Psyche

Selected Papers from the International Conference on Biopesticides 6 (ICOB6)

Guest Editors: Kabkaew L. Sukontason, Mir S. Mulla, Siriwat Wongsiri, John T. Trumble, and Jittawadee R. Murphy

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Editorial

Selected Papers from the International Conference on Biopesticides 6 (ICOB6)

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There is a whole host of chemicals employed in plant protection practices around the world for pest and disease control. Some of the same groups of chemicals are also used for controlling pests and vectors of human diseases. With the advent of DDT some 7 decades ago, a variety of synthetic pesticides were discovered, designed, and evaluated for pesticidal activity. Compounds and moieties having different modes of action were studied and developed for the control of pests and diseases. In the realm of insecticides, the organochlorine insecticides generated great hope and yielded tremendous successes in the area of plant protection and management of some vector-borne diseases. The organochlorines were followed by even more powerful insecticides such as organophosphate, carbamate, and other groups of active agents. Although these insecticides provided excellent control of major pests of crops and humans, they posed considerable environmental problems, such as adverse effects on fish and wildlife, high toxicity to mammals, biomagnification, persistence in water, soil, and food crops. Additionally, the emergence of resistance in target species appears over large areas of the globe, and many of the newly developed control agents became obsolete in a matter of a few years. Responding to these concerns, experts and stake holders in plant protection and disease vector control programs shifted their research focus to the discovery, development, and use of alternative and envirofriendly agents. Thus a new era for the development and practical use of natural and biorational products and biopesticides dawned. Biopesticides include not only plant based bioactive agents but also other natural products of various origins. Since 1995, we have been organizing and holding regional international conferences on the broad subject of biopesticides, where the last one, ICOB6, was held in Chiang Mai, Thailand.

This special issue addresses the role of biopesticides in pest management. The themes include biorational agents, plant-based products, natural products, microbial control agents, antagonistic bacteria, and fungi. From 14 submissions, 6 papers were selected and are published in this special issue, of which 5 were research papers and 1 was a review article. Each paper was reviewed by at least two reviewers and revised according to review comments.

In Intirach et al.'s paper, the authors presented the larvicidal efficacy of essential oils of five plants—*Piper sarmentosum, Foeniculum vulgare, Curcuma longa, Myristica fragrans*, and *Zanthoxylum piperitum*—against laboratory-colonized *Anopheles cracens* mosquitoes, showing 95%–100% larval mortality at concentration of 100 ppm. The strongest larvicidal potential was established from *P. sarmentosum*, followed by *F. vulgare, C. longa, M. fragrans*, and *Z. piperitum*, based on the LC_{50} values. The authors also analyzed the chemical compositions by gas chromatography coupled to mass spectrometry, demonstrating the main component in the oil derived from such plants. Binary mixtures between *P. sarmentosum*, the most effective oil, and the others were proved to be highly efficacious, indicating

synergistic activity. This paper offers some interesting thoughts of the synergistic effects from mixed formulations of different essential oils, which may be helpful in developing effective, economical, and ecofriendly larvicides, as favorable alternatives for mosquito management.

In this special issue, there are two papers studying the fungi or plant extracts against agronomical mites. Bussaman et al.'s paper presented the efficacy of 23 rhizome and leaf extracts against adult female of the Mushroom mite, *Luciaphorus* sp., a destructive pest of several mushroom species. The rhizome extracts derived from *Curcuma xanthorrhiza* and *Zingiber montanum* revealed strong acaricidal activities, followed by *Curcuma longa, Zingiber zerumbet, Kaempferia parviflor* and *Zingiber officinale*. In addition, the leaf extracts of *Ocimum sanctum* and *Melissa officinalis* also caused strong mortality. Such information provided a great potential for future development as natural acaricides for controlling *Luciaphorus* sp. Another paper is by Erdogan et al.'s, reporting the efficacy of biopesticides extracted from five different plants (i.e., *Allium sativum, Rhododendron luteum, Helichrysum arenarium, Veratrum album,* and *Tanacetum parthenium*) against the two-spotted mite, *Tetranychus urticae*, an economic pest causing serious damage to vegetables, flowers, and fruit crops worldwide. The bioassays demonstrated not only the high mortality but also lower numbers of eggs' production.

Research on the fungicide activity of some crude plant extracts or bacteria has also been published. Bussaman et al. evaluated *in vitro* the efficacy of 14 crude leaf extracts against *Colletotrichum gloeosporioides*—a fungus that causes anthracnose disease in tropical fruits. The crude leaf extracts from *Piper sarmentosum*, using the ethanol, methanol, and chloroform as solvents, showed high antifungal activities by inhibiting both mycelium growth and spore germination. Such information provides the potential of these new natural fungicides for management of anthracnose disease. Another paper on fungicide activity of bacteria is provided by Loliam's et al. The authors demonstrated the results of using antagonistic actinomycetes, *Streptomyces rubrolavendulae* S4, against *Phytophthora infestans*, the pathogenic fungi causing the seedling damping off disease in several economic crops in Thailand. This bacterium was proven to induce most effective growth inhibition of fungi tested on potato dextrose agar. In *P. infestans* contaminated peat moss, the survival of tomato and chili seedling was significantly increased for *S. rubrolavendulae* S4 treatment. In addition, *S. rubrolavendulae* S4 showed high efficiency equivalent to fungicide, metalaxyl with no significant difference. The authors propose that this bacterium can prevent the tomato and chili seedling damping off disease in economic plant nurseries.

Only one review article is published in this special issue by Rajashekar et al., focusing on the current state of the botanical insecticides as grain protectants and their mode of action, based on numerous references. On the basis of physiological activities on insects, the plant components has been conventionally classified into 6 groups, namely, repellents, feeding deterrents/antifeedants, toxicants, growth retardants, chemosterilants, and attractants. Focus on the activity of toxicants and grain protectants using essential oils, extracts, and their constituent has sharpened since the 1980s. Some insecticidal active principles of plants are listed. The botanical insecticides that have primarily been used and are commercially available include ryania, rotenone, pyrethrin, nicotine, azadirachtin, and sabadilla. This review proposed that it is possible to develop methods for grain protectants with reduced use of synthetic chemical insecticides.

These papers represent an exciting, insightful observation into the biopesticides point of view. However, research efforts should focus not only on their efficacy but also on mammalian toxicity, mode of action in insects, seed germination, effect on nutritional quality, seedling growth, and stability of the compound. The insecticides of plant origin could be exploited for the development of novel molecules with highly precise targets for sustainable insect pest management in stored grain. We hope that this special issue would attract a major attention of the peers. We would like to express our appreciation to all the authors, reviewers, and the Editor-in-Chief, Dr. Kabkaew L. Sukontason, for great support that made this special issue possible.

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Research Article

Biocontrol of *Phytophthora infestans*, Fungal Pathogen of Seedling Damping Off Disease in Economic Plant Nursery

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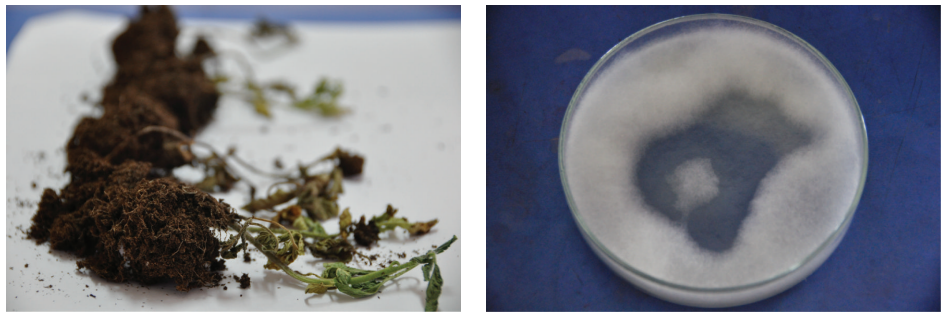
This research aims to control Seedling damping off disease in plants by using antagonistic actinomycetes against the causative fungi. *Phytophthora infestans* was isolated from the infected tomato plant seedling obtained from an economic plant nursery in Amphoe Pak Chong, Nakhon Ratchasima Province, Thailand. The chitinolytic *Streptomyces rubrolavendulae* S4, isolated from termite mounds at the grove of Amphoe Si-Sawat, Kanchanaburi Province, Thailand, was proven to be the most effective growth inhibition of fungal pathogens tested on potato dextrose agar. Tomato and chili seedlings that colonized with antagonistic *S. rubrolavendulae* S4 were grown in *P. infestans* artificial inoculated peat moss. Percents of noninfested seedling in fungal contaminated peat moss were compared to the controls with uninoculated peat moss. In *P. infestans* contaminated peat moss, the percents of survival of tomato and chili seedling were significantly increased ($P < 0.05$) from 51.42 to 88.57 and 34.10 to 76.71 for the *S. rubrolavendulae* S4 treatment, respectively. The *S. rubrolavendulae* S4 also showed high efficiency equivalent to fungicide, metalaxyl with no significant difference ($P > 0.05$). It was clearly demonstrated that *S. rubrolavendulae* S4 can prevent the tomato and chili seedling damping off disease in economic plant nurseries.

1. Introduction

The value of vegetable crops in Thailand was estimated to be around 14,561 million baht in 2009, including tomato and chili. The plantation of these economic crops is done by using reliable seedling producers. Therefore, the economic plant nursery business has been increasing. Disease management has become a major concern during the production of vegetable plug transplants. The seedling damping off disease causes serious problems in economic plant nurseries. Causative pathogenic fungi of seedling damping off disease in plants were reported to be *Pythium* spp., *Phytophthora* sp. [1, 2], *Rhizoctonia solani* [3], *Sclerotium rolfsii* [4], and *Fusarium oxysporum* [5]. *Phytophthora infestans* is the most infamous species of genus which caused pre- and postemergence damping-off and late blight of potato and tomato. Also, peppers, melons, pumpkins, citruses, strawberries, chestnuts, and forest trees are affected by *Phytophthora* species such as *P. cambivora, P. hibernalis, P. citrophthora, P. kernoviae, P. capsici, P. cactorum, P. drechsleri,* and *P. infestans* [6–9]. Chemical fungicides are extensively used in current agriculture and also cause environmental pollution. Nowadays, a method of controlling or preventing the disease is by decreasing hazardous chemical fungicides. Biocontrol is used as an alternative method. The microorganism simultaneously grows together with pathogenic fungi and produced enzyme or organic compounds for suppression fungal growth. Biocontrol with microbial fungicides is being investigated in several academic labs. Seedling damping off disease occurred in economic plant nurseries in Amphoe Pak Chong, Nakhon Ratchasima Province. In this study, the major causative fungal pathogens of the seedling damping disease were investigated. The antagonistic chitinolytic *Streptomyces* against the fungal pathogen was experimented to be used to control the disease in plant nurseries.

2. Materials and Methods

2.1. Isolation and Identification of Plant Pathogenic Fungi. The plant samples were obtained from economic plant



(a) (b)

Figure 1: *Phytophthora infestans* isolated from infected tomato plant seedling (a) and produced white, profusely branching mycelium (b).

nurseries. Roots and stems of tomato seedlings with damping off disease symptoms were washed to remove any excess peat moss. Then, the infested plant parts were surface-sterilized using 5% v/v hypochlorite for 30 seconds, washed with sterile water, and blot-dried on sterile filter paper. Plant pieces were cut into 0.5 cm lengths before being placed onto potato dextrose agar (PDA). The fungal mycelium and spores that grew out of the plant tissues were subcultured and purified on another PDA plate. The pathogenic fungi were identified based on colony morphology and by the characteristics of sporangium and oospores.

2.2. Antagonistic Actinomycetes. The chitinolytic actinomycete was isolated from the termite mounds at the grove of Amphoe Si-Sawat, Kanchanaburi Province, Thailand, by using the soil dilution method. The chitinolytic actinomycete was preliminary classified to be *Streptomyces* sp. based on morphological and physiological characteristics [10]. The chitinolytic actinomycete was found similar to *Streptomyces rubrolavendulae* based on 16S rRNA analysis. The *S. rubrolavendulae* S4 was maintained on nutrient agar slants at 30°C for 3–5 days to make the fresh colony before being used in the next experiment.

