR 5010 at $1x10^8$ cells/mL, 10 µL; *C. longa* crude extracts at 30,000 mg/L, 10 µL, and *C.tropicalis* TISTR 5010 at $1x10^8$ cells/mL, 10 µL resulted in a reduction in the disease severity by 93.2, 90.3, and 89.4%, respectively (Table 10 and Figure 22).

Table 10 Effect of *E. caryophylata* crude extracts at 15,000mg/L, 10 µL, *C. longa* crude extracts at 30,000mg/L,10 µL, *C. utilis* TISTR 5001 at $1x10^8$ cells/mL, and 10 µL *C. tropicalis* TISTR 5010 at $1x10^8$ cells/mL, 10 µL to green mold rot incidence and severity, and reduction of disease incidence and severity, incubated at 25°C for 7 days

Plant extracts + Yeast	Disease Incidence (%)	Reduction of Disease Incidence (%)	Disease severity (%)	Reduction of Disease severity (%)
E. caryophylata x *C. utilis*	6.66 a	90.30	1.87a	96.26
C.longa x *C. utilis*	13.32 ab	80.70	3.43a	93.20
E. caryophylata x *C. tropicalis*	15.55 abc	77.43	4.87a	90.30
C. utilis	17.78 bc	74.18	5.07a	89.87
C. longa x *C. tropicalis*	19.97 bc	71.00	5.30a	89.40
C.tropicalis	22.20 bc	67.77	6.73a	86.61
E. caryophylata	24.42 c	64.55	9.87ab	80.28
C. longa	33.30 d	51.66	20.33b	59.38
Control	68.88e		50.05c	

Values with the same letters in coloumn were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of three replicates.

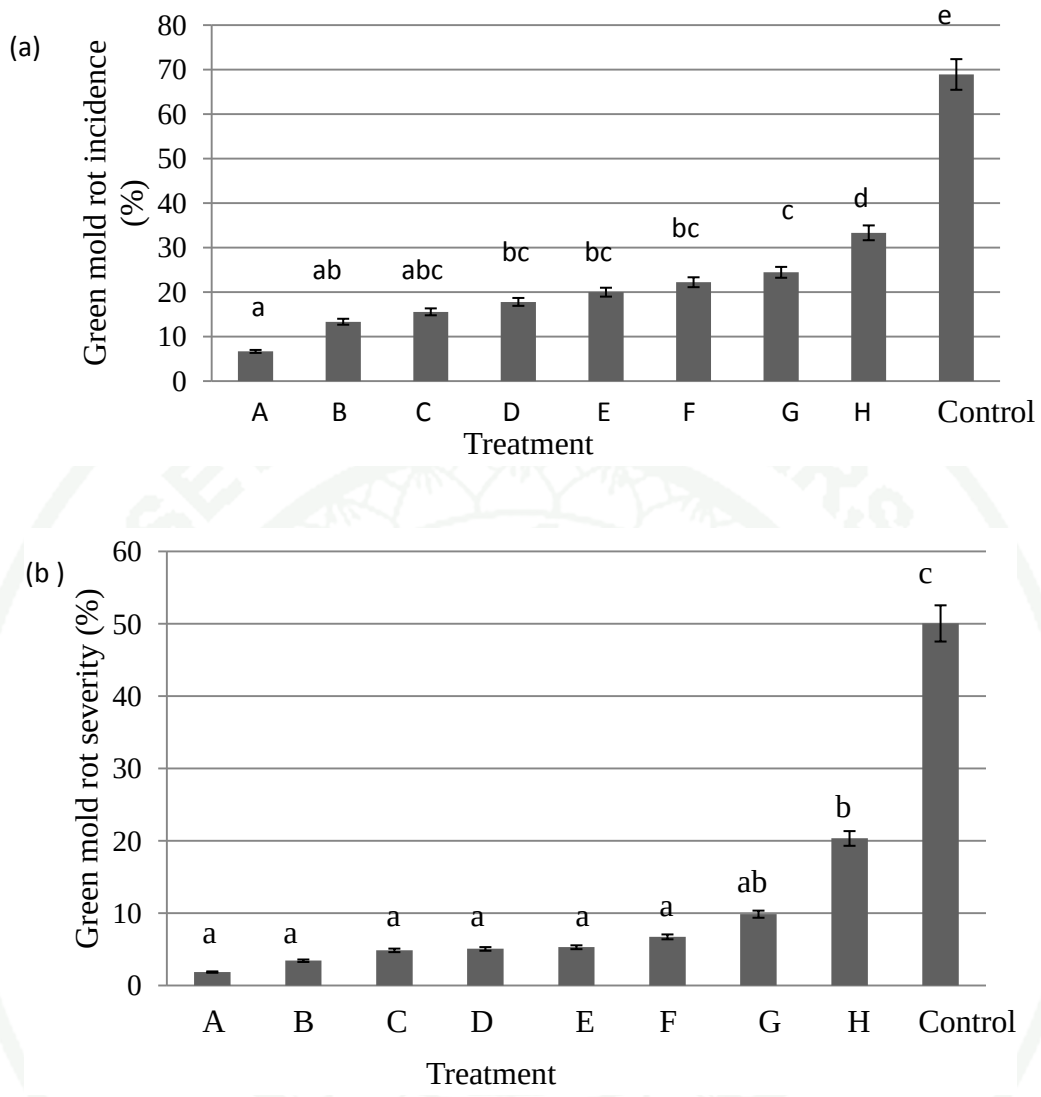


Figure 21 Effect of plant extracts (*E. caryophylata* crude extracts at 15,000 mg/L, and *C. longa* crude extracts at 30,000mg/L) and yeasts (*C. utilis* TISTR 5001 at $1x10^8$ cells/mL and *C. tropicalis* TISTR 5010 at $1x10^8$ cells/mL) on control of green mold rot (*P. digitatum*) incidence (a) and disease severity (b) in wounded citrus fruit, incubated at 25 ^oC and 95% RH for 7 days. A. *E. caryophylata* and *C. utilis;* B. *C. longa* and *C. utilis*; C. *E. caryophylata* and *C. tropicalis* ; D. *C. utilis* ; E. *C. longa* and *C.tropicalis*; F. *C.tropicalis*; G. *E. caryophylata*; H. *C. longa*. Each value is mean of 3 replicates. Bars represent the standard deviations of the mean. Statistical significance determined at $P<0.05$ according to Tukey's HSD test.

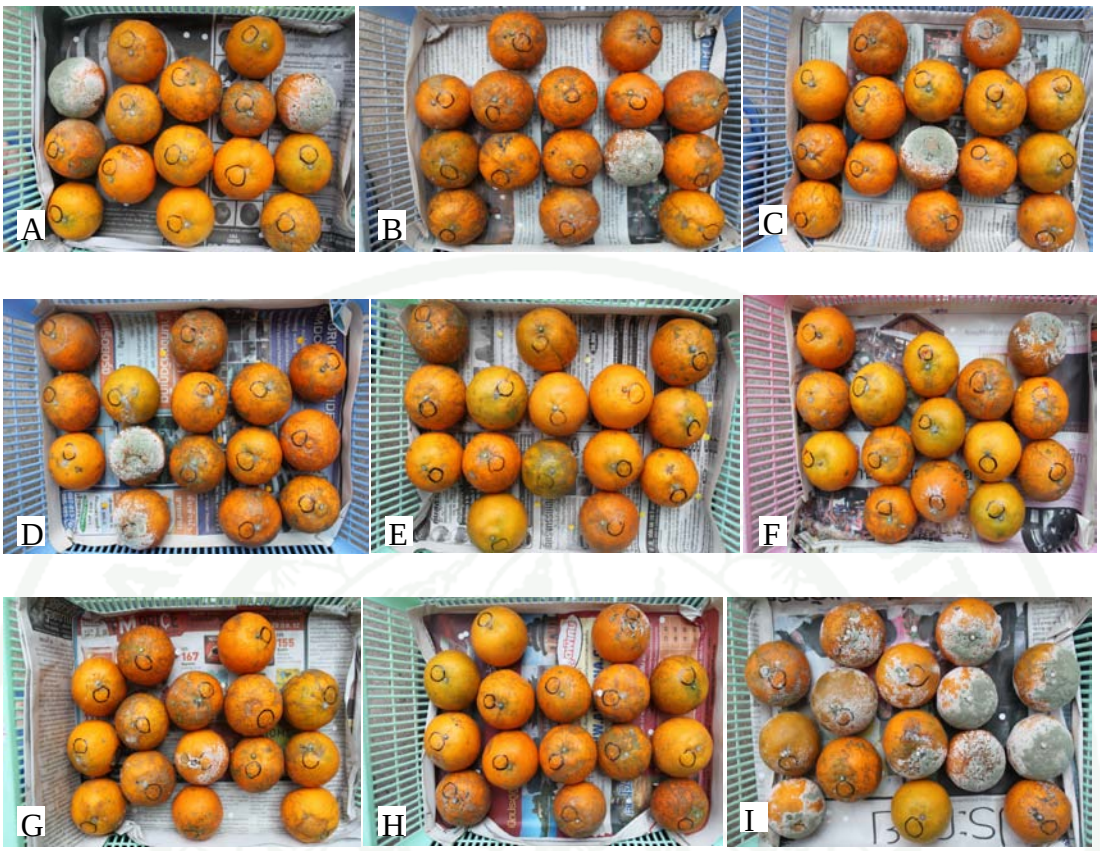


Figure 22 Effect of *E.caryophylata* crude extracts at 15,000mg/L, 10 µl, *C. longa* crude extracts at 30,000mg/L,10 µL (TM), *C. utilis* TISTR 5001 at $1x10^8$ cells/mL, 10µL (Cu) , and *C.tropicalis* TISTR 5010 at $1x10^8$ cells/mL ,10µL (Ct) and their combination to green mold rot, incubated at 25 ^oC for 7 days. A. Treatments of Cu and Clove, B. Ct and Clove, C. Clove, D. Cu and TM, E. Ct and TM, G. Ct, H. Cu, and I. control.

Since combination of *E. caryophylata* crude extracts at 15,000mg/L, 10 µL and *C. utilis* TISTR 5001 $1x10^8$ cells/mL, 10 µL was the best, it was compared with imazalil at several concentrations. It showed that the combination of *E. caryophylata* crude extracts at 15,000mg/L, 10 µL and *C. utilis* TISTR 5001 $1x10^8$ cells/mL, 10µL had higher antifungal activity than imazalil. The combination treatment of *E. caryophylata* crude extracts and *C. utilis* TISTR 5001 $1x10^8$cells/mL demonstrated the disease incidence was the lowest (31.10%) and had highest reduction of disease incidence of 68.9% and Imazalil 150 mg/L was 42.2% (Table 11, Figure 23). It also

reduced the disease severity by 73.39% while in imazalil 150 mg/L by 61.9% (Table 12, Figure 23).