2.3. Antagonistic Test by Dual Culture Technique. The antifungal activity of the *S. rubrolavendulae* S4 against seedling damping off fungi was tested on V8 agar [11] at 30°C using a dual culture technique. The conidia of the *S. rubrolavendulae* S4 were placed on a V8 agar plate in lines. Then, the plates were incubated at 30°C for 5 days to allow growth and sporulation of the *S. rubrolavendulae* S4 prior to inoculation of an agar plug of the pathogenic fungi at the center of the plate. After incubation for 3–5 days at 30°C, the growth inhibition of pathogenic fungi by *S. rubrolavendulae* S4 was investigated. The size of the zone of inhibition developing around the *S. rubrolavendulae* S4 was a measurement of the antagonistic potential against the pathogen. Only the pathogen was used as a positive control and the experiments were repeated three times with three replications for each experiment. Percentage growth inhibition was determined after 3 days incubation by this formula of Skidmore [12]:

$$\text{The percentage of inhibition growth } (\%) = \frac{R - r}{R} \times 100, \quad (1)$$

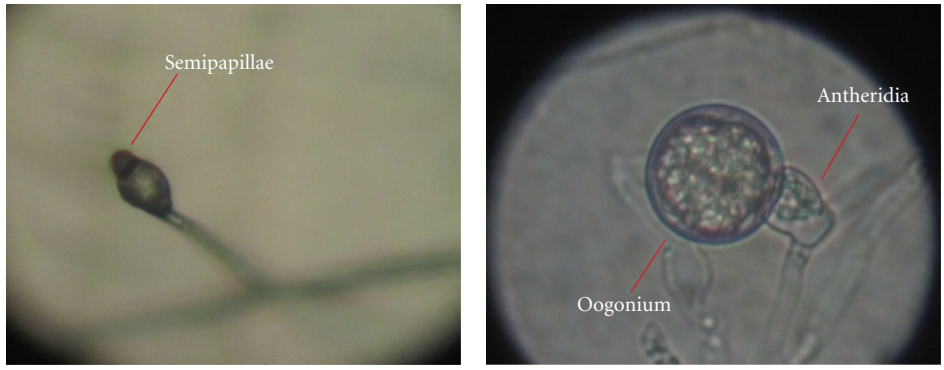
where R represents the radius of the control pathogens growth and r the radius of the pathogen's growth towards the bacterial antagonist.

2.4. Suppression of Seedling Damping Off Fungi under Greenhouse Conditions. Peat mosses were sterilized for 15 mins at 121°C 15 lbs/in^2 three times at 24 h intervals and used as a planting material in this study. The agar plugs, taken from the edge of the young colony of pathogenic fungi, were artificial inoculated into steam-pasteurized peat mosses at the rate of 50 agar plugs/250 g. *S. rubrolavendulae* S4 was cultured in nutrient broth with 1% w/v shrimp shell powder at 30°C for 3 days and used for plant protection experiments. The *S. rubrolavendulae* S4 cell suspension was inoculated into the peat moss at the final concentration of 10^6 cfu/g. The experiment was a 2 × 5 factorial completely randomized design with three replicates. Two kinds of seedling were used: tomato and chili. The 3 sets of 10 seedlings were grown in five types of treated planting material: (1) artificial fungal pathogen infested, (2) artificial fungal pathogen infested but challenged with *S. rubrolavendulae* S4, (3) artificial fungal pathogen infested but treated with fungicide, metalaxyl (Phyto-Q), *S. rubrolavendulae* S4 inoculated, and (4) uninoculated planting material as control. Percentages of the noninfested seedling were then determined.

3. Results

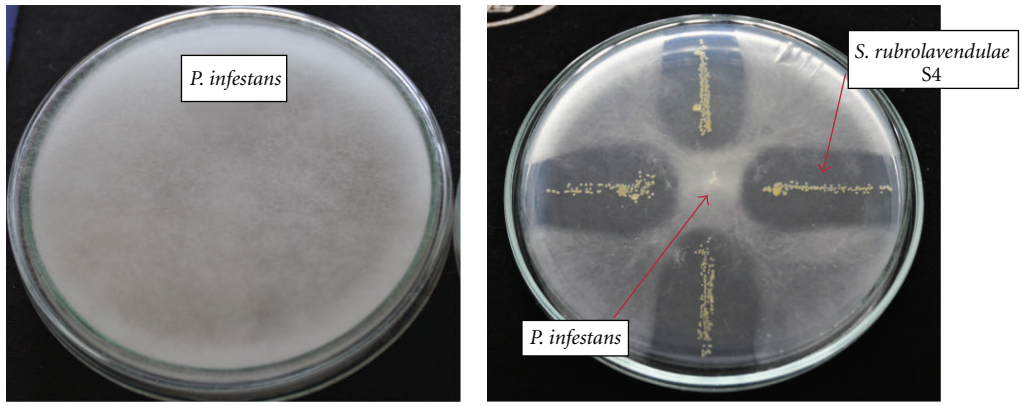
3.1. The Causative Fungal for Damping Off Disease of Seedlings in the Plant Nursery. The major plant pathogenic fungi isolated from the infected tomato plant seedling was identified to be *Phytophthora infestans,* based on morphology in the form of chlamydospores and sporangia which produce zoospores [13]. This isolate can be grown in PDA and produced white, profusely branching and aseptate mycelium without septum (Figure 1).

The leading edge of mycelia plugs was transferred to petri dishes containing peat moss extract, and then incubated



(a) (b)

Figure 2: *Phytophthora infestans*, (a) lemon shape sporangia, (b) amphigynous antheridia of oospores.



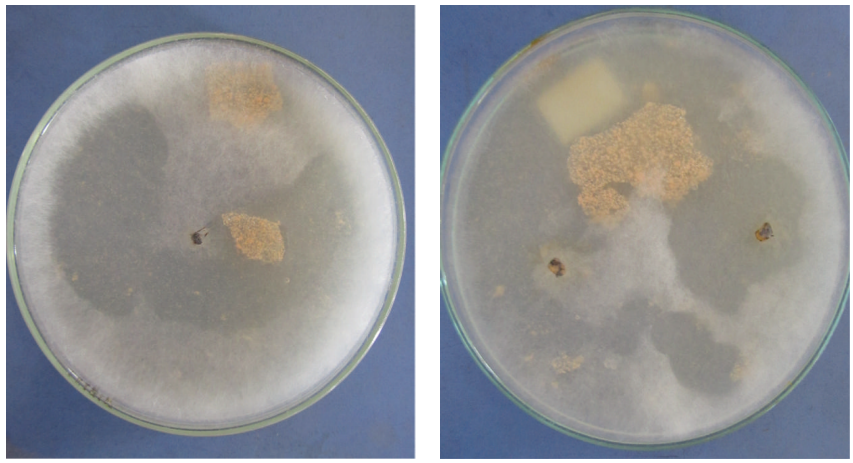
(a) (b)

Figure 3: *In vitro* interactions between *S. rubrolavendulae* S4 and *Phytophthora infestans* on V8 medium. (a) Control plate of *Phytophthora infestans*; (b) *S. rubrolavendulae* S4 against *Phytophthora infestans*.

at room temperature under continuous 40-watt fluorescent illumination for between 1 and 4 days, amphigynous antheridia of oospores. The sporangia in a lemon shape were observed (Figure 2) which are a semipapillae type of sporangia and released zoospores in wet peat moss or water.

3.2. Antagonistic Activity of S. rubrolavendulae S4 against P. infestans. The dual culture method that was used to investigate the antagonistic of the *S. rubrolavendulae* S4 indicated that *S. rubrolavendulae* S4 that was used as antagonistic microorganism for suppression mycelia growth of *P. infestans* on V8 Agar (Figure 3). After being incubated for 3 days at room temperature, the radiuses of *P. infestans* growth on control plate and *P. infestans* growth toward *S. rubrolavendulae* S4 were measured about 4.5 cm and 0.75 cm, respectively. Moreover, the radial growth of *P. infestans* produced a clear zone around the *S. rubrolavendulae* S4 growth indicating the inhibition of the fungal growth. Therefore, 83.33% of growth inhibition has clearly demonstrated that *S. rubrolavendulae* S4 exhibited good growth inhibition of the pathogenic fungi, *P. infestans*.

3.3. Suppression of Tomato and Chili Seedling Damping Off Disease by Antagonistic S. rubrolavendulae S4. The biological suppression of the seedling damping off disease of tomato and chili seedling was performed. *S. rubrolavendulae* S4 cultured in shrimp shell broth at optimum conditions was inoculated into satirized peat moss. Tomato and chili seedling were grown in peat moss and colonized with antagonistic *S. rubrolavendulae* S4 and *P. infestans*. Results from the greenhouse pot experiment demonstrated that *S. rubrolavendulae* S4 significantly inhibited root rot of tomato and chili seedling caused by *P. infestans*. Percents of noninfested seedling in fungal contaminated peat moss were compared to the controls with uninoculated peat moss. In *P. infestans* contaminated peat moss, the percents of survival of tomato and chili seedling were significantly increased ($P < 0.05$) from 51.42 to 88.57 and 34.10 to 76.71 for the isolate S4 treatment, respectively (Table 1). The *S. rubrolavendulae* S4 also showed a high efficiency equivalence to fungicide, metalaxyl with no significant difference ($P > 0.05$). These treated plants looked healthy and increased the percentage of healthy plants showing no symptoms of root rot. The *P. infestans* was reisolated from the infested seedling to confirm the effectiveness of the fungal pathogen.



(a) (b)

Figure 4: *Phytophthora infestans* was observed from peat moss (a) and seeds (b).

Table 1: Efficacy of biocontrol, *S. rubrolavendulae* S4 on suppression of tomato and chili seedling damping off disease caused by *Phytophthora infestans* under greenhouse conditions.

Treatment	Percentage of noninfested tomato seedling	Percentage of noninfested chili seedling
Control	88.56[b]	95.71[c]
P. infestans	51.42[a]	34.10[a]
P. infestans + *S. rubrolavendulae* S4	88.57[b]	76.71[b]
P. infestans + Phyto-Q	94.28[b]	79.99[b]
S. rubrolavendulae S4	87.14[b]	90.29[bc]

[a, b, c] Means within a column with the same letter were not significantly different ($P > 0.05$).

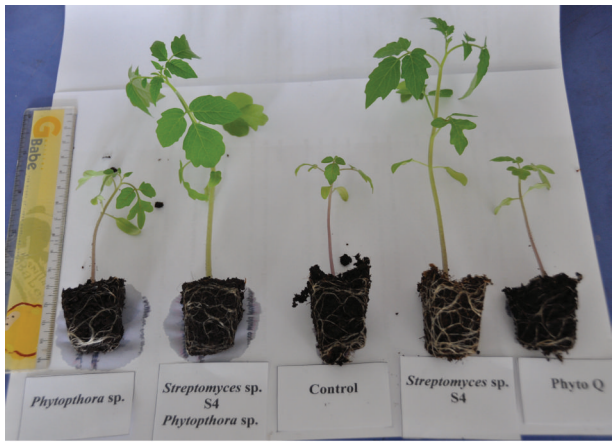


Figure 5: The healthy tomato seedlings grown in peat moss inoculated with *Phytophthora infestans* and *S. rubrolavendulae* S4 in different treatments.

4. Discussion

The plant pathogenic fungi, *Phytophthora infestans,* was isolated from the infected tomato plant seedling in the economic plant nursery. *Phytophthora* often called water mold can be grown in wet soil and produced white, profusely branching, aseptate mycelium, sporangia, and oospores. This *Phytophthora* can spread widely with zoospores and oospores which are produced in sporangium and oogonium, respectively [14].

In sexual spore type, oospores were produced when antheridia was attached to oogonium. Moreover, asexual spore types of *P. infestans* are chlamydospores and sporangia were used as a survival structure. The zoospores were contained in a lemon shape of sporangium (Figure 2(a)).

In the plant fields, greenhouses, and nurseries, chemical fungicides were used for disease management. Metalaxyl and fosetyl-A1 are suggested chemical fungicides to be used against *Phytophthora* species which are dangerous for the environment [15–18]. Therefore, the biological control was applied for disease management that will be safer for health and the environment. Control of *Phytophthora* root rot was achieved by infesting peat moss with *Streptomyces* at the time of planting under greenhouse conditions.

The frequency of healthy plants increased significantly for the susceptible variety, and the average disease severity index decreased significantly for both the resistant and susceptible varieties tested. It was clearly demonstrated that isolate S4 could prevent the tomato and chili seedling damping off disease in the economic plant nursery. In recent studies, the *Trichoderma harzianum*, *Bacillus subtilis*, *Pseudomonas fluorescens,* and *Streptomyces* species were reported as commercial biocontrol agents for controlling *Phytophthora* species (BCA) [2, 19–25]. The mechanisms of parasitism, competition, antibiotic, and enzyme were performed in different antagonistic microorganisms.