Table 11 Comparative treatment among *E. caryophylata* crude extracts at 15,000mg/L, 10µL, *C. utilis* TISTR 5001 1x10^8cells/mL, 10µL, and their combination, with imazalil on green mold rot incidence (%) and reduction of disease incidence on citrus fruits, incubated at 25^{o}C for 7 days

Treatment	Disease Incidence (%)	Reduction of Disease incidence (%)
Imazalil 50 mg/L	95.57 f	4.43
Imazalil100 mg/L	71.10 e	28.90
Imazalil 150 mg/L	57.80 c	42.20
E. caryophyllata	53.30 b	46.70
E. caryophyllata + C.utilis	31.10 a	68.90
C. utilis	64.43 d	35.57
Water (control)	100.00 g	
20% ethanol	100.00 g	0.00

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 3 replicates

Table 12 Comparative treatment between *E. caryophylata* crude extracts at 15,000 mg/L, 10µL , *C. utilis* TISTR 5001 1x10^8 cells/mL, 10µL, and their combination, with imazalil on green mold rot severity (%) and reduction of disease severity on citrus fruits, incubated at 25^{o}C for 7 days

Treatment	Disease Severity (%)	Reduction of Disease severity (%)
Imazalil 50 mg/L	44.00 e	20.63
Imazalil100 mg/L	25.87 c	53.34
Imazalil 150 mg/L	21.10 b	61.94
E. caryophyllata	26.43 c	52.31
E. caryophyllata + C.utilis	14.47 a	73.90
C. utilis	28.43 c	48.70
Water (control)	55.43 f	
20% ethanol	34.77 d	37.28

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each values was mean of 3 replicates

Figure 23 Effect of Imazalil, *E. caryophylata* crude extracts at 15,000mg/L, 10 µL, and *C. utilis* TISTR 5001 1x10^{8}cells/mL, 10 µL, 20% ethanol, 10 µL and water, 10 µL to control of green mold rot severity incubated at 25^{o}C for 7 days

Combination of *E. caryophylata* crude extracts and *C. utilis* TISTR 5001 reduced natural development of green mold rot incidence by 90 % and severity by 86%, while imazalil 150 mg/L, and 20 % ethanol, by 10 % and 5 % of disease incidence and 25.4 %, 8.76 % of disease severity two weeks after storage. (Figure 24 and 25).

E. caryophylata + *C. utilis* Imazalil 150 mg/L

Water 20% ethanol

Figure 24 Effect of *E.caryophylata* crude extracts at 15,000mg/L, and *C.utilis* TISTR 5001 at 1x10^{8}cells/mL for reducing natural rot development on 2 weeks storage compared with water and 20% ethanol

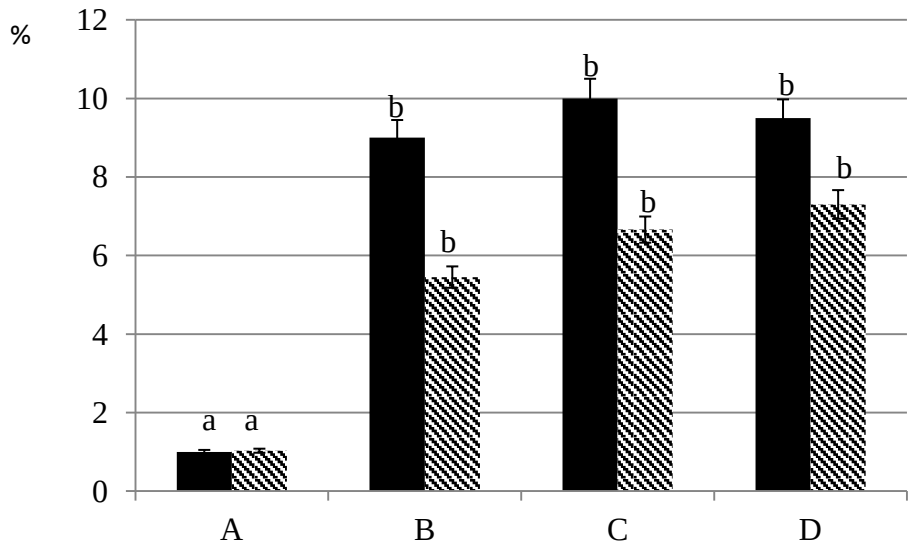


Figure 25 Effect of *E. caryophylata* crude extracts at 15,000mg/L and *C. utilis* TISTR 5001 at $1x10^8$cells/mL for reducing natural rot development of the disease incidence (solid box) and disease severity (shadow box). A. *E. caryophylata* crude extracts at 15,000mg/L and *C. utilis* TISTR 5001 at $1x10^8$cells/mL, B. Imazalil 150 mg/L, C. Water (control), D. 20% ethanol. Each value is mean of 4 replicates. Bars represent the standard deviations of the mean. Statistical significance determined at P<0.05 according to Tukey's HSD test.

6 Fruit Quality

The weight loss was not significant different ($p < 0.05$) among water (control, 3.84%) and *E.caryophyllata* crude extracts 15,000 mg/L and *C. utilis* TISTR 5001 $1x10^8$cells/mL, imazalil 150 mg/L and 20% ethanol by 1.96%, 1.91%, and 2.17% respectively. Similar result was in the juice content, all of juice content in treatment fruits were not significant different (<0.05) compared with before storage. The juice content before storage was 40.61% while *E.caryophyllata* crude extracts 15,000 mg/L and *C. utilis* TISTR 5001 $1x10^8$cells/mL, imazalil 150 mg/L and 20% ethanol by 36.53%, 36.73%, and 36.2% respectively. On the other hand, total soluble solid, fruit

firmness, and ascorbic acid content were higher than before storage, while titratable acidity was decrease 2 week after storage (Table 13).

Population of *P. digitatum* on citrus fruits was observed by cutting the citrus peel in the wounded area around 1 x 1 cm and shook in 10 mL of sterile water, and then cultured on PDA medium. The population of *P. digitatum* in 1, 2, and 3 day(s) was the lowest in the treatment of combination of *E.caryophyllata* crude extracts 15,000 mg/L, 10 µL and *C. utilis* TISTR 5001 $1x10^8$cells/mL, 10 µL by 1.4×10^4, 8.6×10^4, and 2.1×10^5 respectively, while population of *P. digitatum* in individual treatment was highest (Figure 26).

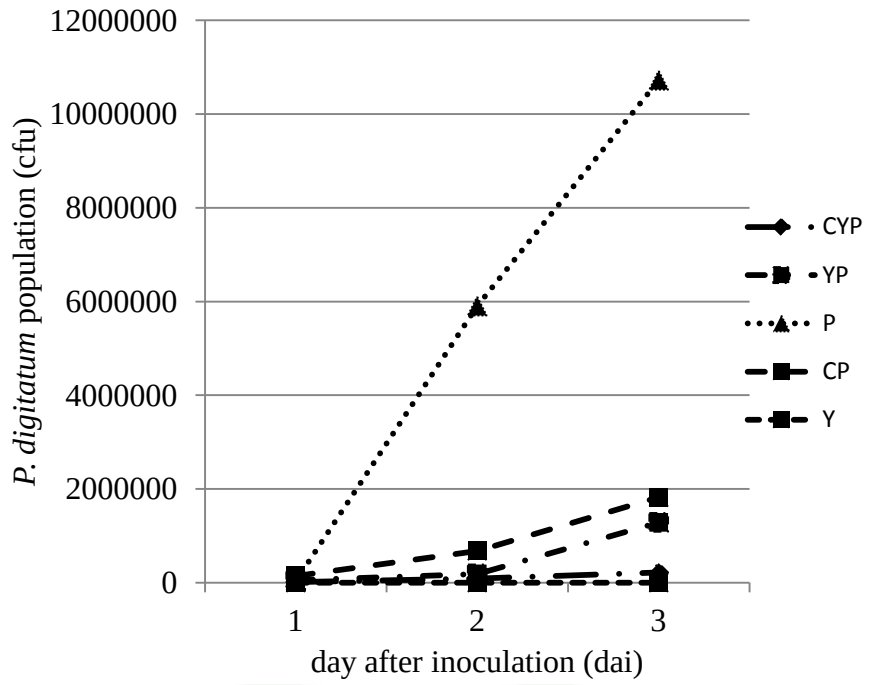


Figure 26 *P. digitatum* colonization on citrus fruits 1, 2, and 3 day(s) after treated with yeasts and plant extracts, incubated at 25°C. C= *E.caryophyllata* crude extracts 15,000 mg/L, 10 µL, Y= *C. utilis* TISTR 5001 $1x10^8$cells/mL, 10 µL, P= *P.digitatum* $1x10^5$ conidia/mL, 10 µL

Table 13 Effect of *E. caryophylata* and *C. utilis* TISTR 5001 on postharvest qualities of citrus fruits

Treatment	Weight loss(%)	Fruit Firmness (kg)	Juice content (%)	Total Soluble Solid (%)	Titratable Acidity (%)	Ascorbic Acid Content (mg/100g)
E. caryophylata and *C. utilis*	1.96 a	1.59 b	36.53 a	9.15 b	0.33 a	16.61 b
Imazalil 150 mg/L	1.91 a	1.60 b	36.73 a	9.80 b	0.19 a	15.01 b
Water	2.17 a	1.51 b	36.20 a	9.70 b	0.18 a	15.09 b
Ethanol 20%	3.84 a	1.57 b	37.74 a	9.65 b	0.19 a	16.15 b
Before storage		1.34 a	40.61 a	5.90 a	1.73 b	12.15 a

Values with the same letters in coloumn were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each value was mean of 4 replicates.

Population of *C. utilis* TISTR 5001 on citrus fruits was observed by cutting the citrus peel in the wounded area around 1 x 1 cm and shook in 10 mL of sterile water, and then cultured on PDA medium showed the lowest at the treatment of mixture of *C. utilis* TISTR 5001 ($1x10^8$cells/mL, 10 µL) and *E. caryophyllata* crude extracts (15,000mg/L, 10 µL) at 1, 2, and 3 days after inoculation by $4x10^3$, $2x10^5$, and $2.4x10^5$ cfu respectively. While *C. utilis* treatment alone without any other treatment had the highest population, population were 5.3×10^4, 3.9×10^5, and 7×10^5 cfu respectively (Figure 27).

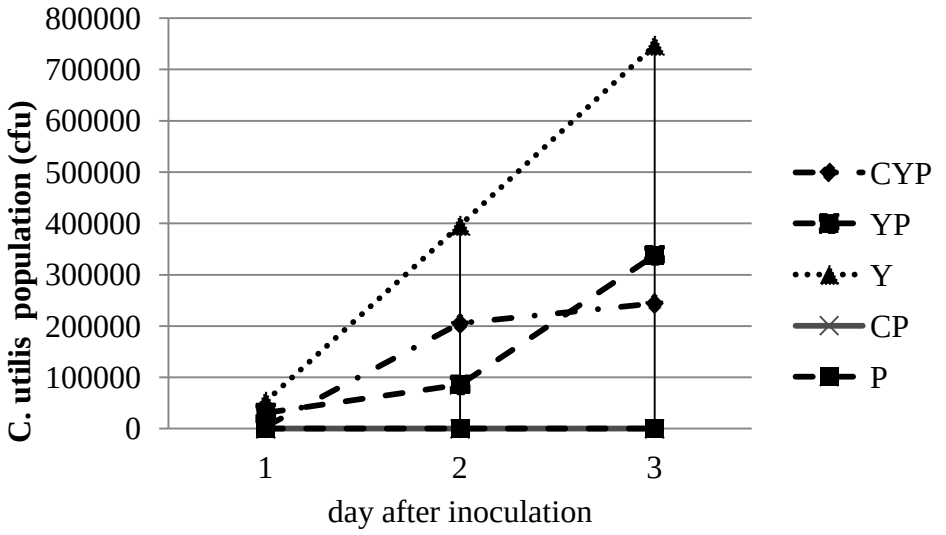


Figure 27 *C. utilis* colonization on citrus fruits 1, 2, and 3 day(s) after treated by yeasts and plant extracts and incubated at 25°C, C= *E.caryophyllata* crude extracts 15,000 mg/L, 10 µL, 10 µL, Y= *C. utilis* TISTR 5001 $1x10^8$cells/mL, 10 µL, P= *P.digitatum* $1x10^5$ conidia/mL, 10 µL

Citrus peels were cultured both on PDA and YMA media to observe *P. digitatum* and yeasts colonization in the wound site after 2, 4, 6, and 8 h after application. *P. digitatum* colonization was revealed to be the lowest on citrus treated with the combination of *E. caryophylata* crude extracts at 15,000 mg/L, 10 µL, and *C. utilis* TISTR 5001 $1x10^8$ cells/mL, 10 µL (Figure 28). Survival of *C. utilis* was found to be the second on fruits treated with *E. caryophylata* crude extracts at 15,000 mg/L, 10 µL, and *C. utilis* TISTR 5001 $1x10^8$ cells/mL, 10 µL, while population of

C. utilis was highest in treatment of *C. utilis C. utilis* TISTR 5001 1x10^8 cells/mL, 10 µL and *P. digitatum* 1x10^5 conidia/mL, 10 µL (Figure 29) As higher colonization of the antagonist resulted in lower the pathogen colonization in citrus wound, thus depicting a strong relationship (R^2 = 0.8287) (Figure 30).