The extracellular cell wall degrading enzymes including chitinases, cellulases, amylases, and 1, 3-β-glucanases are found in the genus *Streptomyces* [26–28]. The high chitinases and cellulases activities were produced by *S. rubrolavendulae* S4.

The 51.42 and 34.10 percentage of survival of tomato and chili seedling were observed in pots containing *P. infestans*, respectively (Table 1). After 3–5 days of cultivation in pots, the tomato and chili appeared to contain seedling damping off disease. A soften, decayed, weakened, and die seedling were expressed in pregerminated and postgerminated chili and tomato seeds. Then, peat mosses or seeds were transferred onto PDA plates and cultured at room temperature for 3 days (Figure 4). After that the similarity characteristics to *Phytophthora infestans* were observed from that plate. An aseptate mycelium, lemon shape of sporangium, and oogonium were observed under a light microscope (10X). The results indicated that the expression of damping off disease in chili and tomato seedlings is caused by *Phytophthora infestans*. After 50 days of cultivation, the healthy tomato and chili transplants were performed in treatments with added *S. rubrolavendulae* S4. These transplants showed higher height and weight than other treatments (data not show) (Figure 5). The bioactive natural compounds and plant hormones can be produced from several *Streptomyces* species and affect the host plant as plant growth promotion bacteria [29, 30]. The results clearly demonstrated that seedling damping off disease of tomato and chili in economic plant nurseries can be controlled by *S. rubrolavendulae* S4.

5. Conclusion

The chitinolytic *S. rubrolavendulae* S4 had a strong-antagonistic activity against *Phytophthora infestans*, isolated from the infected tomato plant seedlings. Therefore, *S. rubrolavendulae* S4 can be used as a good biocontrol for seedling damping off disease in economic plant nurseries. In *P. infestans* contaminated peat moss, the biocontrol increased the percentage of surviving tomato and chili seedling from 51.42 to 88.57 and 34.10 to 76.71, respectively. The mass cell production of *S. rubrolavendulae* S4 in an appropriate medium will be conducted as future work.

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The authors would like to thank Green and Clean Vegetables Limited, the economic plant nursery in Amphoe Pak Chong, Nakhon Rachasima Province, for supporting the plant samples.

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Research Article

Investigations on the Effects of Five Different Plant Extracts on the Two-Spotted Mite *Tetranychus urticae* Koch (Arachnida: Tetranychidae)

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Two-spotted mite, *Tetranychus urticae* Koch (Arac.: Tetranychidae), is an economic pest worldwide including Turkey, causing serious damage to vegetables, flowers, and fruit crops. In recent years, broad-spectrum insecticides/miticides have been used to control this pest in Turkey. Control is difficult mainly due to resistance to conventional pesticides. This study was conducted to determine efficacy of pesticides extracted from five different plants [i.e., *Allium sativum* L. (Amaryllidaceae), *Rhododendron luteum* S. (Ericaceae), *Helichrysum arenarium* L. (Asteraceae), *Veratrum album* L. (Liliaceae), and *Tanacetum parthenium* L. (Asteraceae)] against this mite. Bioassays were tested by two different methods to determine the effects of varying concentrations. Experiments were performed using 3 cm diameter leaf disk from unsprayed bean plants (*Phaseolus vulgaris* L.). In addition, the effects of the extracts on reproduction and oviposition were investigated. The extract yielded high mortality. In the lowest-concentration bioassays, the adult mites laid lower numbers of eggs compared to the untreated control. No ovicidal effect was observed.

1. Introduction

Diseases and insect pests are the major limiting factors in the production of high quality agricultural products. Although conventional pesticides have become an indispensable tool in controlling some pests economically, rapidly, and effectively, extensive use of insecticides may lead to a number of undesirable side effects including the development of insect resistance and resurgence of primary and secondary pests outbreaks. Also they can have adverse effects on nontarget organisms and general environmental contamination [1–4]. The other problems with synthetic insecticides are environmental pollution and insect resistance. According to Nas [5] interest in the application of botanical pesticides for crop protection is on the rise. Many researchers are experimenting and developing alternative plant extracts as pesticides to be used against pest insects.

Plants have the richest source of renewable natural pesticides. Specifically, plant extracts provide a safe and viable alternative to synthetic pesticides and are compatible with the use of beneficial organisms, pest-resistant plants, and to preserving a healthy environment in an effort to decrease reliance on synthetic pesticides. There are many benefits of using botanical pesticides such as reduced environmental degradation, increased safety for farm workers, increased food safety, reduction in pesticide resistance, and improved profitability of production.

As a result, many plant compounds, the majority of which are alkaloids and terpenoids, have now been known to affect insects' behaviour, growth and development, reproduction, and survival [6–9]. Many investigations have recently been performed in relation to effects of plants such as *Chrysanthemum roseum* Web. and Mohr. (Compositae), *Nicotiana tabaccum* L. (Solanaceae), *Derris elliptica* Benth (Fabaceae), neem tree, *Azadirachta indica* A. Juss (Meliaceae), *Melia azaderach* L. (Meliaceae), and *Xanthium strumarium* L. (Solanaceae) on insects [10–13]. The seed kernel extract of neem, known as azadirachtin, has been most

thoroughly tested, and it has been extracted in larger quantities than the other components of neem [14, 15]. High rates of mortality have been found on the two spotted mites fed on the leaves treated with *A. indica* extract. In addition, the same extract significantly reduced the reproductive capacity of mites and the survival of the progeny of treated females greatly diminished in comparison to the control [16].

T. urticae is a very important pest worldwide, causing serious damage to vegetables, flowers, and fruit crops. Many crops must be protected with synthetic acaricides during hot and dry seasons that favor severe outbreaks of *T. urticae*. It is able to transmit many of plants viruses [17].

R. luteum and *V. album* are poisonous plants. It is recorded that the extract of *V. album* has been used as insecticide or rodenticide since the Roman times. Also, today, plants containing toxic alkaloid are used successfully as insecticides and fungicides [18]. In one of the studies evaluating the effectiveness of plant extracts against house flies as indicated, *V. album* inhibited the development of the larvae and the high toxicity [19].

H. arenarium, T. parthenium, and *A. sativum* are important medicinal plants. *H. arenarium*, an infusion of the bright yellow flowers, is used in the treatment of gallbladder disorders and as a diuretic in treating rheumatism and cystitis. It is a component in *zahraa*, an herbal tea used for medicinal purposes in some countries [20]. *A. sativum* and *T. parthenium* have a broad spectrum of biological activity. They have been used for anti-inflammatory, antibacterial, and antifungal activities [21]. It is determined that the extract of *T. vulgare* inhibited the development of *Dermanyssus gallinae* (Mesostigmata: Dermanyssidae). In addition, the same plant extract cultivated showed that it is effective on *T. urticae* [22]. The extract of garlic leaves caused high mortality and reduced reproductive capacity on *T. urticae* [23]. According to the literature, no Works have been published on the acaricidal activity of *H. arenarium*. This study was undertaken in the laboratory at the Central Plant Protection Research Institute in 2009, and the miticidal effect of five plant extracts on *T. urticae* was tested.

2. Material and Methods

2.1. Plants and Preparation of Extracts. This study covered five plant species; *R. luteum*, *H. arenarium*, *A. sativum*, *V. Album,* and *T. parthenium* were tested as an alternative miticidal. Their leaves and stems were collected when plants were at the flowering stage during the years 2008 and 2009. Only the fruit garlic plant was used for this purpose. Ethanol was used as a solvent to extract the required material from five plants for use as an acaricide. The method of Brauer and Devkota [24] was used in preparation of five plants' ethanolic extract.

The materials were stored in the laboratory to dry up. The dried materials were grounded using a blender, and ethanol was added to the dried powder for 72 hours. This mixture was extracted in 5-6 hours using a Soxhlet machine. The ethanol was removed from the extract in a rotary evaporator (50–60°C). For each plant sample 200 g of dried materials were used to prepare the extract.

2.2. Mites. As a test organism, *T. urticae* was reared on green bean plants, *Phaseolus vulgaris*. The bean plants used in the experiment were grown in a greenhouse.

2.3. Effects of the Extracts of Five Plants on Tetranychus urticae. In all the experiments, first instar larvae and 3-day-old adults were used. Four concentrations and an untreated control were used for all bioassays. Test samples for bioassay were resuspended in distilated water with TritonX.100 at a rate of 0.1 mL/L. Vaseline was used so as to prevent the mites from escaping. Experiments were carried out using (3 cm diameter) leaf discs of green bean leaves. The leaf disks were placed on a moistened filter paper disk and each disk was infested with 10 individuals. Each treatment was replicated 10 times. The concentrations used for mites were 1%, 3%, 6%, and 12% [16].

2.4. Effect on Eggs. Green bean leaf discs were placed into petri dishes on moistened filter paper and females of the same age were put on leaf discs. The eggs were counted after two days. Ten eggs were placed in every petri dish and the other eggs removed. Then the eggs were sprayed with different concentrations of extract (17–20 μL/cm^2) using a small hand-held sprayer. The numbers of hatched larvae were recorded.

2.5. Effect of the Extracts on Larvae and Adults

2.5.1. Leaf-Dipping Method. Green bean leaf discs were treated by dipping them into extract solutions of known concentrations, then left to dry for 30 minutes. The treated leaf discs and individual mites were placed in the petri dishes (9 cm in diameter) that were lined with moistened filter paper. The results were assayed after 1, 3, and 6 days by counting the number of living adults and larvae.

2.5.2. Leaf-Spraying Method. Green bean leaf discs were placed into Petri dishes on moisturized filter paper. Ten adults were placed in every Petri dish. Then eggs were sprayed with different concentrations of extract (17–20 μL/cm^2) using a small hand-held sprayer. The results were assayed after 1, 3, and 6 days by counting the number of living adults.

2.6. Effect on Egg-Laying Capacity. Green bean leaf discs were dipped for 3–5 seconds in prepared concentrations (1, 3, 6, and 12%), then they were dried for 30 minutes and placed in petri dishes with ten adults. After 48 hours of feeding on treated green bean leaves, mites were given untreated green bean leaves. The experiment was repeated 10 times. Daily monitoring was done for fourteen days and the total number of eggs was recorded [25].

The experiments were conducted in a climate chamber at 25-26°C and under long daylight (18 h : 6 h, light : dark). The effect was calculated according to Abbott [26]. The obtained reasults were submitted to a variance analysis and the mean values were compared by Duncan's test ($P = 0.05$) calculated by the program SPSS 13.6). Mortality rate was calculated as; mortality = after treatment the number of died mites/before treatment the number of mites · 100).

Table 1: Effect (mean ± SE) and mortality (%) of extracts obtained from different five plants on *T. urticae*.

Treatment		Leaf-dipping method				Leaf-spraying method	
		Larvae		Adult		Adult	
	Concentration (%)	Mortality (%)	Effect (%)	Mortality (%)	Effect (%)	Mortality (%)	Effect (%)
H. arenarium	1	46	31.59 ± 4.00^{c}	37	25.32 ± 4.10^{c}	52	39.76 ± 5.18^{c}
	3	53	41.59 ± 5.47^{bc}	47	37.22 ± 6.77^{bc}	66	59.88 ± 4.65^{b}
	6	58	46.09 ± 2.53^{b}	51	42.36 ± 5.61^{b}	76	71.82 ± 1.76^{ab}
	12	71	62.72 ± 2.28^{a}	64	56.85 ± 5.63^{a}	85	82.38 ± 1.92^{a}
A. sativum	1	46	31.30 ± 5.01^{b}	29	16.43 ± 2.43^{b}	66	59.76 ± 4.45^{b}
	3	50	37.80 ± 5.96^{b}	34	27.59 ± 5.17^{ab}	69	65.45 ± 5.16^{ab}
	6	56	43.37 ± 5.95^{b}	45	34.35 ± 6.76^{a}	77	72.79 ± 4.38^{a}
	12	68	58.35 ± 6.31^{a}	49	39.49 ± 5.07^{a}	78	73.92 ± 3.16^{a}
V. album	1	50	35.83 ± 4.33^{c}	51	29.07 ± 4.71^{c}	47	33.57 ± 4.12^{c}
	3	65	54.93 ± 5.22^{b}	61	42.41 ± 6.33^{b}	59	49.72 ± 3.39^{b}
	6	75	65.37 ± 3.15^{ab}	78	51.57 ± 5.37^{a}	70	62.58 ± 2.98^{b}
	12	77	70.55 ± 2.44^{a}	79	79.02 ± 3.76^{a}	81	75.77 ± 3.81^{a}
T. parthenium	1	49	38.22 ± 5.83^{c}	64	54.49 ± 4.34^{c}	47	33.61 ± 4.14^{c}
	3	64	54.06 ± 3.14^{b}	77	69.58 ± 1.52^{b}	60	49.75 ± 3.41^{b}
	6	75	67.89 ± 2.56^{a}	85	82.41 ± 1.94^{a}	71	62.62 ± 2.96^{b}
	12	82	76.54 ± 3.51^{a}	88	83.47 ± 1.95^{a}	81	75.68 ± 3.77^{a}
R. luteum	1	44	31.87 ± 3.31^{b}	27	31.66 ± 4.50^{b}	37	25.81 ± 2.94^{c}
	3	48	35.38 ± 4.05^{b}	44	34.02 ± 3.62^{b}	42	43.23 ± 3.40^{b}
	6	74	66.14 ± 4.50^{a}	53	44.35 ± 4.43^{b}	58	50.31 ± 3.28^{ab}
	12	81	75.62 ± 3.03^{a}	67	63.66 ± 2.44^{a}	68	61.97 ± 3.75^{a}
Control		22	0	15	0	15	0

Within columns, means ± SE followed by the same letter are not significantly different (DUNCAN's multiple F-test $P < 0.05$).

3. Results and Discussion

3.1. Effect on Eggs. All of the eggs treated were found to have hatched. It is determined that the ethanolic extracts of *R. luteum, H. arenarium, A. sativum*, *V. album*, and *T. parthenium* did not have an ovicidal effect. The hatched larvae continued to develop as it was in the control.

3.2. Effect of the Extracts on Larvae

3.2.1. Leaf-Dipping Methods. From Table 1, it can be observed that ethanol extracts of five plants had a significant mortality and the highest effect on *T. urticae* larvae. In all of the plant extracts, the highest effect occurred at a concentration of 12% while the smallest effect was at 1%. The increased concentration led to increased larval mortality. Statistical analysis showed $P < 0.05$ importance between the treatments. The extract of *T. parthenium* showed the highest effect on the *T. urticae* larvae. The smallest effect was at the extract of *A. Sativum*.

3.3. Effect of the Extracts on Adult

3.3.1. Leaf-Dipping Methods. As shown in Table 1, for the adults placed on leaf discs treated with different plant of extracts, the highest effect was determined at a concentration of 12% the extract of *T. parthenium*. Among the plant extracts, the extract of *T. parthenium* indicated the highest mortality. On the other hand, the smallest mortality was found at the extract of *A. sativum.* The increased concentration led to increased adult mortality.

3.3.2. Leaf Spraying Method. For the larvae placed on leaf discs treated with different plant of extracts at concentration of %12, mortality at the extract of *H. arenarium, A. sativum, V. album*, *T. parthenium*, and *R. luteum* was 85, 78, 81, 81, and 68%, respectively. In all of the extracts the highest effect was determined at a concentration of 12% while the smallest effect was at 1% (Table 1).

In both methods, similar results were obtained and there was not a significant difference on the mortality when leaf-dipping method was compared with direct spraying on the plant.

3.4. Effect on Egg-Laying Capacity. The numbers of eggs laid by mites feeding on extract-treated bean leaves were found to be statistically significant ($P < 0.05$) for all extracts with the maximum number of eggs obtained from the control. The lowest number of eggs was found at the 12% concentration of the extract of *R. luteum,* and the number of eggs laid was reduced significantly by increasing concentration (Table 2).

Ethanolic extracts were made from different plants and their effects were tested on two-spotted mite for the first time

Table 2: Effect of extracts from obteined different five plants on egg laying capacity of *T. urticae*.

Concentrations (%)	Treatment				
	H. arenarium	*A. sativum*	*V. album*	*T. parthenium*	*R. luteum*
	Number of eggs (mean ± SE)				
Control	162.5 ± 11.80^{c}	162.5 ± 11.80^{c}	162.5 ± 11.80^{c}	162.5 ± 11.80	162.5 ± 11.80^{c}
1	145.5 ± 5.91^{c}	184.0 ± 12.10^{b}	152.6 ± 10.50^{c}	158.0 ± 12.1^{b}	152.6 ± 10.50^{c}
3	94.5 ± 6.0^{b}	154.3 ± 10.3^{b}	137.6 ± 13.43^{c}	153.3 ± 10.3^{b}	137.6 ± 13.40^{c}
6	81.8 ± 6.40^{b}	115.2 ± 9.13^{a}	108.9 ± 19.9^{b}	136.2 ± 9.12^{b}	88.9 ± 19.92^{b}
12	62.5 ± 6.33^{ab}	98.2 ± 8.60^{a}	96.4 ± 2.52^{b}	87.2 ± 8.60^{a}	18.4 ± 2.50^{a}

Within columns, means ± SE followed by the same letter are not significantly different (DUNCAN's multiple F-test $P < 0.05$).

in the world. It was observed that some extracts showed a high rate of mortality and reduced fecundity on *T. urticae.*

There were no references in the literature of other studies using four plant extracts ethanolic extract on *T. urticae* except that *A. sativum*. However, other plant extracts have been investigated and the findings for *T. urticae* are similar to those of our study. Neem seed kernel extracts and its formulation are reported to influence mortality, repellency, and fecundity of mites [27–29]. It was found out that the two commercial preparations of neem seed extracts (Margosan-0 and Neem Azal S, Neem Azal T/S) were effective on *T. urticae* [16, 30]. Several herbal extracts of *Achillea millefolium* L. (Asteraceae), *Taraxacum officinale*s F. H. (Asteraceae), *Matricaria chamomilla* L. (Asteraceae), and *Salvia officinalis* L. (Lamiaceae) demonstrated strong inhibition of the feeding activity of mites [31, 32]. It was determined that the extracts of yew showed a high mortality, decrease in female fecundity and shortened longevity [33, 34]. Shi et al. [35] revealed that the extract of *Bassia scoparia* (L.) A. J. Scott. (Chenopadiaceae) showed contact and systemic effects, and it caused high rates of mortality in all the three species (*T. urticae, T. cinnabarinus*, and *T. viennensis*). Pure azadirachtin reduced the reproductive capacity and feeding of *T. urticae* [36]. Crude foliar extracts of 67 species from six subfamilies of Australian Lamiaceae showed both contact and systemic toxicity to these mites [37]. The extracts of wild tomato leaf showed strong repellency effect on *T. urticae* [38]. The acaricidal activities of plant extracts on *T. urticae* were tested. The mortalities were high in extracts *Albizia coreana* Twig., *Pyracantha angustifolia* F. (Rosaceae), and *Ligustrum japonicum* Thunb. (Oleaceae) within 48 h treatment [39]. Attia et al. [23] revealed that the extract of garlic led to a rise in female mortality and a reduction in fecundity with the increasing of concentration. Essential oils of *Artemisia absinthium* L. (Asteraceae) and *Tanacetum vulgare* L. (Asteraceae) were extracted by three methods, a microwave-assisted process (MAP), distillation in water (DW), and direct steam distillation (DSD), and tested for their toxicity as contact acaricides to *T. urticae.* DSD and DW extracts of *T. vulgare* were more toxic (75.6 and 60.4% mite mortality, resp., at 4% concentration) to *T. urtica* than to the MAP extract (16.7% mite mortality at 4% concentration) [22]. The ethanol extracts *of Croton rhamnifolius* H.B.K. (Euphorbiaceae) *C. sellowi, C. jacobinensis, and C. micans* had a high mortality on *T. urticae,* whereas *C. sellowi* extract showed the highest effect [40]. Garlic extract showed a mortality at 48–57% on *T. urticae* [41]. Wang et al. [42] revealed that the crude extract of walnut leaf had some contact and systemic effect on *T. cinnabarinus* and *T. viennensis*.

It was found out that the extract of *V. album* and *T. parthenium* had a high rate mortality and reduced fecundity for *T. urticae.* Ethanolic extracts of *V. album* and *T. parthenium* can be useful to control *T. urticae* populations on vegetable plants grown through Integrated Pest Management (IPM) and organic systems of agriculture.

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Review Article

Botanicals as Grain Protectants

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Prevention of food losses during postharvest storage is of paramount economic importance. Integrated pest management is now a widely accepted strategy in pest control including postharvest infestation control which involves the use of chemical (contact/residual) insecticides along with fumigants. The use of synthetic chemical insecticides is either not permitted or used restrictively because of the residue problem and health risks to consumers. In view of the above, there is a need for plants that may provide potential alternatives to the currently used insect control agents as they constitute a rich source of bioactive molecules. Available literature indicates that plant could be source for new insecticides. Therefore, there is a great potential for a plant-derived insecticidal compounds. This paper focuses on the current state of the botanical insecticides as grain protectants and its mode of action.

1. Introduction

Food grain losses due to insect infestation during storage are a serious problem, particularly in the developing countries [1, 2]. Losses caused by insects include not only the direct consumption of kernels, but also accumulation of exuviae, webbing, and cadavers. High levels of the insect detritus may result in grain that is unfit for human consumption and loss of the food commodities, both, in terms of quality and quantity. Insect infestation-induced changes in the storage environment may cause warm moist "hotspots" that provide suitable conditions for storage fungi that cause further losses. It is estimated that more than 20,000 species of field and storage pests destroy approximately one-third of the world's food production, valued annually at more than $100 billion among which the highest losses (43%) occurring in the developing world [3, 4]. The quantitative and qualitative damage to stored grains and grain product from the insect pests may amount to 20–30% in the tropical zone and 5–10% in the temperate zone [5, 6]. Food grain production in India has reached 250 million tonnes in the year 2010-2011, in which nearly 20–25% food grains are damaged by stored grain insect pests [7, 8]. The efficient control and removal of stored grain pests from food commodities has long been the goal of entomologists throughout the world.

The major pests of stored grain and pulses of the Indian subcontinent are classified in to two groups, namely, primary pests: those which are capable of penetrating and infesting intact kernel of grain and have immature stages develop within kernel of grain and secondary pests which cannot infest the whole grain but feed on as broken kernels, debris, high moisture weed seeds, and grain damaged by primary pests. In general, the immature stages of the secondary pest species are found external to the grain. It is often thought that secondary invaders cannot initiate infestation. The important primary pests are the rice weevil, *Sitophilus oryzae* (L.), granary weevil, *Sitophilus granaries* (L.), (Coleoptera: Curculionidae), lesser grain borer, *Rhyzopertha dominica* (F.), (Coleoptera: Bostrichidae), Khapra beetle, *Trogoderma granarium* (Everts), (Coleoptera: Dermestidae), and the pulse beetle *Callosobruchus chinensis* (L.) (Coleoptera: Bruchidae). The secondary pests are rust-red flour beetle, *Tribolium castaneum* (Herbst), (Coleoptera: Tenebrionidae), rusty grain beetle, *Cryptolestes ferrugineus* (L.), (Coleoptera: Cucujidae),

sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), (Coleoptera: Silvanidae), mites, (Acarina: Tetranychidae) *Liposcelis corrodens*, and (Psocoptera: Liposcelidae).

2. Infestation Control by Pesticides and Their Side Effects

Since the 1950s, synthetic insecticides have been used extensively in grain facilities to control stored product insect pests. Fumigants such as methyl bromide, phosphine, cyanogens, ethyl formate, or sulfuryl fluoride rapidly kill all life stages of stored product insects in a commodity or in a storage structure. Fumigation is still one of the most effective methods for the prevention of stored product losses from insect pests. But pests develop resistance, not stored products were showing a slow upsurge in fumigation resistance [9]. Resistance to phosphine is so high in Australia and India, it may cause control failures [10, 11]. Methyl bromide has been identified as a major contributor to ozone depletion [12] and has been banned in developed countries, and developing nations have committed to reducing the use by 20% in 2005 and phase out in 2015. Contact insecticides such as malathion, chlorpyrifos, or deltamethrin are sprayed directly on grain or storage structure for protection from infestation for several months. The incidence of insecticide resistance is a growing problem in stored-product protection. Resistance to one or more insecticides has been reported in at least 500 species of insects and mites [13].

Champ [14] reported that resistance to pesticides used to protect grain and other stored food stuffs is widespread and involves all groups of pesticides and most of the important pests. Some of the contact insecticides have become ineffective because of widespread resistance in insect population. Resistance to malathion is widespread in Canada, USA and Australia [15]. Stored product insects pests were found to be resistant against different insecticides including the cyclodienes, chlorpyrifos, cyanophos, carbamates, carbaryl, cypermethrin, dichlorodiphenyltrichloroethane, deltamethrin, diazinon, dichlorovos, ethylene bromide, ethyl formate organophosphates, permethrin, pyrethrins, and propoxur.