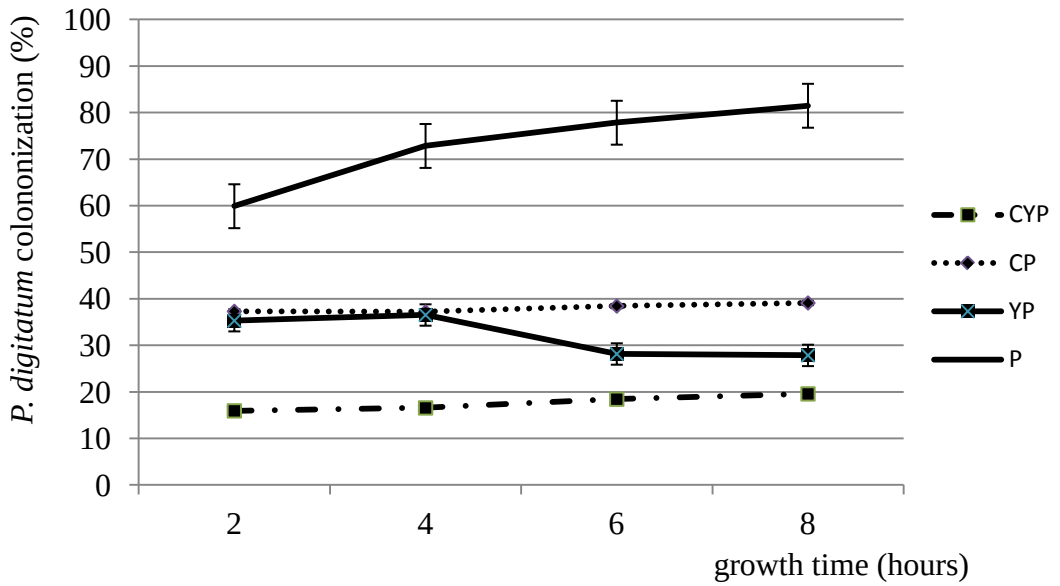


Figure 28 *P. digitatum* colonization on citrus peel, treated with *E. caryophylla* crude extracts at 15,000 mg/L, 10 µL and *C. utilis* TISTR 5001 1x10^{8}cells/mL, 10 µL at 2, 4, 6, and 8 h after inoculation at 25^{o}C. Each value is mean of five replicates. C= *E.caryophyllata* crude extracts 15,000 mg/L, 10 µL, Y= *C. utilis* TISTR 5001 1x10^{8}cells/mL, 10 µL P= *P.digitatum* 1x10^5 conidia/mL, 10 µL

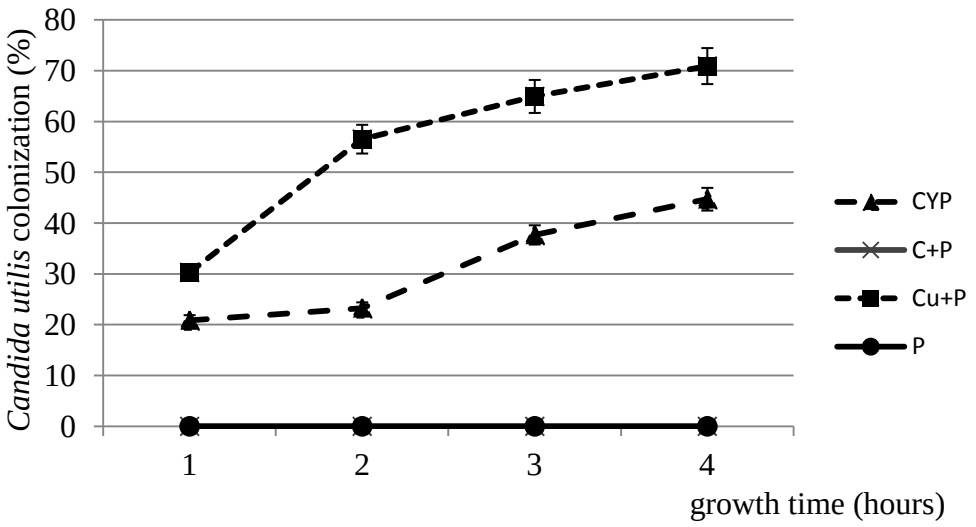


Figure 29 *C. utilis* colonization on citrus peel, treated with *E. caryophylla* crude extracts at 15,000 mg/L, 10 µL and *C. utilis* TISTR 5001 1x10^{8}cells/mL, 10 µL at 2, 4, 6, and 8 h after inoculation at 25^{o}C. Each value is mean of five replicates. C= *E.caryophyllata* crude extracts 15,000 mg/L, 10 µL, Y= *C. utilis* TISTR 5001 1x10^{8}cells/mL, 10 µL P= *P.digitatum* 1x10^5 conidia/mL, 10 µL

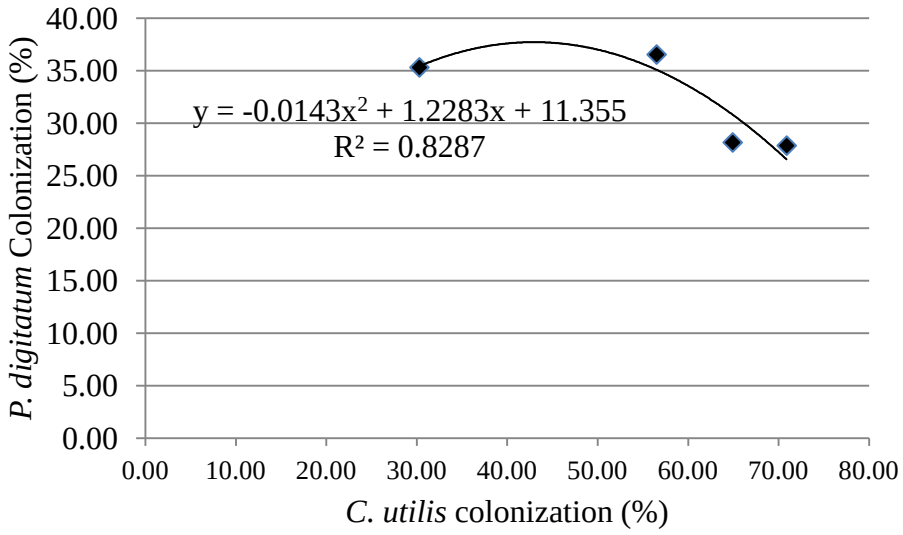


Figure 30 Relationship between *P. digitatum* and *C. utilis* colonization on citrus peel. Each value is mean of 5 replicates

The possibility of mode of action of *E.caryophyllata* crude extracts and *C. utilis* TISTR 5001 to inhibit *P. digitatum* growth had been tested on PDA by poisonous food technique. The result showed that *P. digitatum* was cultured on PDA plus *E.caryophyllata* 5000 mg/L or PDA plus *E.caryophyllata* 5000 mg/L+ 2% citrus juice was completely inhibited mycelium growth, while *P. digitatum* which was cultured on PDA or/and plus 2% citrus juice was inhibited by *C. utilis* and *E.caryophyllata* by 64.83 and 16.42% respectively (Table 14).

The result showed that *P. digitatum* which was cultured with *C. utilis* on PDA and PDA + 2% citrus juice grew normally (Figure 31 and 32), but when *P. digitatum* was cultured on PDA plus *E. caryophylata* crude extracts at 5,000mg/L and 2% citrus juice grew but subsequent died (Figure 36 C and D). It was indicated by cytoplasmic coagulation and vesiculation of hyphal content (Fig. 36 E and F). However, this result was better than *P. digitatum* growth on PDA plus 5000 mg/L *E.caryophyllata* without citrus juice. *P. digitatum* did not germinate (Figure 34B) and the number of alive conidia also reduced (Figure 34C). On the other hand, *Candida utilis* was still alive as shown by staining method (Figure 35).

Table 14 Effect of *C. utilis* TISTR 5001, *E. caryophylata* crude extracts at 5,000mg/L, and their combination on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated by 3 cm, incubated at 25^{o}C for 7 days

Treatment	inhibition zone (%)
PDA	64.83 b
PDA+2% citrus juice	16.42 a
PDA+5,000 mg/L *E.caryophyllata*	100.00 c
PDA+5,000 mg/L *E.caryophyllata* +2% citrus juice	100.00 c

Values with the same letters were not significant different from each other based on the Tukey'S HSD Multiple range test (p=0.05). Each value was mean of 3 replicates

Figure 31 *P. digitatum* on PDA (A). The microscopic view of *P. digitatum* hyphae (1000x), under natural light source (B) and Fluorescent light source (C), The fluorescent of *P. digitatum* hypal indicated that *P. digitatum* was alive (showed by an arrow). Bar indicated 10 µm

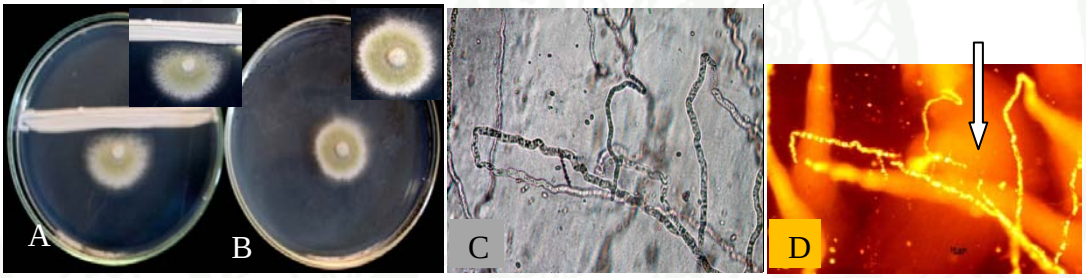


Figure 32 A. Dual culture *P. digitatum* and *Candida utilis* TISTR 5001 on PDA , B. control. The hyphae of *P. digitatum* treated with the fluorescent stain with nile red, viewed by fluorescence microscope, C. the microscopic view (400x), under natural light source, and D. fluorescent light source. The fluorescent of *P. digitatum* conidia indicated that the conidia were alive (showed by an arrow). Bar indicated 10 µm

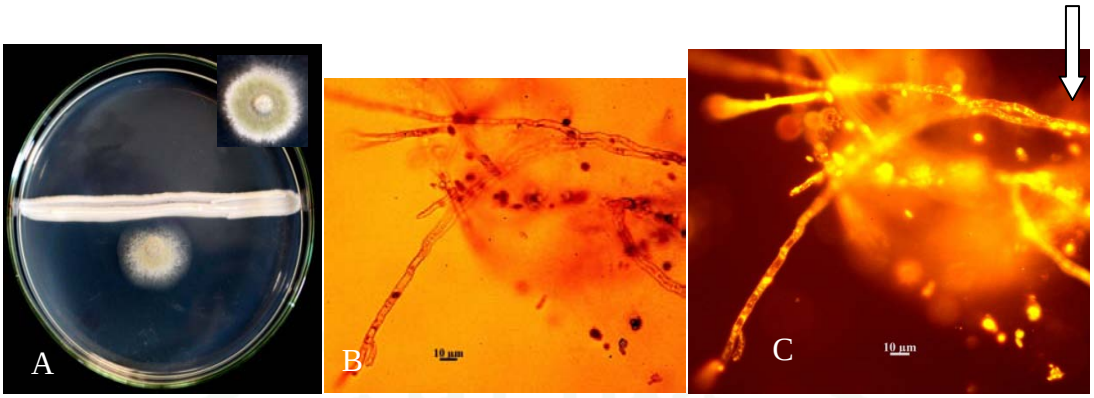


Figure 33 A. Dual culture *P. digitatum* and *Candida utilis* TISTR 5001 on PDA + 2% citrus juice. The hyphae of *P. digitatum* treated with the fluorescent stain with nile red, viewed by fluorescence microscope, B. the microscopic view (400x), under natural light source, and C. fluorescent light source. The fluorescent of *P. digitatum* conidia indicated that the conidia were alive (showed by an arrow). Bar indicated 10 µm..

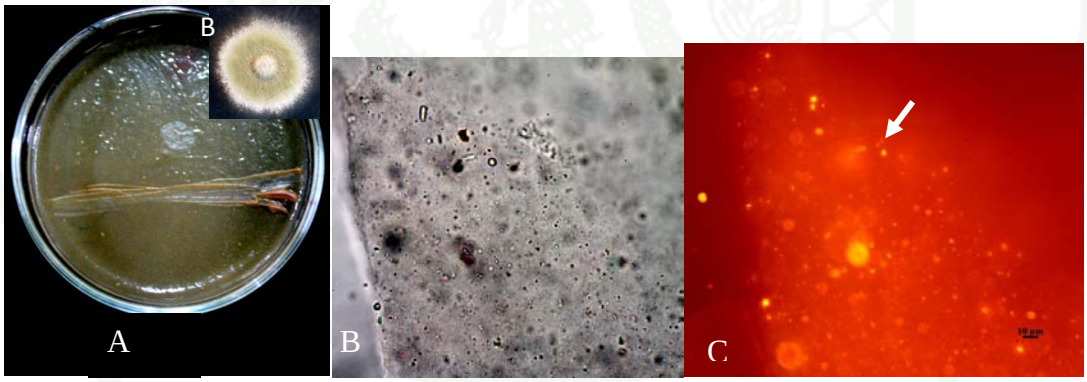


Figure 34 A. Dual culture *P. digitatum* and *Candida utilis* TISTR 5001 on PDA + 5000 mgL^{-1} *E.caryophyllata* crude extracts, B. Control. The microscopic view of *Candida utilis* TISTR 5001 (400x), C. under natural light source, and D. Fluorescent light source. The fluorescent of *P. digitatum* conidia indicated that the conidia were alive (showed by an arrow). Bar indicated 10 µm.

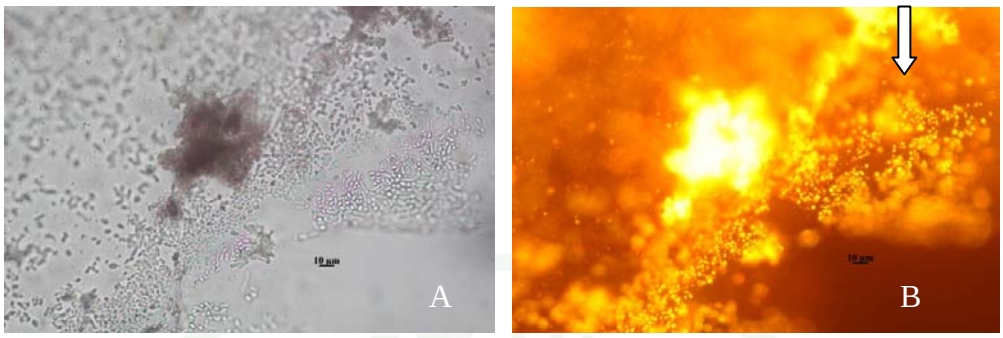


Figure 35 The *Candida utilis* TISTR 5001were cultured on PDA + *E.caryophyllata* crude extracts. The microscopic view of *Candida utilis* TISTR 5001 (400x), A. under natural light source, and B. Fluorescent light source. The fluorescent of *C. utilis* TISTR 5001 cells indicated that the conidia were alive (showed by an arrow). Bar indicated 10 µm

7 Bioassay of antifungal compound on the TLC plate

E.caryophyllata crude extracts was loaded on TLC (thin layer chromatography) plate for separation of antifungal compound. The location of antifungal compound was at Rf 0.73 (Figure 38), indicated by clear zone or white area where the fungi that tested did not grow. The fungus were used namely C*ladosporium cladosporoides* (Figure 38A) and *P. digitatum* (Figure 38B). on the other hand the location of active compound showed blue colour after sprayed with anisaldehide (Figure 38 C). In addition the position clearly showed on UV lamp with 254 nm and 354 nm (Figure 39 A-B).

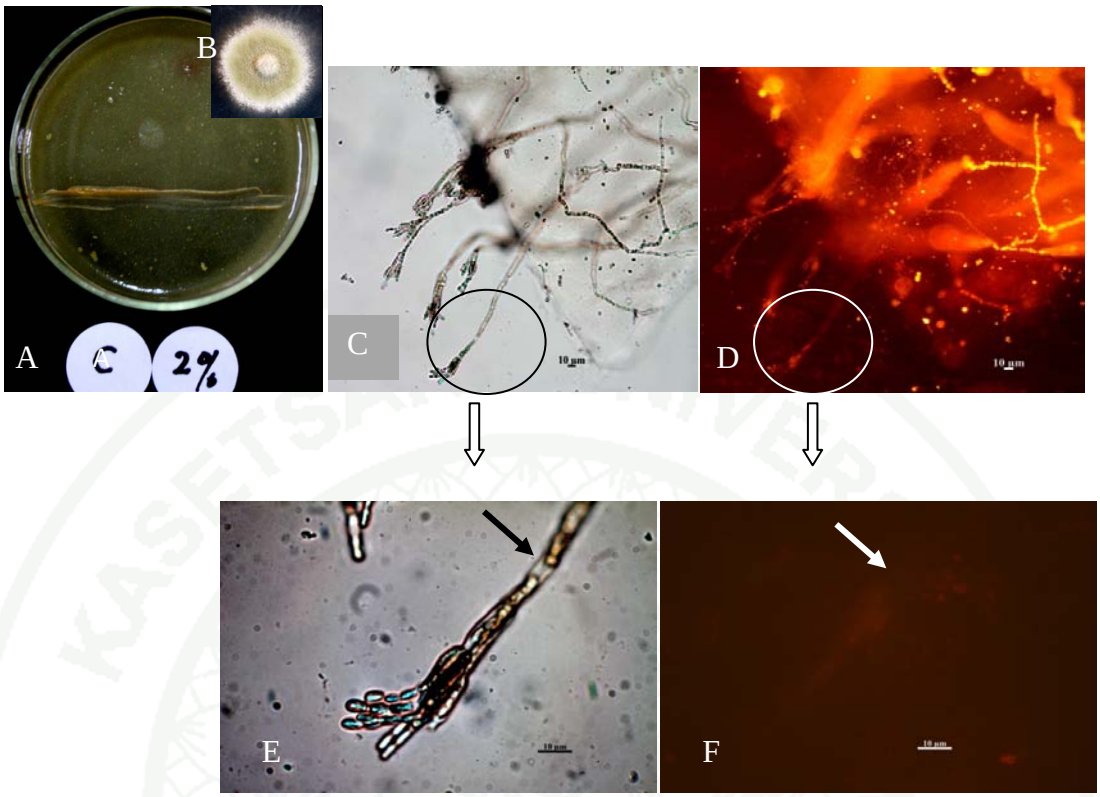


Figure 36 A. Dual culture *P. digitatum* and *Candida utilis* on PDA +5000 mgL^{-1} *E.caryophyllata* crude extracts + 2% citrus juice. B. Control. The microscopic view of *P. digitatum* under natural light source, C. 200x, and E. 1000x, and D. fluorescent light source (200x), and F. 1000x. Cytoplasmic coagulation and vesiculation on hyphae were indicated by circle . The gold fluorescent absent in the hypha of *P. digitatum* indicated that the pathogen was dead (showed by a white arrow). Bar indicated 10 µm

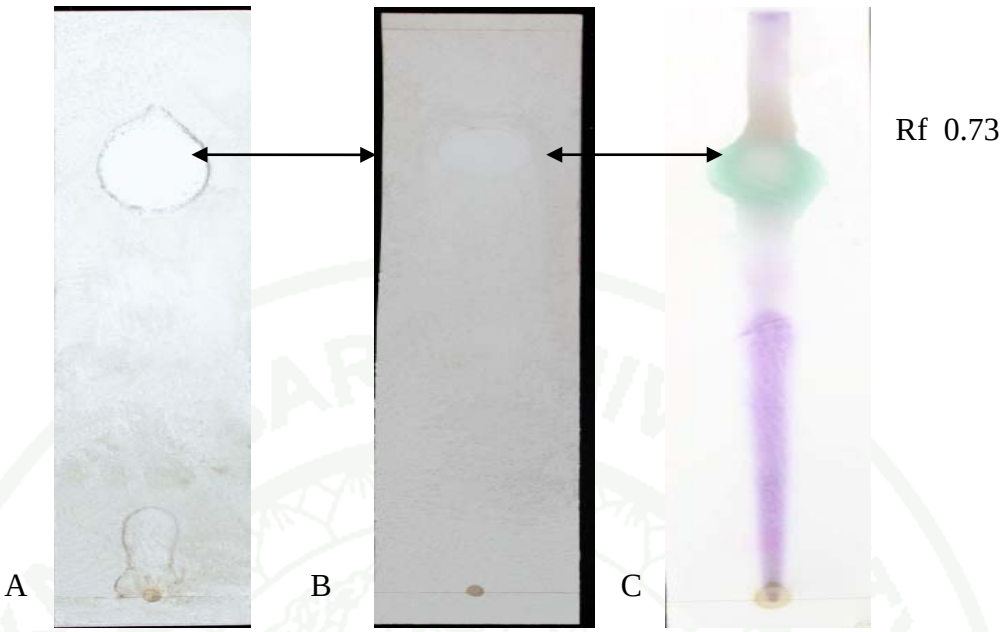


Figure 37 A. Antifungal activity observed by direct spraying of spore of C*ladosporium cladosporoides* spores and B. *P. digitatum*, and C. anisaldehyde on *Eugenia caryophyllata* crude extracts. Solvent system Acetone: Dichloromethane: Toluene = 10:60:30