Although chemical insecticides are effective, their repeated use has led to residual toxicity, environmental pollution and an adverse effect on food besides side effect on humans [16, 17]. Their uninterrupted and indiscriminate use not only has led to the development of resistant strains but also accumulation of toxic residues on food grains used for human consumption that has led to the health hazards [18]. In view of all these problems, several insecticides have either been banned or restricted in their use.

3. Botanicals as Alternative to Synthetic Pesticides

The increasing serious problems of resistance and residue to pesticides and contamination of the biosphere associated with large-scale use of broad spectrum synthetic pesticides have led to the need for effective biodegradable pesticides with greater selectivity. This awareness has created a worldwide interest in the development of alternative strategies, including the discovery of newer insecticides [19, 20]. However, newer insecticides will have to meet entirely different standards. They must be pest specific, nonphytotoxic, nontoxic to mammals, ecofriendly, less prone to pesticide resistance, relatively less expensive, and locally available [21]. This has led to re-examination of the century-old practices of protecting stored products using plant-derivatives, which have been known to resist insect attack [5, 22–24]. Plant derived materials are more readily biodegradable, less likely to contaminate the environment and nay be less toxic to mammals. There are many examples of very toxic plant compounds. Therefore, today, researchers are seeking new classes of naturally occurring insecticides that might be compatible with newer pest control approaches [2, 25, 26].

Since ancient times, there have been efforts to protect harvest production against pests. The Egyptian and Indian farmers used to mix the stored with fire ashes [83, 84]. The ancient Romans used false hellebore (*Veratrum album*) as a rodenticide, the Chinese is credited with discovering the insecticidal properties of Derris species, whereas pyrethrum was used as an insecticide in Persia and China [4]. In many parts of the world, locally available plants are currently in wide use to protect stored products against damage caused by insect infestation [80, 85–87]. Indian farmers used neem leaves and seed for the control of stored grain pests [88]. In northern Cameroon, cowpeas are traditionally mixed with sieved ash after threshing and the mixture put into mud granaries or clay jars [89]. In eastern Africa, leaves of the wild shrub *Ocimum suave* and the cloves of *Eugenia aromatic* are traditionally used as stored grain protectants [90]. In Rwanda, farmers store edible beans in a traditional closed structure (imboho) and whole leaves of *Ocimum canum* are usually added to the stored foodstuff to prevent insect damage within these structures [75]. Owusu [91] suggested natural and cheaper methods for the control of stored product pests of cereals, with traditionally useful Ghanaian plant materials. In some south Asian countries, food grains such as rice or wheat are traditionally stored by mixing with 2% turmeric powder [92, 93]. The use of oils in stored-products pest control is also an ancient practice. Botanical insecticides such as pyrethrum, derris, nicotine, oil of citronella, and other plant extracts have been used for centuries [27, 94, 95]. More than 150 species of forest and roadside trees in India produce oilseeds, which have been mainly used for lighting, medicinal purposes, and also as insecticides from ancient times to early 20th century [96]. Turmeric, garlic, *Vitex negundo*, gliricidia, castor, *Aristolochia*, ginger, *Agave americana*, custard apple, *Datura*, *Calotropis*, *Ipomoea,* and coriander are some of the other widely used botanicals to control and repel crop pests [81, 97].

Talukder [5] has listed 43 plant species as insect repellents, 21 plants as insect feeding deterrents, 47 plants as insect toxicants, 37 plants as grain protectants, 27 plants as insect reproduction inhibitors, and 7 plants as insect growth and development inhibitors. Eighteen species showed insecticidal

potential, and antiovipositional properties against *Sitophilus oryzae* [98].

4. Classification of Botanical Insecticides

On the basis of physiological activities on insects, Jacobson [3] conventionally classified the plant components into 6 groups, namely, repellents, feeding deterrents/antifeedants, toxicants, growth retardants, chemosterilants, and attractants. Focus on the toxicants and grain protectants on activity of essential oil, extracts, and its constituents has sharpened since the 1980s.

4.1. Repellents. The repellents are desirable chemicals as they offer protection with minimal impact on the ecosystem, as they drive away the insect pest from the treated materials by stimulating olfactory or other receptors. Repellents from plant origins are considered safe in pest control; minimise pesticide residue; ensure safety of the people, food, and environment [1, 5, 99]. The plant extracts, powders, and essential oil from the different bioactive plants were reported as repellent against stored grain insect pests [1, 91, 100–102]. For example, the essential oil of *Artemisia annua* was found as repellent against *Tribolium castaneum* and *Callosobruchus maculates* [103].

4.2. Antifeedants/Feeding Deterrents. Antifeedants, sometimes referred to as "feeding deterrents" are defined as chemicals that inhibit feeding or disrupt insect feeding by rendering the treated materials unattractive or unpalatable [104, 105]. Some naturally occurring antifeedants, which have been characterized, include glycosides of steroidal alkaloids, aromatic steroids, hydroxylated steroid meliantriol, triterpene hemiacetal, and others [3, 106]. Essential oil constituents such as thymol, citronellal and α-terpineol are effective as feeding deterrent against tobacco cutworm, *Spodoptera litura* synergism, or additive effects of combination of monoterpenoids from essential oils have been reported against *Spodoptera litura* larvae [107]. The screening of several medicinal herbs showed that root bark of *Dictamnus dasycarpus* possessed significant feeding deterrence against two stored-product insects [108].

4.3. Toxicants. Research on new toxicants of plant origin has not declined in recent years despite the increased research devoted to the discovery of synthetic insecticides [25]. Worldwide reports on plant derivates showed that many plant products are toxic to stored product insects [6, 16, 27, 55, 82, 91, 109–114]. Talukder [32] listed the use of 43 plant species expressing toxicant effects of different species of stored-products insects. Pascual-Villalobos and Robledo [115] carried out screening of plant extracts from 50 different wild plant species of southeastern Spain for insecticidal activity towards *Tribolium castaneum* and reported that four species, namely, *Anabasis hispanica, Senecio lopezii, Bellardia trixago,* and *Asphodelus fistulosus* were found be promising. Two major constituents of the essential oil of garlic, *Allium sativum*, methyl allyl disulfide and diallyl trisulfide were to be potent toxicant and fumigants against *Sitophilus zeamais* and *Tribolium castaneum* [116]. Rahman [117] reported that nicotine, an active component of *Nicotiana tabacum,* is a strong organic poison which acts as a contact-stomach poison with insecticidal properties. This compound is, of course, very toxic to humans as well. The essential oil vapours distilled from anise, cumin, eucalyptus, oregano, and rosemary were also reported as fumigantants and caused 100% mortality of the eggs of *Tribolium confusum* and *Ephestia kuehniella* [118]. Many species of the genus *Ocimum* oils, extracts, and their bioactive compounds have been reported to have insecticidal activities against various insect species [59, 119]. A list of many known toxicants from plant origin, reported as effective on stored-product insect-pest management, is given in Table 1.

4.4. Natural Grain Protectants. From very early times, plant materials have been used as natural protectants of stored grains. Worldwide reports indicate that when mixed with stored grains, leaf, bark, seed powder, or oil extracts of plants reduce oviposition rate and suppress adult emergence of stored product insects, and also reduce seed damage rates [25, 40, 46, 87, 119–122]. In 1989, Jacobson [123] noted that the most promising natural grain protectants were generally observed in the plant families, Annonaceae, Asteraceae, Canellaceae, Labiatae, Meliaceae, and Rutaceae.

The Indian neem plant is the most well-known example and its various parts, namely, leaves, crushed seeds, powdered fruits, oil, and so forth, have been used to protect stored grain from infestation [1, 124, 125]. The neem oil and kernel powder gave effective grain protection against stored grain insect pests like *Sitophilus oryzae, Tribolium cataneum, Rhyzopertha dominica,* and *Callosobruchus chinensis* at the rate of 1 to 2% kernel powder or oil [126]. The neem oil adhered to grain forms uniform coating around the grains against storage pests for a period of 180–330 days [127]. Yadava and Bhatnagar [128] reported that a dried leaves of *Azadirachta indica* have been mixed with stored grains for protection against insects. Azadirachtin is an active principle from the neem plant, which is an effective grain protectant against insect infestation [129]. Rajashekar et al. [7] reported that root powder extracts of *Decalepis hamiltonii* have been mixed with stored grains for protection against various stored grain insect pests. Eighteen species offered protection to wheat up to 9 months without affecting seed germination [98].

In parts of eastern Africa, leaves of some plants and allelochemicals including azadirachtin, nicotine, and rotenone have traditionally been used as grain protectants [5, 130]. The powders of *Rauvolfia serpentina, Acorus calamus,* and *Mesua ferrea* are used as a grain protectant against *Rhyzopertha dominica* [131]. In a survey in northern semiarid regions of Ghana only 16 plants were identified as being used as grain protectants [132]. In Africa, the grain protectant potential of different plant derivatives, including plant oils against major stored-product pests were also found to be very promising and reduced the risks associated with the use of insecticides [82, 121]. In northern Cameroon, the

Table 1: List of plant species reported to show insecticidal activity.

Plant species	Family	Plant part	References
Acorus calamus	Acoraceae	O, RO	[27]
Allium sativum	Alliaceae	P	[28]
Annona squamosa	Annonaceae	L	[29]
Aphanamixis polystachya	Meliaceae	SC, SE	[25]
Azadirachta indica	Meliaceae	O, SP, LP	[30]
Baccharis salicifolia	Asteraceae	O	[31]
Bassia longifolia	Sapotaceae	E	[5]
Brassica spp.	Cruciferae	L, ZE	[32]
Cajanus cajan	Fabaceae	O	[33]
Calophyllum inophyllum	Clusiaceae	O	[34]
Calotropis procera	Apocynaceae	LP	[35]
Carum carvi	Apiaceae	FE	[36]
Cinnamomum aromaticum	Lauraceae	B	[37]
Citrus	Rutaceae	O	[38]
Curcuma longa	Zingiberaceae	P	[39]
Chenopodium ambrosioides	Amaranthaceae	FE, O	[40]
Cocos nucifera	Arecaceae	O	[25]
Convolvulus arvensis	Convolvulaceae	LE	[41]
Conyza dioscoridis	Asteraceae	ZE	[41]
Coriandrum sativum	Apiaceae	SE, O	[42]
Datura alba	Solanaceae	LP	[43]
Decalepis hamiltonii	Asclepiadaceae	XP	[7]
Eichhornia crassipes	Pontederiaceae	LE	[44]
Elaeis guineensis	Arecaceae	O	[45]
Elaeis guineensis	Palmaceae	O	[46]
Embelia ribes	Myrsinaceae	FE, O	[47]
Eucalyptus globules	Myrtaceae	LP, M	[48]
Foeniculum vulgare	Apiaceae	FE	[49]
Glycine max	Fabaceae	O	[50]
Jatropha gossypifolia	Euphorbiaceae	SE	[51]
Juniperus virginiana	Cupressaceae	O	[52]
Lantana camara	Verbenaceae	TE	[45]
Lonchocarpus spp.	Leguminosae	O	[53]
Lupinus albus	Fabaceae	SE	[54]
Lupinus termis	Leguminosae	SE	[54]
Melia azedarach	Meliaceae	O, E	[55]
Mentha citrate	Lamiaceae	O	[56]
Nicotiana tabacum	Solanaceae	E	[57]
Ocimum canum	Lamiaceae	LP	[58]
Ocimum kilimandscharicum	Lamiaceae	O	[59]
Piper nigrum	Piperaceae	O, E	[60, 61]
Polygonum hydropiper	Polygonaceae	L	[62]
Pongamia glabra	Fabaceae	O, E	[61]
Psidium guajava	Myrtaceae	L, LP	[63]
Ryania speciosa	Flacourtiaceae	YE	[64]
Sapindus trifoliatus	Sapindaceae	SP	[65]
Schleichera trijuga	Sapindaceae	O	[66]
Sesamum orientale	Pedaliaceae	O	[5]
Sesamum indicum	Pedaliaceae	O	[67]
Syzygium aromaticum	Myrtaceae	O	[68]
Tagetes erecta	—	X, Y	[69]
Tanacetum cinerariaefolium	Asteraceae	O, P	[55]
Thujopsis dolabrata	Cupressaceae	E	[64]
Trigonella foenumgraecum	Fabaceae	SE	[70]
Vitex negundo	Lamiaceae	L	[71]

Note. L: leaves, B: bark, F: fruits, S: seeds, O: oil, P: powder, E: extract, M: vapour, R: Rhizome, T: plant, V: flower, X: root, and Y: stem, (Source: [5, 6]).

essential oils of plants *Xylopia aethiopica, Vepris heterophylla,* and *Luppia rugosa* are used for protection of stored grains from attack of stored grain insect pests [114]. The components of citrus peels were used as grain protectant against *Callosobruchus maculatus* [133]. Coconut oil has been found effective against *Callosobruchus chinensis,* for a storage period of six months, when applied to *Vigna radiata* (green gram) at 1% [134]. Formulations of menthol were used as protection of pulse grain from attack of *Callosobruchus Chinensis* [135]. Spinosad, a naturally occurring insecticide from the actinomycete, *Saccharopolyspora spinosa,* has high efficacy, a broad insect pest spectrum, low mammalian toxicity, and minimal environmental profile is unique among existing products currently used for stored-grain protection [136].