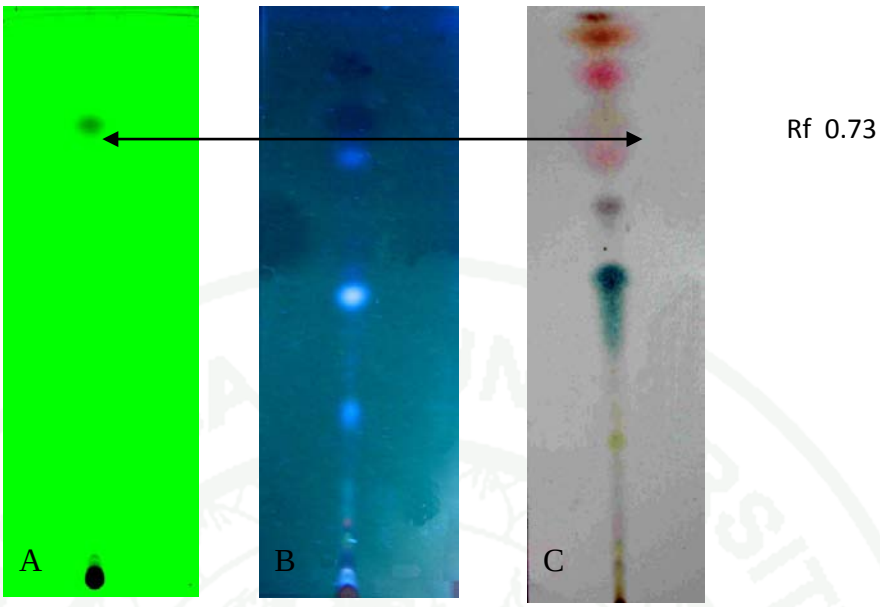
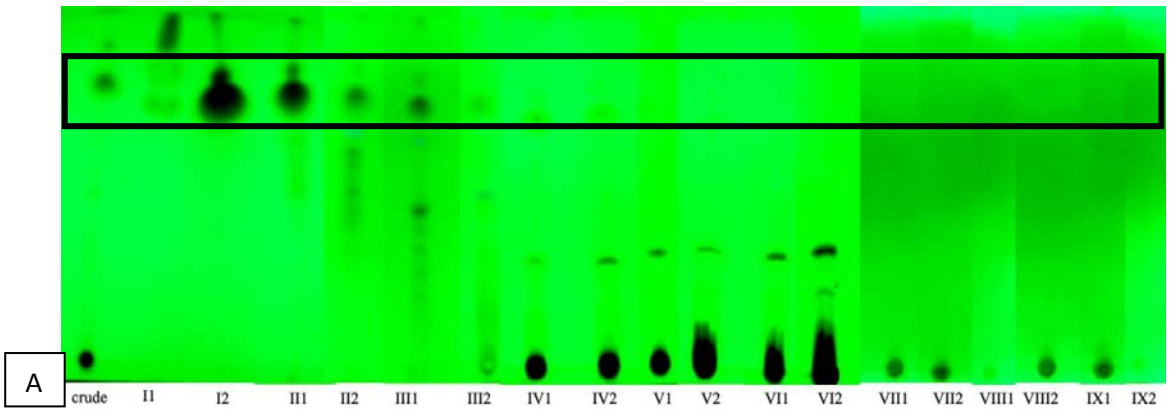
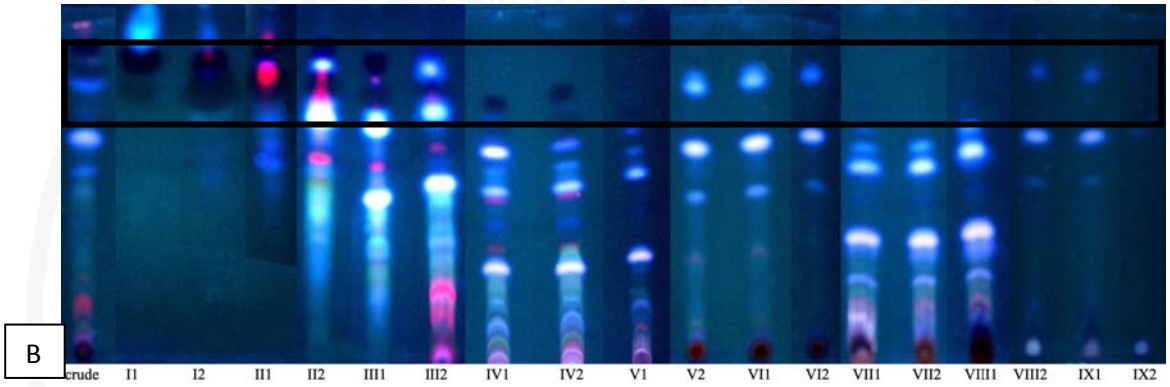


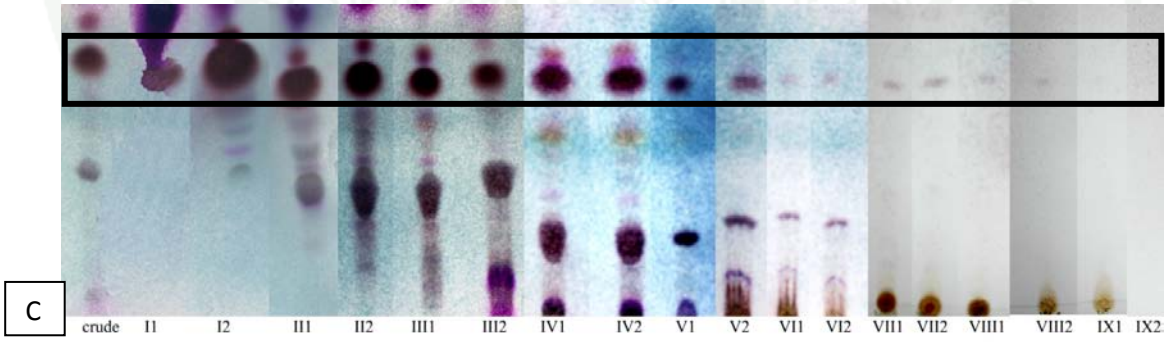
Figure 38 *Eugenia caryophyllata* crude extracts on TLC plate show active compound in dark colour under UV lamp 254 nm (A), 365 (B) and Anisaldehyde (C). Solvent system Acetone: Dichloromethane: Toluene = 10:60:30

7.1 Separation of antifungal compound by column chromatography (CC).

Fractionation for separating the active antifungal compound of *Eugenia caryophyllata* crude extracts was done by elution with organic solvent mixtures of hexane, ethyl acetate, and methanol. Eighteen fractions were collected and the bioassay tests were done by separating on TLC plate. The active compound of *Eugenia caryophyllata* crude extracts showed the dark color under UV lamp 254 nm (Figure 40A) infractions number I-1 to IV-2, and dark blue color under 365 nm (Figure 40B), more over the active compound show dark purple after spray with anisaldehide (Figure 40 C), showed inhibition zone after sprayed with *Cladosporium cladosporoides* (Figure 40D).







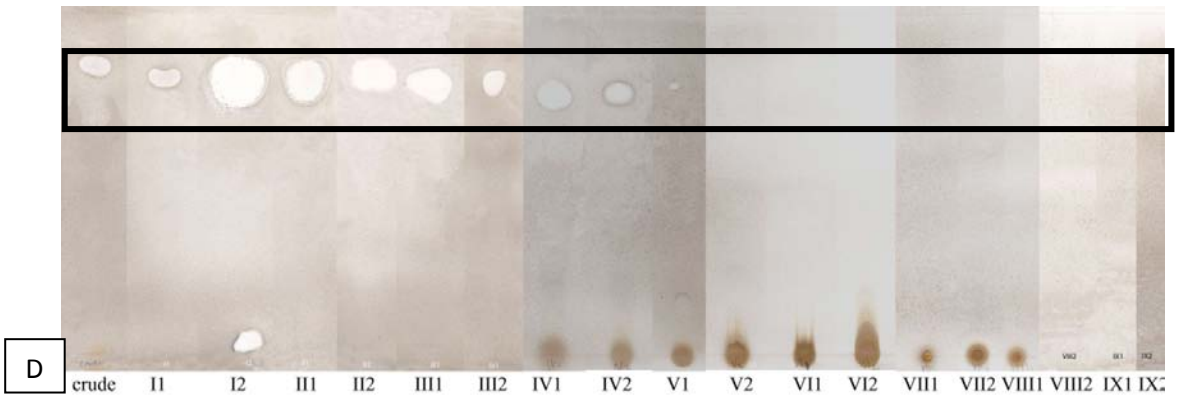


Figure 39 A. *Eugenia caryophyllata* fractions on TLC plate show active compound in dark colour under UV lamp 254 nm, B. 365, C. anisaldehyde, and D. *Cladosporium* sp inhibition zone . Lane 1= crude extracts, Lane 2 (I1, I2)= fraction eluted with 100% hexane, Lane 3 (II1,II2)= fraction eluted with hexane: ethyl acetate = 75%: 25%, Lane 4 (III1,III2) = fraction eluted with hexane: ethyl acetate = 50%: 50%, Lane 5 (IV1,IV2)= fraction eluted with hexane: ethyl acetate = 25%: 75%, Lane 6(V1, V2)= fraction eluted with ethyl acetate 100%, Lane 7 (VI1,VI2)= fraction eluted with ethyl acetate: methane= 75%: 25%, Lane 8 (VII1,VII2)= fraction eluted with ethyl acetate: methane= 50%: 50%, Lane 9 (VIII1,VIII2)= fraction eluted with ethyl acetate: methane= 25%: 75%, Lane 10 (IX1, IX2)= fraction eluted with ethyl methane 100%. The position of active compound at Rf value of 0.73 were in Line1 to 5 and use for further analysis.

The mixture of fractions number I-1 to IV-2 was tested on TLC plated and sprayed with *P. digitatum.* The result showed that the inhibition zone as same position as crude extracts (Figure 39).

Crude Fraction I.1 – IV.2

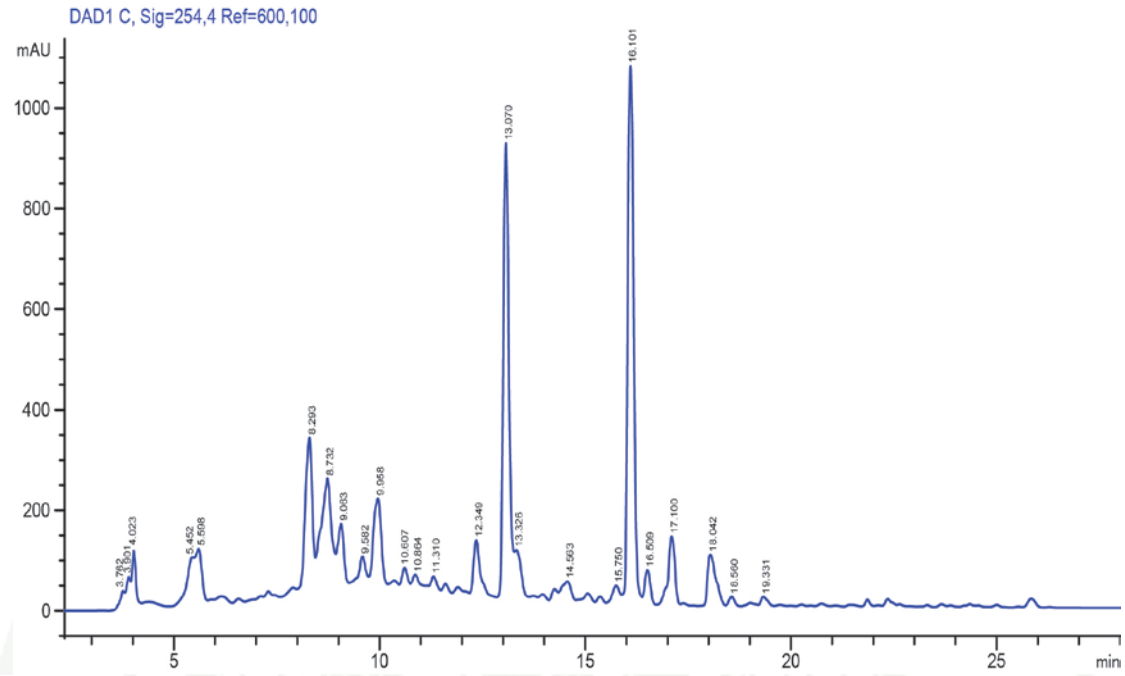
Figure 40 *Eugenia caryophyllata* fractions I.1 – IV.2 on TLC plate show active compound in *Cladosporium* sp inhibition zone

8 Identification of antifungal compound

8.1 Analyzed by High Performance Liquid Chromathography (HPLC) the HPLC chromatogram

E. caryophyllata crude extracts and *E. caryophyllata* crude purified extract was tested by using HPLC. In the chromatogram of the *E.caryophyllata* bud extract had two major peaks at 13.070 minutes and 16.101 minutes (Figure 41 A), while in purified of the *E. caryophyllata* bud extract only had one peak at 16.085 minutes. All of peaks were selected for trappings (Figure 41 B). The UV spectra of both extracts were identical with maxima at 230 nm and the reference at 600nm (Figure 42)

A



B

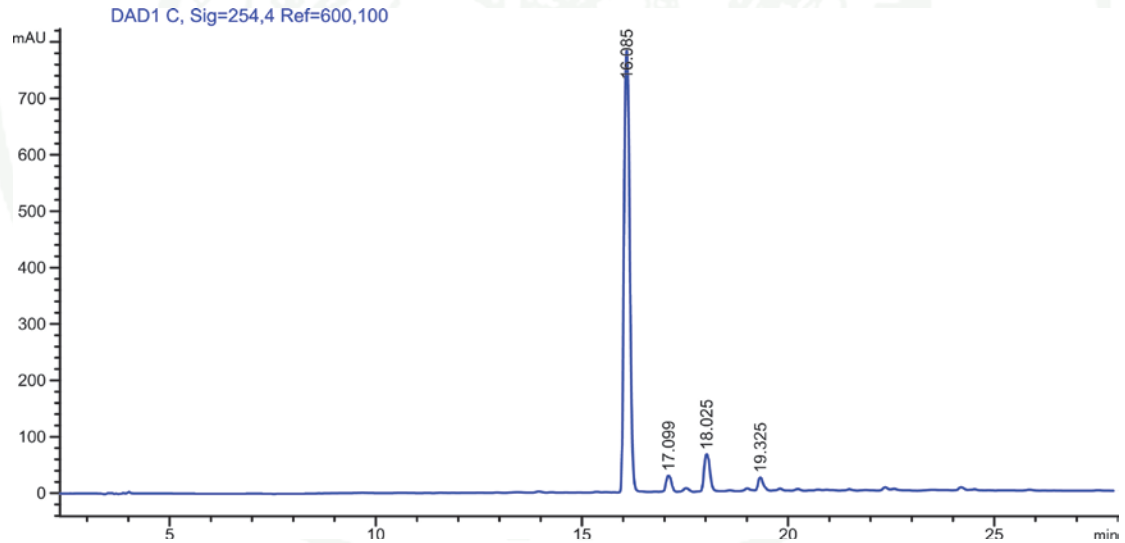


Figure 41 A. HPLC profiles from *Eugenia caryophyllata* crude extracts and B. fraction of *Eugenia caryophyllata* crude extracts

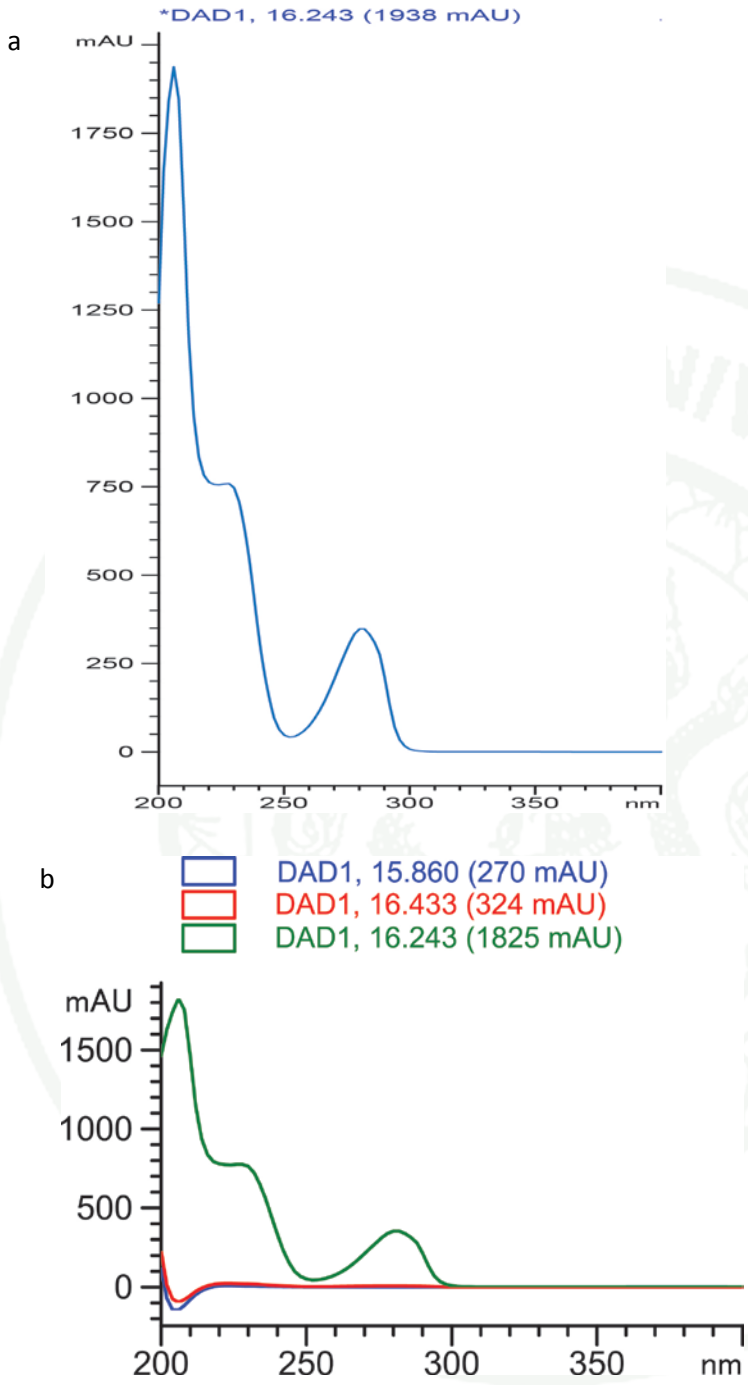


Figure 42 a. UV spectra of the peaks shown in the HPLC chromatogram of *Eugenia caryophylla* extract and b. reference

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Discussion

Eugenia caryophyllata and *Curcoma longa* crude extracts were established to be the promising crude extracts to control *P. digitatum in vitro*. The antifungal activities of clove may be due to its active compound, eugenol, which is the main constituent of clove oil (approximately 83.02%). Clove extract possesses stronger antifungal activities against *A. flavus* and *P. citrinum* than against *R. nigricans.* The suggested mechanism for this is eugenol which increases the permeability of the cells and causes irregular branching of hyphae in the apical part and the loss of linearity, collapsed and squashed due to the lack of cytoplasm (Xing *et al*, 2011). Laksanaphisut and Sangchote (2010) reported crude extracts of turmeric powder in 20% of ethanol at 30,000 ppm reduced disease incidences of green mold disease on treated fruit by 25%.

Bardin *et al*., (2003), reported that combined applications of a biocontrol agent and synthetic chemicals or plant materials often provide better plant protection than individual treatments. The combined application of *Paeonia suffruticosa* (medicinal plant) and *T. harzianum* was disclosed to be more effective than either treatment with a single agent to control *Rhizoctonia* damping-off (Lee *et al*., 2008). The result agree with Lee *et al*. (2011) who tested fifty five species of medicinal plant materials for their antifungal activity *in vitro* against *Rhizoctonia solani* AG 2-1 to improve the biocontrol efficacy of *T. harzianum.* Six species were found to be effective against *R. solani* AG 2-1. Among these six medicinal plant materials, *Eugenia caryophyllata* flower bud, and *Cinnamomum loureirii* stem bark were the most effective against *R. solani* AG 2-1 mycelial growth, with an inhibitory efficacy of 73.7% and 71.1%, respectively.

This study clearly showed that integrated treatment of *E.caryophyllata* crude extracts and *Candida utilis* TISTR 5001 significantly reduced green mould rot of *P. digitatum* on the citrus fruits. It was indicated that *E. caryophyllata* crude extracts and *C. utilis* TISTR 5001 have fungicidal properties to control green mould rot disease on citrus fruits. Bardin *et al*., (2003), reported that combined applications of a

biocontrol agent and synthetic chemicals or plant materials often provide better plant protection than individual treatments. The combined application of *Paeonia suffruticosa* (medicinal plant) and *T. harzianum* was more effective than each treatment to control *Rhizoctonia* damping-off (Lee *et al*., 2008). The result agree with Lee, *et al*. (2011) who tested fifty five species of medicinal plant materials for their antifungal activity *in vitro* against *Rhizoctonia solani* AG 2-1 to improve the biocontrol efficacy of *T. harzianum*. Six species were found to be effective against *R. solani* AG 2-1. Among these six medicinal plant materials, *Eugenia caryophyllata* flower bud and *Cinnamomum loureirii* stem bark have high efficacy against *R. solani* AG 2-1 mycelial growth which inhibited by 73.7% and 71.1% respectively.

All treatments showed weight loss 2 weeks after storage but the percentage of juice as same as juice content before storage. Storing citrus without any cover, layering or coating at 25^{o}C caused loss of weight, because of evaporated, but not effect to the juice content. Total soluble solid, fruits firmness, and ascorbic acid content were increase compared with those before storage. On the other hand titratable acidity was decrease 2 weeks after storage. The mechanism of increasing total soluble solid and ascorbic acid involved more than concentration by dehydration as there is active accumulation of solid in fruits, the process including sugar movement, accumulation and/or storage in fruits (Mc. Cornack, 1975). Generally, all treatmens followed a similar pattern to accumulate ascorbic acid during ripening.

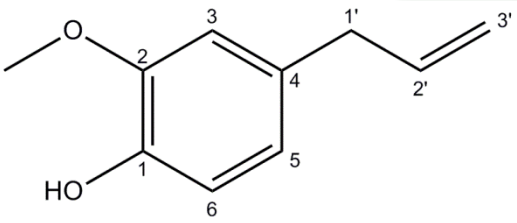
The *P. digitatum* showed no growth on PDA plus 5000 mg/L *E.caryophyllata* crude extracts for seven days, approved that crude extracts was effective to control green mould rot of citrus fruits a short period (days), nonetheless, its efficacy should be improved when protection was required for long periods. Moreover, *C. utilis* TISTR 5001 cells grew on PDA plus 5000 mg/L *E.caryophyllata* crude extracts, this result acclaimed that *E.caryophyllata* crude extracts had no growth effect to the *C. utilis* TISTR 5001 then it could control *P. digitatum* for long period. Cell death because its loss of cell contents and the initiation of autolysis. Besides this, the permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Armstrong, 2006). The damage was irreversible and the

changes showed general disorganization of the cytoplasm as well as cytoplasm leakage, probably caused by loss of conidial membrane integrity which could eventually lead to fungal cell death (Bakkali, 2008). In this regard, Denyer (1990) affirms that leakage of intracellular material was a general phenomenon provoked by many antimicrobial substances. Perhaps eugenol an active ingredient in *E.caryophyllata* induces either hyperpolarization of the inner mitochondrial membrane or mitochondrial swelling or both. Usually, cells in which mitochondria are destabilized and finally broken down will decrease in the coupling efficiency of the electron-transport chain and therefore can generate ROS intermediates which can lead to oxidative stress (Martindale and Holbrook, 2002). In addition, Park *et al*. (2007) studied effect of clove oil on *Trichophyton mentagrophytes* under transmission electron microscope, the inner mitochondrial membranes were partially destroyed, with complete destruction of the cell wall.