4.5. Chemosterilants/Reproduction Inhibitors. Many researchers reported that plant parts, oil, extracts, and powder mixed with grain-reduced insect oviposition, egg hatchability, postembryonic development, and progeny production [137–139]. Lists of 43 plant species have been reported as reproduction inhibitors against stored product insects [32]. Reports have also indicated that plant derivatives including the essential oils caused mortality of insect eggs [82]. Many ground plant parts, extracts, oils, and vapour also suppress many insects [6, 7].

4.6. Insect Growth and Development Inhibitors. Plant extracts showed deleterious effect on the growth and development of insects and reduced larval pupal and adult weight significantly, lengthened the larval and pupal periods, and reduced pupal recovery and adult eclosion [140]. Rajasekaran and Kumaraswami [141] reported that grains coated with plant extracts completely inhibited the development of insect

Table 2: List of insecticidal active principles of plants.

Active principle	Plant species	Insect toxicity	Insect species	References
Anonaine	*Annona reticulate*	Contact	*Callosobruchus chinensis*	[72]
Azadirachtin	*Azadirachta indica*	Contact: IGR	Stored grain pests, aphids	[30]
E-Anethole	*Foeniculum vulgare*	Contact	*Sitophilus oryzae*, *Callosobruchus chinensis*	[49]
β-Asarone	*Acorus calamus*	Contact;	Stored grain pests	[73]
Z-Asarone	*Acorus calamus; Acorus gramineus*	Contact	*Sitophilus zeamais*	[26]
Bornyl acetate	*Chamaecyparis obtuse*	Contact	*Sitophilus oryzae*	[27]
Camphor	*Ocimum kilimandscharicum*	Contact	*Sitophilus oryzae*	[59]
(+)-3-Carene	*Baccharis salicifolia*	Contact	*Tribolium castaneum*	[59]
Carvacrol	*Thujopsis dolabrata*	Contact; fumigant	*Sitophilus oryzae*, *Callosobruchus chinensis*	[60]
Carvone	*Carum carvi*	Contact	*Sitophilus oryzae*, *Rhyzopertha dominica*	[74]
1,8 Cineole	*Eucalyptus*	Contact; fumigant	*Rhyzopertha dominica* *Tribolium castaneum*	[38]
Cinnamaldehyde	*Cinnamomum aromaticum*	Contact	*Tribolium castaneum*, *Sitophilus zeamais*	[37]
Dioctyl hexanedioate	*Conyza dioscoridis*	Contact	*Tribolium castaneum*, *Sitophilus granaries*	[41]
Eugenol	*Citrus*	Fumigant	*Sitophilus oryzae*	[38]
Estragole	*Foeniculum vulgare*	Contact	*Sitophilus oryzae* *Lasioderma serricorne*	[31]
(+)-Fenchone	*Foeniculum vulgare*	Contact	*Sitophilus oryzae* *Lasioderma serricorne*	[31]
Hexa decane	*Chenopodiumambrosioides*	Contact	*Tribolium castaneum*, *Sitophilus granaries*	[41]
Hexadecanoic acid	*Convolvulus arvensis*	Contact	*Sitophilus oryzae*, *Rhyzopertha dominica.*	[41]
Linalool	*Ocimum canum* Sims	Fumigant	*Tribolium castaneum*, *Sitophilus granaries*	[75]
Limonene	*Citrus*	Contact	*Tribolium castaneum*	[27]
(−)-Limonene	*Baccharis salicifolia*	Contact; fumigant	*Tribolium castaneum*	[31]
Nicotine	*Nicotiana tabacum*	Contact	Mites, aphids, thrips, leafhopper	[39]
Pyrethrin I and II	*Tanacetum cinerariaefolium*	Contact; stomach poison	Stored grain pests, crop pests	[76]
β-Pinene	*Baccharis salicifolia*	Contact	*Tribolium castaneum,*	[27]
α-Pinene	*Baccharis salicifolia*	Fumigant	*Tribolium castaneum,*	[27]
Rotenone	*Lonchocarpus* sp.	Contact; stomach poison	Crop pests, lace bugs, *Sitophilus oryzae*	[55]
Ryania	*Ryania speciosa*	Contact; stomach poison	Potato beetle, aphids, lace bugs, stored grain pests	[77]
Sabadilla	*Schoenocaulon officinale*	Contact; stomach poison	Stinks, thrips, squash bugs, leaf hoppers, caterpillars	[78]
Spinosyn A and D	*Saccharopolyspora spinosa*	Stomach poison	Stored grain pests	[79]

like *Sitophilus oryzae.* Plant derivatives also reduce the survival rates of larvae and pupae and adult emergence [101]. Development of eggs and immature stages inside grain kernel were also inhibited by plant derivatives [102]. The crude extract also retarded development and caused mortality of larvae, cuticle melanisation, and high mortality in adults [142].

5. Some Important Phytochemicals with Insecticidal Properties

The botanical insecticides that have primarily been used and are commercially available include ryania, rotenone, pyrethrin, nicotine, azadirachtin, and sabadilla (Tables 2 and 3).

Table 3: Insecticidal activity and mammalian toxicity of some natural insecticides.

Natural insecticides	Insect toxicity*	Mammalian toxicity Oral (rat) LD_{50} (mg/kg b.w.)
Anethole	C, S	2090
β-asarone	C, S	275
Azadirachtin	IGR, R	13000
Carvacrol	C	810
1,8-Cineole	C, F	2480
Cinnamaldehyde	C	1160
Cuminic aldehyde	C, S	1390
Eugenol	C, F	500
Nicotine	C	50
Pyrethrin I and II	C, S	1200
Rotenone	S	350
Ryania	C, S	750
Sabadilla	C, S	5000
Spinosad	C	3738

*C: contact, S: stomach poison, F: fumigant, IGR: insect growth regulator, and R: repellent [80–82].

5.1. Ryania. The active components of ryania are derived from the roots and woody stems of the plant *Ryania speciosa*, native to Trinidad [143]. Ryania has low mammalian toxicity, with a median lethal dose (LD_{50}) of 750 mg/kg and works as both contact and stomach poison. It has long residual activity among the botanical insecticides. This botanical insecticide has a unique mode of action and affects muscles by binding to the calcium channels in the sarcoplasmic reticulum. This causes calcium ion flow into the cells, and death follows very rapidly [20]. Ryania works best on caterpillars (i.e., codling moth, corn earworm); however, is it also active on a wide range of insects and mites, including potato beetle, lace bugs, aphids and squash bug [144].

5.2. Rotenone. Rotenone is derived from the roots of the two plants: *Lonchocarpus* sp. and *Derris* sp. are both legumes originally from the East Indies, Malaya and South America. Rotenone is a moderately of the toxic botanical insecticides, with an LD_{50} of 132 mg/kg to mammals [81]. In fact, rotenone is more toxic to mammals than both carbaryl and malathion, two commonly used synthetically derived insecticides. Also, rotenone is extremely toxic to fish [55]. This botanical insecticide works as both contact and stomach poison. Rotenone is slower acting than most other botanical insecticides, taking several days to kill pests; however, pests stop feeding almost immediately. It degrades rapidly in air and sunlight. Rotenone blocks respiration by electron transport on the complex I. Rotenone shows broad spectrum of activity on many insects and mite pests, including leaf-feeding beetles, caterpillars, lice, mosquitoes, ticks, fleas, and fire ants [145].

5.3. Pyrethrin/Pyrethrum. Pyrethrin I and II are derived from the seeds or flower of *Chrysanthemum cinerariaefolium* [55, 146] which is grown in Africa, Ecuador, and Kenya. Pyrethrin has a low mammalian toxicity. However, cats are highly susceptible to pyrethrin poisoning. The LD_{50} of pyrethrin is 1200 to 1,500 mg/kg [81, 147, 148]. Pyrethrin is one of the oldest household insecticides still available and is fast acting, providing almost immediate "knockdown" of insects following an application. It works as both a contact and a stomach poison. The material has a very short residual activity-degrading rapidly under sunlight, air and moisture, which means that frequent applications may be required. Pyrethrin can be used up until harvest, as there is no waiting interval required between initial application and harvest of food crops [149].

The way pyrethrin kills insects (mode of activity) is by disrupting the sodium and potassium ion-exchange process in insect nerves and interrupting the normal transmission of nerve impulses. Pyrethrin has activity on wide range of insects and mites, including flies, fleas, beetles, and spider mites [150].

5.4. Nicotine. Nicotine, which is derived from *Nicotiana tabacum,* is toxic to mammals among the botanical insecticides with an LD_{50} between 50 and 60 mg/kg [55, 151]. It is extremely harmful to humans. Nicotine, a fast-acting nerve toxin, works as a contact poison. It kills insects (and humans) through bonding to receptors at the nerve synapses (junctures), causing uncontrolled nerve firing, and by mimicking acetylcholine (Ach) at the nerve-muscle junctions in the central nervous system [152].

Certain plant types, such as roses, may be harmed or injured by nicotine sprays. Nicotine is most effective on soft-bodied insects and mites, including aphids, thrips, leafhoppers, and spider mites. Many caterpillars are resistant to nicotine [153].

5.5. Azadirachtin. Azadirachtin is derived from the tree *Azadirachta indica,* grown in India and Africa [55]. Azadirachtin has an extremely low mammalian toxicity and is least toxic of the commercial botanical insecticides, with an LD_{50} of 13,000 mg/kg. Azadirachtin is considered a contact poison; however, it has "some" systemic activity in plants when applied to the foliage. The material is generally nontoxic to beneficial insects and mites. Azadirachtin has broad mode of activity, working as a feeding deterrent, insect-growth regulator, repellent, and sterilant; and it may also inhibit oviposition [55, 154]. The material is active on a broad range of insects, including stored grain pests, aphids, caterpillars and mealybugs [30].

5.6. Sabadilla. Sabadilla is derived from the seeds of plant *Schoenocaulon officinale,* which is grown in Venezuela. Sabadilla is one of the least toxic registered botanical insecticides, with mammalian LD_{50} of 5,000 mg/kg. Sabadilla works as contact toxicant and a stomach poison. Similar to other botanical insecticides, the material has minimal residual activity and degrades rapidly in sunlight and moisture

(rainfall). Sabadilla works by affecting nerve cell membranes, causing loss of nerve function, paralysis, and death [146]. It is effective against caterpillars, leaf hoppers, thrips, stink, and squash bugs.

5.7. Avermectins. Avermectins, which are macrocyclic lactones, are derived from the actinomycete, *Streptomyces avermitilis* [155], lethal dosage of 50% in range of 10–11.3 mg/kg for rat. This molecule is most effective against agricultural pests with lethal concentration of 90% (LC_{90}) in the range of 0.02 ppm for mites and has somewhat least toxicity to stored products pests. It is effective on internal parasites of domestic animals [156]. Avermectins block the neurotransmitter GABA at the neuromuscular junction in insects and mites. Visible activity, such as feeding and egg laying, stops shortly after exposure, though death may not occur for several days [157].

5.8. Spinosads. Spinosad is a mixture of spinosyn A and spinosyn D and was originally isolated from the soil Actinomycete, *Saccharopolyspora spinosa* [158]. Spinosad is recommended for the control of a very wide range of caterpillars, leaf miners and foliage-feeding beetles. Spinosyns have a novel mode of action, primarily targeting binding sites on nicotinic acetylcholine receptors that are distinct from those at which other insecticides exert activity, leading to disruption of acetylcholine neurotransmission [79, 159].

5.9. (Z) Asarone. (Z) Asarone is natural insecticide isolated from *Acorus calamus* L. [26]. This molecule is more effective against adults of *Sitophilus oryzae, Lasioderma serricorne,* and *Callosobruchus chinensis* and shows both fumigant and contact toxicity. Some studies show that the molecules possess *in vivo* carcinogenic effects [160] and *in vitro* mutagenic activites [161]. Further, this molecule induces structural chromosome aberration in human lymphocytes *in vitro* [162]. Due to its mammalian toxicity [81, 163], the molecule is unsafe for grain treatment.

6. Challenges to the Utilization of Botanicals Pesticides

Many plant species contain secondary metabolites that are potent against several pest species. Not only are some of the plants (e.g., the neem trees) of major interest as sources of phytochemicals for more environmentally benign grain/crop protection. Phytochemical products can increase income of rural farmers and promote safety and quality of food and life in general [8, 164].

The successful utilization of botanicals can help to control many of the world's destructive pests and diseases, as well as reduce erosion, desertification, deforestation, and perhaps even reducing human population by acting as spermaticide (although this will be considered a major negative effect by many cultures and religions) [165]. Although the possibilities of using botanical pesticides seem almost endless, many details remain to be clarified. Many obstacles must be overcome and many uncertainties clarified before their potential can be fully realized. These limitations seem surmountable; however, they present exciting challenges to the scientific and economic development communities. Solving the following obstacles and uncertainties may well bring a major new resource which will benefit much of the world. These obstacles include:

(i) lack of experience and appreciation of the efficacy of botanicals for pest control. There are still doubts as to the effectiveness of plant-derived products (both "home-made" and commercial products) due to their slow action and lack of rapid knockdown effect;

(ii) genetic variability of plant species in different localities;

(iii) difficulty of registration and patenting of natural products and lack of standardization of botanical pesticide products;

(iv) economic uncertainties occasioned by seasonal supply of seeds, perennial nature of most botanical trees, and change in potency with location and time with respect to geographical limitations;

(v) handling difficulties as there is no method for mechanizing the process of collecting, storing, or handling the seeds or leaves or flowers from some of the perennial trees;

(vi) instability of the active ingredients when exposed to direct sunlight;

(vii) competition with synthetic pesticides through aggressive advertising by commercial pesticides dealers and commercial-formulated botanicals are more expensive than synthetic insecticides and are not as widely available;

(viii) rapid degradation, although desirable in some respects, creates the need for more precise timing or more frequent applications;

(ix) Data on the effectiveness and long-term (chronic) mammalian toxicity are unavailable for some botanicals, and tolerances for some have not been established.

7. Conclusion

Many authors have evaluated the insecticidal (grain protectant) properties of plant products on various species of stored product insect pests. The results clearly show that it is possible to develop methods for grain protectants with reduced use of synthetic chemical insecticides. The main advantages of botanical pesticides are ecofriendly, easily biodegradable, nontoxic to nontarget organisms, and many plant-derived natural products acting against insects could be produced from locally available raw materials. They have been numerous botanical pesticides studied at the laboratory level. Research efforts should focus not only on their efficacy, but also on mammalian toxicity, mode of action in insects, seed germination, effect on nutritional quality, seedling growth, and stability of the compound. The insecticides of

plant origin could be exploited for the development of novel molecules with highly precise targets for sustainable insect pest management in stored grain.

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Research Article

Effect of Crude Leaf Extracts on *Colletotrichum gloeosporioides* (Penz.) Sacc.

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Colletotrichum gloeosporioides (Penz.) Sacc. is a fungus that causes anthracnose disease in tropical fruit plants, resulting in damages of the fruit plants and low yield and quality of fruits. The use of chemical fungicides is common for management of this disease, but it also results in the development of fungal resistance to the chemicals. Therefore, this study aims to *in vitro* evaluate the efficacy of 14 crude leaf extracts against *C. gloeosporioides*. The results showed that *Piper sarmentosum* leaf extracts, using 80% of ethanol, methanol, and chloroform as solvents, were found to have very high antifungal activities. Crude methanol extract of *P. sarmentosum* leaves could effectively inhibit the growth of fungal mycelium (100%), followed by crude chloroform extract (81.85%) and 80% ethanol extract (45.50%). Maximum inhibition of *C. gloeosporioides* spore germination could be obtained after application with crude methanol extract of *P. sarmentosum* leaves and crude chloroform extract of *Mentha cordifolia* leaves at 1.25 and 2.5%, respectively. In conclusion, crude extracts of *P. sarmentosum* leaves were found to be highly effective for inhibiting both *C. gloeosporioides* mycelium growth and spore germination, and they have a potential as the new natural fungicides for management of anthracnose disease.

1. Introduction

Colletotrichum gloeosporioides (Penz.) Sacc. is a causative agent for anthracnose disease in many tropical fruit trees such as mango and papaya. This disease is very harmful and can cause spoilage and rotting of fruit plants, resulting in low yield and poor quality of the fruits [1]. The use of chemical fungicides is the most common choice for management of anthracnose disease, but this also causes the development of fungal resistance [2]. In addition, continuous and inappropriate use of chemical fungicides to manage anthracnose disease is not considered to be the long-term solution because this can increase the investment expenses, the risk of having high levels of toxic residues, and also the concerns in human health and environmental settings [3]. Due to these reasons, there are several attempts to search for alternative measures to control the anthracnose disease effectively. Recent efforts have focused on the development of environmentally safe, long-lasting, and effective biocontrol methods for management of anthracnose diseases. The utilization of natural products, especially the plant extracts, has been shown to be effective against many plant pathogens and considered to be safe for consumers and environments [4]. A number of plant species have been reported to possess natural substances that are toxic to a variety of plant pathogenic fungi [5, 6]. The extracts derived from *Curcuma longa* (leaf and rhizome), *Tagetes erecta* (leaf), and *Zingiber officinales* (rhizome) were shown to have antifungal activities against fungal anthracnose by completely inhibiting conidial germination of *C. gloeosporioides* [7]. The aqueous leaf extracts of custard apple (*Annona reticulate* L.) and papaya could inhibit spore formation and germination of *Rhizopus stolonifer* and also conidial formation of *C. gloeosporioide*s [8]. In addition, *C. capsici* mycelial growth

and spore germination were found to be suppressed by crude leaf extracts of *Piper betle* L. using methanol, chloroform, and acetone as solvents [9]. Hence, in this study, the *in vitro* antifungal activities of 14 leaf extracts were evaluated against *C. gloeosporioides*, a causative agent of mango anthracnose disease.

2. Materials and Methods

2.1. Fungal Culture. *C. gloeosporioides* was isolated from the upper surface of infected mango and cultured using potato dextrose agar (PDA) medium at 25°C.

2.2. Plant Materials and Extractions. Leaves from 14 different plant species were collected locally or bought at local markets of Maha Sarakham province which is in the northeast region of Thailand (Table 1). Leaf samples were thoroughly washed using tap water, air-dried at room temperature for 3 to 4 h, and finally dried in a hot-air oven at 45–50°C for 1 to 2 days depending on the plant species. Dried leaf samples were ground using small grinder, then placed in polyethylene bags, and stored at 4°C until required. For each sample, 50 g of leaf powder were added to 150 mL of methanol (M), 80% ethanol (E), or chloroform (F) (thus ratio between leaf powder and solvent was 1 : 3). The mixtures were agitated for 72 h on rotary shaker (130 rpm). The obtained extracts were centrifuged at 8,000 rpm for 10 minutes, filtered through Whatman filter paper no. 1, and transferred to 250 mL round-bottom flasks. Finally, these 42 extracts were evaporated using rotary evaporator at 45°C. Concentrated extracts were allowed to dry in hot-air oven, weighed again, and kept at 4°C until required for antifungal assays.

2.3. Screening of Leaf Extracts against C. Gloeosporioides Mycelial Growth. Forty two crude leaf extracts were *in vitro* tested for their efficacy against *C. gloeosporioides* mycelia growth using the poisoned food technique [10]. All crude leaf extracts were reconstituted to have the concentration of 5%. Then 1 mL of each extract was used for mixing with 19 mL of warm PDA and poured into 9 cm sterile Petri dish. After solidification, the plates were inoculated with the 6 mm agar piece containing a week old *C. gloeosporioides* mycelia. For each crude leaf extracts, the experiments were performed in three replicates. PDA plates mixed with carbendazim (commercial fungicide at 0.005%) and sterile distill water were served as positive and negative controls, respectively. The inoculated plates were incubated at 30°C, and the diameters of fungal colonies were measured every day for 5 days.

Inhibition of mycelial growth was calculated using the following formula [11]:

$$\%\ \text{Inhibition} = \frac{X - Y}{X} \times 100, \quad (1)$$

X: diameter of fungal colony grown on negative control plate, Y: diameter of fungal colony grown on plates containing crude leaf extracts.

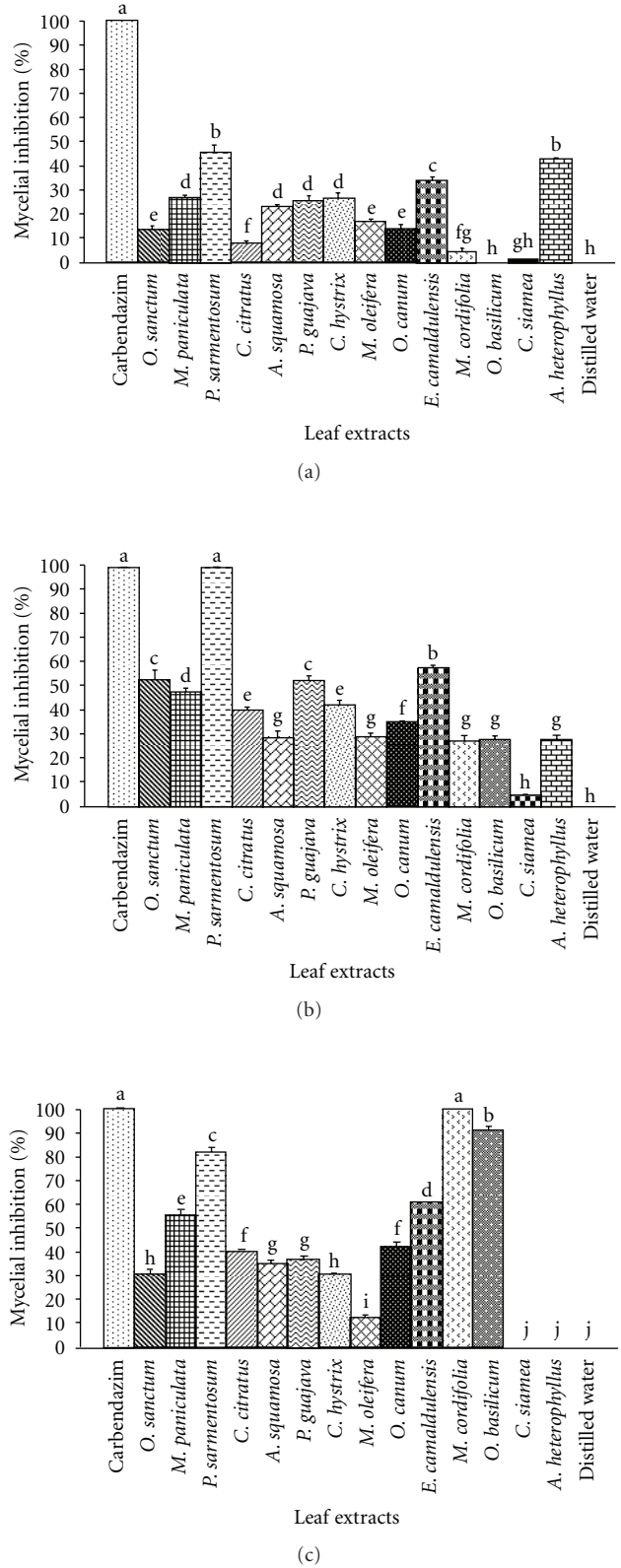

Figure 1: Inhibition of *C. gloeosporioides* mycelial growth by crude leaf extracts using (a) 80% ethanol, (b) methanol, and (c) chloroform as solvents. Bars (mean ± SE) with the same letter(s) are not significantly different as determined by LSD test at $P < 0.05$.

Table 1: List of plants.