The mode of action of antagonist yeast on postharvest pathogen was complex. It might involve nutrient competition (Droby *et al.*, 1989) site exclusion and direct parasitism (Ippolito *et al.*, 2000), production of hypha enzyme (El-Ghaouth *et al.*, 1998) and induce resistance (Arras, 1996). Wisnewski *et a.*,1991, found the tenacious attachment of *P. guilermondii* to pathogen hypha and the production of β -1,3 glucanase by the yeast. However, many researchers supported competition for nutrient was the main mode of action of antagonistic yeast against pathogen (Droby *et al.*, 1989). The yeast cell colonizes rapidly and competes effectively with the pathogen for nutrient in wound, resulting in the nutritional starvation of the pathogen (Mc Laughin *et al.*, 1990).

The ^{1}H NMR and the ^{13}C NMR provided further evidence for the confirmation of the structure of the isolated compound. Base on the mass spectral data, it was concluded that the isolated compound was eugenol. Eugenol is a phenylpropene, an allyl chain-substituted guaiacol. Eugenol is a member of the class of chemical compounds. It is a clear to pale yellow oily liquid extracted. 1H NMR spectrum of the major peak at 16. 085 minutes showed characteristic signals of a phenylpropanoids as 4-Allyl-2-methoxyphenol and has atoher name 2-Methoxy-4-(2-

propenyl)phenol, Eugenic acid, Caryophyllic acid, 1-Allyl-3-methoxy-4-hydroxybenzene, Allylguaiacol, 2-Methoxy-4-allylphenol, 4-Allylcatechol-2-methyl ether, 2-methoxy-4-(2-propen-1-yl)phenol (Table 15 and Figure 43).



Chemical Formula: $C_{10}H_{12}O_2$
Molecular Weight: 164,20

Figure 43 Chemichal formula and molecular weight of eugenol

Table 15 ^{13}C and ^{1}H Chemical shift of NMR spectra of *Eugenia caryophyllata* extracts

Atomnumber	^{13}C	^{1}H
1	143.85	-
2	146.39	-
3	111.06	6.73 a
4	131.86	-
5	121.12	6.72 a
6	114.21	6.89
1‘	39.83	3.37
2‘	137.78	6.00
3‘	115.46	5.11
OMe	55.79	3.91

CONCLUSION AND RECOMMENDATION

Conclusion

A combination of *Candida utilis* TISTR 5001 and *Eugenia caryophyllata* crude extract was the best combination to attain a reduction to the disease incidence and the disease severity. *P. digitatum* colonization was the lowest on citrus treated with the combination of *E. caryophylata* crude extract and *C. utilis* TISTR 5001. Survival of *C. utilis* TISTR 5001 was the highest on fruits treated with *E. caryophylata* crude extract and *C. utilis* TISTR 5001. Combination of *E. caryophylata* crude extract and *C. utilis* TISTR 5001 significantly reduced the natural development of green mold of citrus fruits. The combination had no effect to fruits quality. Based on HPLC and NMR (^{1}H and ^{13}C) confirmed that the active compound of *E. caryophylata* was eugenol. Hence, the results indicate a combination of the plant extracts and yeasts posses antifungal activity that can be exploited as an ideal treatment for future plant disease management.

The combination of *Eugenia caryophylata* crude extract and *Candida utilis* had a more potent antifungal activity than Imazalil at 150 mg/L. *E.caryophyllata* crude extract was effective to control green mould rot of citrus fruits for short period and *C. utilis* can control *P. digitatum* for long period.

Recommendation

E. caryophylata crude extract and *C. utilis* TITR 5001 can be an ideal treatment for future plant disease management. Furthermore, further study should be focused on the combination of this study revealed that a combination of *E. caryophylata* crude extract and *C. utilis* TITR 5001 with other control strategies, such as waxing, atmosphere condition, carrier and adhesive material to develop an effective approach for postharvest disease control of citrus fruits. This combination might be used as a drench treatment in packaging line, but still need more detail research especially how to apply the combination.

Plant extracts are not durable in the effective stability, better serve the plant crude extracts stored in dark bottles and stored in a refrigerator.

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APPENDIX

Appendix Table 1 Analysis of variance of i*n vitro* screening of ethanol concentrations at different percentages on their inhibition of hyphal growth of *P.digitatum*

Source of variation	df	Sum of square	Mean Square	F
Treatment	19	8972.58	472.24	30.29 **
Error	40	8972.58	15.59	
Total	59	9596.09		

df = degree of freedom

** = significant at P<0.01

Appendix Table 2 Analysis of variance of i*n vitro* screening of 7 plant crude extracts in wide concentrations dissolved in 20% ethanol amended in PDA on their inhibition of hyphal growth of *P.digitatum* at 25^{o}C for 24 h

Source of variation	df	Sum of square	Mean Square	F
Block	2	240.86	120.43	1.62**
Treatment	31	42852.52	1382.34	18.62**
Plant extracts (P)	7	34060.84	4865.83	65.54**
Concentration (C)	3	3441.68	1147.23	15.45**
PxC	21	5350.00	254.76	3.43**
Error	62	4602.86	15.59	
Total	95	47696.23		

df = degree of freedom

** = significant at P<0.01

Appendix Table 3 Analysis of variance of i*n vitro* screening of 7 plant crude extracts dissolved in 20% ethanol at different concentrations amended in PDA plus 2% citrus juice on their inhibition of hyphal growth of *P.digitatum* at 25^{o}C for 24 h for 7 days

Source of variation	df	Sum of square	Mean Square	F
Block	2	6.49	3.24	0.15ns
Treatment	31	89273.91	2879.80	128.65**
Plant extracts (P)	7	69154.59	9879.23	441.33**
Concentration (C)	3	9813.01	3271.00	146.12**
P x C	21	10306.31	490.78	21.92**
Error	62	1387.89	22.39	
Total	95	90668.29		

df = degree of freedom
ns = non significant
** = significant at P<0.01

Appendix Table 4 Analysis of variance of disease incidence (%) of green mold rot on citrus fruits which were treated with *P. digitatum* (1x10^{5} conidia/ml, 10 µL), imazalil (500 mg/L), *E. caryophyllata* crude extract (15,000 mg/L,10 µL), *C. longa* crude extract (30,000 mg/L,10 µL), *C. citratus* crude extract and *M. charantia* crude extract (20,000 mg/L, 10 µL) incubated at 25^{o}C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	19	22818.11	1200.95	24.56**
Error	40	1955.56	48.89	
Total	59	24773.66		

df = degree of freedom, ** = significant at P<0.01

Appendix Table 5 Analysis of variance of disease severity (%) of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$ conidia/ml, 10 µL), and imazalil (500 mg/L), *E. caryophyllata* crude extract (15,000 mg/L,10 µL), or *C. longa* crude extract (30,000 mg/L,10 µL), *C. citratus* crude extract and *M. charantia* crude extract (20,000 mg/L, 10 µL) incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	19	23335.78	1228.19	22.32**
Error	40	2201.31	55.03	
Total	59	25537.08		

df = degree of freedom

** = significant at P<0.01

Appendix Table 6 Analysis of variance of disease incidence (%) of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$conidia/ml, 10 µL), and ethanol 20%, or *E. caryophyllata* crude extract (15,000 mg/L,10 µL), *C. longa* crude extract (30,000 mg/L,10 µL), and *M. charantia* crude extract (20,000 mg/L, 10 µL) incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	2658.94	886.31	2.73**
Error	8	2598.49	324.81	
Total	11	5257.43		

df = degree of freedom

** = significant at P<0.01

Appendix Table 7 Analysis of variance of disease severity of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$conidia /ml, 10 µL), and ethanol 20%, or *E. caryophyllata* crude extract (15,000 mg/L,10 µL), *C. longa* crude extract (30,000 mg/L,10 µL), and *M. charantia* crude extract (20,000 mg/L, 10 µL) incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	154.96	51.65	0.23*
Error	8	1834.57	229.32	
Total	11	1989.53		

df = degree of freedom

* = significant at P<0.05

Appendix Table 8 Analysis of variance of disease incidence (%) of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$ conidia/ml, 10 µL), and ethanol 20%, or *E. caryophyllata* crude extract (15,000 mg/L,10 µL), and *C.longa* crude extract (30,000 mg/L,10 µL, incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	2	19759.28	9879.64	45.69**
Error	9	1945.99	216.22	
Total	11	21705.28		

df = degree of freedom

** = significant at P<0.01

Appendix Table 9 Analysis of variance of disease severity (%) of green mold rot on citrus fruits which were treated with *P. digitatum* ($1x10^5$ conidia /ml, 10 µL), and ethanol 20%, or *E. caryophyllata* crude extract (15,000 mg/L,10 µL), and *C.longa* crude extract (30,000 mg/L,10 µL, incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	2	2842.75	1421.38	121.33**
Error	9	105.44	11.72	
Total	11	2948.19		

df = degree of freedom

** = significant at P<0.01

Appendix Table 10 Analysis of variance of effect of yeast on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated by 3cm, incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	6	4965.92	827.65	3.48**
Error	21	4990.71	237.65	
Total	27	9956.64		

df = degree of freedom

** = significant at P<0.01

Appendix Table 11 Analysis of variance of effect of yeast(s) individual and individual or in combination on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated by 3 cm, incubated at 25^{o}C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	9	144208.07	16023.12	745.12**
Error	40	860.16	21.50	
Total	49	145068.23		

df = degree of freedom

** = significant at P<0.01

Appendix Table 12 Analysis of variance of effect of *E. caryophylata* crude extract at 15,000mg/L, 10 µl, *C. longa* crude extract at 30,000mg/L,10 µl, *C. utilis* TISTR 5001 at 1x10^{8} cells/mL, and 10 µl *C. tropicalis* TISTR 5010 at 1x10^{8} cells/mL, 10 µl of green mold rot incidence, incubated at 25^{o}C for 7 days

Source of variation	df	Sum of square	Mean Square	F	
Block	2	319.66	159.83	1.68	ns
Treatment	8	7923.66	990.46	10.44	**
Error	16	1518.20	94.89		
Total	26	9761.51			

df = degree of freedom

ns = non significant

** = significant at P<0.01

Appendix Table 13 Analysis of variance of effect of *E. caryophylata* crude extract at 15,000mg/L, 10 µl, *C. longa* crude extract at 30,000mg/L,10 µl, *C. utilis* TISTR 5001 at $1x10^8$ cells/mL, and 10 µl *C. tropicalis* TISTR 5010 at $1x10^8$ cells/mL, 10 µl of green mold rot severity, incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F	
Block	2	432.07	216.03	1.65	ns
Treatment	8	5608.28	701.04	5.35	**
Error	16	2095.13	130.95		
Total	26	8135.48			

df = degree of freedom
ns = non significant
** = significant at P<0.01

Appendix Table 14 Analysis of variance of comparative treatment among *E. caryophylata* crude extract at 15,000mg/L, *C. utilis* TISTR 5001 $1x10^8$cells/mL, and thereof with imazalil of green mold rot incidence (%) on citrus fruits, incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	460.09	153.36	43.22**
Error	12	42.58	3.55	
Total	15	502.67		

df = degree of freedom
** = significant at P<0.01

Appendix Table 15 Analysis of variance of comparative treatment among *E. caryophylata* crude extract at 15,000mg/L, *C. utilis* TISTR 5001 $1x10^8$cells/mL, and thereof with imazalil of green mold rot severity (%) on citrus fruits, incubated at 25^oC for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	31199.31	10399.77	80.57**
Error	12	1548.92	129.08	
Total	15	32748.22		

df = degree of freedom

** = significant at P<0.01

Appendix Table 16 Analysis of variance of comparative treatment between *E. caryophylata* crude extract at 15,000mg/L, *C. utilis* TISTR 5001 $1x10^8$cells/mL, and their combination, with imazalil of green mold rot incidence (%) on citrus fruits, incubated at 25^oC for 7 days

Source of variation	df	Sum of square	Mean Square	F
Block	2	77.06	38.53	0.89 ns
Treatment	8	13214.04	1651.76	38.18**
Error	16	692.14	43.26	
Total	26	13983.24		

df = degree of freedom

** = significant at P<0.01

Appendix Table 17 Analysis of variance of comparative treatment between *E. caryophylata* crude extract at 15,000mg/L, *C. utilis* TISTR 5001 $1x10^8$cells/mL, and their combination with imazalil of green mold rot severity (%) on citrus fruits, incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F	
Block	2	127.44	63.72	1.05	ns
Treatment	8	3613.64	451.71	7.45	**
Error	16	970.18	60.63		
Total	26	4711.21			

df = degree of freedom

ns = non significant at P<0.05

** = significant at P

Appendix Table 18 Analysis of variance of effect of *E. caryophylata* crude extract at 15,000mg/L, and yeast (*C. utilis* TISTR 5001 at $1x10^8$cells/mL) for reducing natural rot development of the disease incidence

Source of variation	df	Sum of square	Mean Square	F	
Treatment	3	998.75	332.92	67.71	**
Error	12	59.00	4.92		
Total	15	1057.75			

df = degree of freedom

** = significant at P<0.01

Appendix Table 19 Analysis of variance of effect of *E. caryophylata* crude extract at 15,000mg/L, and yeast (*C. utilis* TISTR 5001 at $1x10^8$cells/mL) for reducing natural rot development of the disease severity 2 weeks after storage

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	460.09	153.36	43.22**
Error	12	42.58	3.55	
Total	15	502.67		

df = degree of freedom

** = significant at P<0.01

Appendix Table 20 Analysis of variance of *P. digitatum* colonization on citrus fruits 1 day after treated by yeasts and plant extracts and incubated at 25°C

Source of variation	df	Sum of square	Mean Square	F
Block	4	2.37992E+11	5.95E+10	15.00**
Treatment	4	31569366400	7.89E+09	1.99*
Error	16	63444470400	3.97E+09	
Total	24	55724323600		

df = degree of freedom

** = significant at P<0.01

* = significant at P<0.05

Appendix Table 21 Analysis of variance of *P. digitatum* colonization on citrus fruits 2 days after treated by yeasts and plant extracts and incubated at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Block	4	6.96992E+14	1.74E+14	21.51**
Treatment	4	1.06313E+14	2.66E+13	3.28*
Error	16	1.29594E+14	8.1E+12	
Total	24	4.69093E+13		

df = degree of freedom

** = significant at P<0.01

* = significant at P<0.05

Appendix Table 22 Analysis of variance of *P. digitatum* colonization on citrus fruits 3 days after treated by yeasts and plant extracts and incubated at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Block	4	2.16545E+15	5.41E+14	21.53**
Treatment	4	3.35133E+14	8.38E+13	3.33*
Error	16	4.02402E+14	2.52E+13	
Total	24	1.9711E+14		

df = degree of freedom

** = significant at P<0.01

* = significant at P<0.05

Appendix Table 23 Analysis of variance of *C. utilis* colonization on citrus fruits 1 day after treated by yeasts and plant extracts and incubated at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Block	4	1.69E+10	4.22E+09	6.01**
Treatment	4	2.12E+09	5.29E+08	0.75ns
Error	16	1.12E+10	7.03E+08	
Total	24	7.81E+09		

df = degree of freedom

** = significant at P<0.01

ns = non significant at P<0.05

Appendix Table 24 Analysis of variance of *C. utilis* colonization on citrus fruits 2 days after treated by yeasts and plant extracts and incubated at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Block	4	1.03E+12	2.58E+11	7.37**
Treatment	4	5.61E+10	1.4E+10	0.40ns
Error	16	5.6E+11	3.5E+10	
Total	24	4.72E+11		

df = degree of freedom

** = significant at P<0.01

ns = non significant at P<0.05

Appendix Table 25 Analysis of variance of *C. utilis* colonization on citrus fruits 3 days after treated by yeasts and plant extracts and incubated at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Block	4	7.84E+12	1.96E+12	16.57**
Treatment	4	1.1E+12	2.74E+11	2.31*
Error	16	1.89E+12	1.18E+11	
Total	24	1.77E+12		

df = degree of freedom

** = significant at P<0.01

* = significant at P<0.05

Appendix Table 26 Analysis of variance of *P. digitatum* colonization on citrus peel, treated with *E. caryophylla* crude extract at 15,000 mg/L and *C. utilis* TISTR 5001 1x10^{8}cells/mL at 2 h after inoculation at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	4855.46	1618.49	6.99**
Error	17	3938.22	231.66	
Total	20	8793.68		

df = degree of freedom

** = significant at P<0.01

Appendix Table 27 Analysis of variance of *P. digitatum* colonization on citrus peel, treated with *E. caryophylla* crude extract at 15,000 mg/L and *C. utilis* TISTR 5001 1x10^8cells/mL at 4 h after inoculation at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F	
Treatment	3	8227.75	2742.58	4.61	**
Error	17	10113.65	594.92		
Total	20	18341.41			

df = degree of freedom

** = significant at P<0.01

Appendix Table 28 Analysis of variance of *P. digitatum* colonization on citrus peel, treated with *E. caryophylla* crude extract at 15,000 mg/L and *C. utilis* TISTR 5001 1x10^8cells/mL at 6 h after inoculation at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	10185.69	3395.23	6.18**
Error	17	9339.599	549.39	
Total	20	19525.29		

df = degree of freedom

** = significant at P<0.01

Appendix Table 29 Analysis of variance of *P. digitatum* colonization on citrus peel, treated with *E. caryophylla* crude extract at 15,000 mg/L and *C. utilis* TISTR 5001 1x10^{8}cells/mL at 8 h after inoculation at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F	
Treatment	3	10979.73	3659.91	16.49	**
Error	17	3771.51	221.85		
Total	20	14751.24			

df = degree of freedom

** = significant at P<0.01

Appendix Table 30 Analysis of variance of *C. utilis* colonization on citrus peel, treated with *E. caryophylla* crude extract at 15,000 mg/L and *C. utilis* TISTR 5001 1x10^{8}cells/mL at 2 h after inoculation at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	3490.74	1163.58	12.33**
Error	17	1604.60	94.39	
Total	20	5095.34		

df = degree of freedom

** = significant at P<0.01

Appendix Table 31 Analysis of variance of *C. utilis* colonization on citrus peel, treated with *E. caryophylla* crude extract at 15,000 mg/L and *C. utilis* TISTR 5001 1x10^{8}cells/mL at 4 h after inoculation at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	10719.77	3573.25	46.93**
Error	17	1294.27	76.13	
Total	20	12014.04		

df = degree of freedom

** = significant at P<0.01

Appendix Table 32 Analysis of variance of *C. utilis* colonization on citrus peel, treated with *E. caryophylla* crude extract at 15,000 mg/L and *C. utilis* TISTR 5001 1x10^{8}cells/mL at 6 h after inoculation at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	15016.15	5005.39	23.81**
Error	17	3573.05	210.18	
Total	20	18589.20		

df = degree of freedom

** = significant at P<0.01

Appendix Table 33 Analysis of variance of *C. utilis* colonization on citrus peel, treated with *E. caryophylla* crude extract at 15,000 mg/L and *C. utilis* TISTR 5001 1x10^8cells/mL at 8 h after inoculation at 25^{o}C

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	18419.80	6139.93	26.33**
Error	17	3964.26	233.19	
Total	20	22384.06		

df = degree of freedom

** = significant at P<0.01

Appendix Table 34 Analysis of variance of effect of *E. caryophylata* and *C. utilis* TISTR 5001 on weight loss (%) of citrus fruits after storage on 25^{o}C, RH 100% for 2 weeks

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	93.15	31.05	15.06**
Error	15	30.92	2.06	
Total	19	124.07		

df = degree of freedom

** = significant at P<0.01

Appendix Table 35 Analysis of variance of effect of *E. caryophylata* and *C. utilis* TISTR 5001 on juice content (%) of citrus fruits after storage on 25^{o}C, RH 100% for 2 weeks

Source of variation	df	Sum of square	Mean Square	F
Treatment	4	26947.50	6736.87	516.22**
Error	15	195.75	13.05	
Total	19	27143.26		

df = degree of freedom

** = significant at P<0.01

Appendix Table 36 Analysis of variance of effect of *E. caryophylata* and *C. utilis* TISTR 5001 on total soluble solid (%) of citrus fruits after storage on 25^{o}C, RH 100% for 2 weeks

Source of variation	df	Sum of square	Mean Square	F
Treatment	4	44.23	11.06	50.57**
Error	15	3.28	0.22	
Total	19	47.51		

df = degree of freedom

** = significant at P<0.01

Appendix Table 37 Analysis of variance of effect of *E. caryophylata* and *C. utilis* TISTR 5001 on fruit firmness (%) of citrus fruits after storage on 25oC, RH 100% for 2 weeks

Source of variation	df	Sum of square	Mean Square	F
Treatment	4	0.19	0.05	12.73**
Error	15	0.06	0.01	
Total	19	0.25		

df = degree of freedom

** = significant at P<0.01

Appendix Table 38 Analysis of variance of effect of *E. caryophylata* and *C. utilis* TISTR 5001 on ascorbic acid content (mg/L) of citrus fruits after storage on 25^{o}C, RH 100% for 2 weeks

Source of variation	df	Sum of square	Mean Square	F
Treatment	4	48.11	12.03	8.68**
Error	15	20.77	1.39	
Total	19	68.89		

df = degree of freedom

** = significant at P<0.01

Appendix Table 39 Analysis of variance of effect of *E. caryophylata* and *C. utilis* TISTR 5001 on titratable acidity (%) of citrus fruits after storage on 25°C, RH 100% for 2 weeks

Source of variation	df	Sum of square	Mean Square	F
Treatment	4	7.29	1.83	52.23**
Error	15	0.53	0.04	
Total	19	7.82		

df = degree of freedom

** = significant at P<0.01

Appendix Table 40 Analysis of variance of effect of *C. utilis* TISTR 5001, *E. caryophylata* crude extract at 5,000mg/L, and their combination on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated by 3cm, incubated at 25°C for 7 days

Source of variation	df	Sum of square	Mean Square	F
Treatment	3	43633.42	14544.50	60.03**
Error	1	242.28	242.28	
Total	16	43875.69		

df = degree of freedom

** = significant at P<0.01

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