Scientific name	Family	Common name
Cymbopogon citratus Stapf.	Gramineae	Takhrai, lemongrass
Citrus hystrix DC.	Rutaceae	Leech lime
Murraya paniculata (L.) Jack.	Rutaceae	Orange jessamine, satin-wood
Ocimum basilicum Linn.	Labiatae	Horapa, sweet basil, common basil
Ocimum canum Linn.	Labiatae	Hairy basil
Moringa oleifera Lamk.	Moringaceae	Horse radish tree
Annona squamosa Linn.	Annonaceae	Sugar apple
Ocimum sanctum Linn.	Malvaceae	Holy basil, sacred basil
Psidium guajava Linn.	Myrtaceae	Guava
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Red river gum, red gum
Artocarpus heterophyllus Lam.	Moraceae	Jackfruit tree
Piper sarmentosum Roxb. Ex Hunter.	Piperaceae	Chaplu
Mentha cordifolia Opiz.	Lamiaceae	Kitchen mint, marsh mint
Cassia siamea (Lamk.) Irwin and Barneby	Fabaceae	Cassod tree, siamese senna

2.4. Effect of Leaf Extracts Prepared at Different Concentrations on C. Gloeosporioides Mycelial Growth and Spore Germination. Twelve of the leaf extracts that were found to have high levels of activities against *C. gloeosporioides* mycelial growth were selected for further testing at lower concentrations. Various concentrations of selected crude leaf extracts were prepared (2.5, 1.25, 0.625, 0.3, 0.2, 0.1, and 0.05%) and *in vitro* tested against *C. gloeosporioides* mycelial growth (as described above) and spore germination. Inhibition of spore germination was examined by spreading 100 μL of *C. gloeosporioides* spore suspension (10^5 spores/mL) on PDA plates containing each leaf extracts. Carbendazim and sterile distill water were served as positive and negative controls, respectively. Plates were incubated at 30°C and monitored for 7 days.

2.5. Statistical Analysis. All data were subjected to analysis of variance (ANOVA) using the general linear models procedure (SAS Institute, Cary, NC, USA). The data of the percentages of mycelial inhibition were arcsine transformed before analysis. The means of % mycelial inhibition of all treatments were compared and determined using the LSD test at $P \leq 0.05$.

3. Results

3.1. Screening of 42 Crude Leaf Extracts against C. Gloeosporioides Mycelial Growth. Different solvents used for extraction could result in different levels of *in vitro* antifungal activities of the crude leaf extracts (5%) as measured by poisoned food technique. The antifungal activities of leaf extracts using 80% ethanol, methanol, and chloroform as solvents were found to range between 0.77–45.50%, 4.35–100% and 12.37–100%, respectively (Figure 1). Even though all crude leaf extracts exhibited certain levels of activities against *C. gloeosporioides* mycelia, the 80% ethanol extract of *O. bacilicum* and chloroform extracts of *A. heterophyllus* and *C. siamea* did not effectively prevent mycelial growth. *C. siamea* leaves that were extracted using 80% ethanol and methanol were found to have very low antifungal activities at 0.77 and 4.35%, respectively (Figure 1).

Crude leaf extracts using 80% ethanol as solvent were shown to have rather low antifungal activities (less than 50%), as shown by that *P. sarmentosum, A. heterophyllus,* and *E. camaldulensis* could prevent the growth of *C. gloeosporiodes* mycelia at 45.50, 42.75, and 33.85%, respectively (Figure 1(a)). Interestingly, crude methanol extracts of *P. sarmentosum* leaves exhibited the highest inhibition activities against *C. gloeosporioides* mycelialgrowth (100%), followed by *E. camaldulensis* (57.75%), *O. sanctum* (52.75%), and *P. guajava* (52.75%). However, the other methanol leaf extracts were found to have levels of antifungal activities less than 50% (Figure 1(b)). There were 5 chloroform extracts that were found to have more than 50% inhibition activities against *C. gloeosporioides* mycelial growth, including *M. cordifolia* (100%), *P. sarmentosum* (81.75%), *E. camaldulensis* (60.25%), *M. paniculata* (55.50%), and *O. bacilicum* (91.10%) (Figure 1(c)).

3.2. Effect of Leaf Extracts Prepared at Different Concentrations on C. Gloeosporioides Mycelial Growth and Spore Germination. Twelve crude leaf extracts (derived from 7 plant species), including the extracts of *P. sarmentosum* and *E. camaldulensis* in all solvents, the extract of *A. heterophyllus* in 80% ethanol, the extracts of *O. sanctum* and *P. guajava* in methanol, and the extracts of *O. bacillicum* and *M. paniculata* in chloroform were prepared at various concentrations (2.5, 1.25, 0.625, 0.3, 0.2, 0.1, and 0.05%) and determined for their efficacy against *C. gloeosporioides* mycelia growth and spore germination.

Although at lower concentrations these plant extracts exhibited lower antifungal activities, some plant extracts remained effective (Tables 2 and 3). In particular, when compared to carbendazim (commercial fungicide), the crude methanol extract of *P. sarmentosum* and chloroform extract of *M. cordifolia* at 2.5% could significantly inhibit the

Table 2: Effect of 12 selected crude leaf extracts prepared at different concentrations on *Colletotrichum gloeosporioides* mycelial growth.

Leaf extracts	% inhibition of *Colletotrichum gloeosporioides* mycelial growth* Concentration (%)						
	2.5	1.25	0.625	0.3	0.2	0.1	0.05
M. paniculata/C	54.45 ± 0.71^{cA}	52.75 ± 0.35^{efB}	33.40 ± 0.85^{fC}	17.25 ± 1.06^{fD}	16.50 ± 2.12^{cD}	0.57 ± 0.18^{cdE}	0.00 ± 0.00^{dE}
A. heterophyllus/E	7.45 ± 1.10^{hA}	3.85 ± 0.50^{jkB}	1.95 ± 0.78^{jkC}	0.27 ± 0.03^{hD}	0.00 ± 0.00^{fD}	0.00 ± 0.00^{eD}	0.00 ± 0.00^{dD}
O. sanctum/M	3.85 ± 0.21^{iA}	1.12 ± 0.53^{klB}	0.00 ± 0.00^{kC}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
P. guajava/M	35.25 ± 1.06^{eA}	20.50 ± 0.70^{iB}	0.00 ± 0.00^{kC}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
O. basilicum/C	57.25 ± 1.06^{cA}	56.50 ± 0.70^{eA}	36.35 ± 2.33^{eB}	21.50 ± 0.7^{eC}	9.87 ± 0.18^{dD}	0.37 ± 0.17^{deE}	0.00 ± 0.00^{dE}
M. cordifolia/C	97.60 ± 0.84^{aA}	75.75 ± 1.76^{cB}	56.50 ± 2.12^{cC}	37.50 ± 0.7^{cD}	19.12 ± 1.24^{bE}	3.85 ± 0.50^{bF}	37.50 ± 0.71^{cG}
P. sarmentosum/E	16.50 ± 2.12^{gA}	5.30 ± 0.98^{jB}	3.35 ± 0.92^{jB}	0.27 ± 0.03^{C}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
P. sarmentosum/M	100.00 ± 0.00^{aA}	88.25 ± 1.76^{bB}	80.00 ± 1.41^{bC}	56.50 ± 0.71^{bD}	19.10 ± 2.70^{bE}	3.85 ± 0.51^{bF}	1.37 ± 0.10^{bF}
P. sarmentosum/C	77.75 ± 1.80^{bA}	68.00 ± 2.83^{dB}	42.25 ± 1.06^{dC}	30.85 ± 1.63^{dD}	9.50 ± 2.12^{dE}	0.00 ± 0.00^{eF}	0.00 ± 0.00^{dF}
E. camaldulensis/E	29.75 ± 2.48^{fA}	26.50 ± 4.95^{hB}	23.85 ± 0.21^{gC}	16.75 ± 0.35^{fD}	5.85 ± 0.51^{eE}	0.82 ± 0.71^{cF}	0.00 ± 0.00^{dF}
E. camaldulensis/M	49.50 ± 2.12^{dA}	37.25 ± 1.06^{gB}	11.50 ± 2.12^{iC}	8.85 ± 0.50^{gCD}	7.75 ± 0.35^{deD}	0.00 ± 0.00^{eE}	0.00 ± 0.00^{dE}
E. camaldulensis/C	55.25 ± 2.50^{cA}	50.75 ± 1.06^{fB}	15.25 ± 2.47^{hC}	8.35 ± 0.50^{gD}	1.37 ± 0.11^{fE}	0.00 ± 0.00^{eE}	0.00 ± 0.00^{dE}
Carbendazim (0.005%)	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
Distilled water	0.00 ± 0.00^{jA}	0.00 ± 0.00^{lA}	0.00 ± 0.00^{kA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{eA}	0.00 ± 0.00^{dA}

C: chloroform, M: methanol, E: 80% ethanol.
*Percentages of inhibition within the row followed by the same uppercase letter are not significantly different at $P < 0.05$ as determined by LSD test. Percentages of inhibition within the column followed by the same lowercase letter are not significantly different at $P < 0.05$ as determined by LSD test.

Table 3: Effect of 12 selected crude leaf extracts prepared at different concentrations on *Colletotrichum gloeosporioides* spore germination.

Leaf extracts	% inhibition of *Colletotrichum gloeosporioides* spore germination* Concentration (%)						
	2.5	1.25	0.625	0.3	0.2	0.1	0.05
M. paniculata/C	51.10 ± 2.68^{cdA}	42.00 ± 4.24^{deB}	32.35 ± 5.16^{efC}	16.25 ± 3.88^{eD}	13.00 ± 2.82^{eD}	5.00 ± 1.41^{efE}	0.00 ± 0.00^{eE}
A. heterophyllus/E	42.50 ± 2.12^{eA}	38.50 ± 3.50^{efA}	21.50 ± 0.71^{gB}	13.20 ± 2.54^{eC}	4.50 ± 0.71^{fgD}	0.00 ± 0.00^{fE}	0.00 ± 0.00^{eE}
O. sanctum/M	33.35 ± 1.06^{fA}	24.00 ± 1.41^{gB}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{eC}	10.00 ± 0.00^{gC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}
P. guajava/M	50.66 ± 1.83^{cdA}	35.25 ± 5.39^{fB}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{gC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}
O. basilicum/C	65.00 ± 7.07^{bA}	58.60 ± 1.97^{cAB}	47.20 ± 5.37^{dBC}	36.33 ± 0.95^{dC}	22.00 ± 1.41^{dC}	9.00 ± 0.00^{eD}	0.00 ± 0.00^{eD}
M. cordifolia/C	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	85.30 ± 6.64^{bB}	67.00 ± 8.48^{bC}	59.33 ± 3.74^{bC}	42.50 ± 0.71^{cD}	31.12 ± 2.65^{cE}
P. sarmentosum/E	56.75 ± 3.88^{cA}	48.00 ± 2.83^{dB}	37.30 ± 1.83^{eC}	20.00 ± 2.83^{eD}	12.20 ± 1.13^{eE}	8.00 ± 1.42^{eE}	0.00 ± 0.00^{eF}
P. sarmentosum/M	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	90.50 ± 6.36^{bB}	72.20 ± 5.94^{bC}	65.15 ± 6.85^{bD}	57.00 ± 8.48^{bD}	36.10 ± 5.51^{bE}
P. sarmentosum/C	100.00 ± 0.00^{aA}	87.50 ± 2.12^{bB}	66.70 ± 3.25^{cC}	55.60 ± 1.97^{cD}	28.00 ± 5.65^{cE}	19.12 ± 1.24^{dF}	14.00 ± 5.66^{dF}
E. camaldulensis/E	48.10 ± 5.15^{deA}	41.60 ± 4.80^{efA}	31.20 ± 1.13^{efB}	18.00 ± 1.41^{eD}	10.50 ± 0.71^{eD}	4.00 ± 1.42^{efDE}	0.00 ± 0.00^{eE}
E. camaldulensis/M	54.35 ± 5.16^{cdA}	43.33 ± 2.36^{deB}	29.50 ± 0.71^{fC}	18.20 ± 1.13^{eD}	10.00 ± 1.41^{efE}	0.00 ± 0.00^{fF}	0.00 ± 0.00^{eF}
E. camaldulensis/C	47.30 ± 3.81^{deA}	40.00 ± 4.24^{efA}	28.16 ± 2.60^{fgB}	17.00 ± 1.41^{eBC}	8.00 ± 0.00^{efCD}	0.00 ± 0.00^{fD}	0.00 ± 0.00^{eD}
Carbendazim (0.005%)	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
Distilled water	0.00 ± 0.00^{gA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{gA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{eA}

C: chloroform, M: methanol, E: 80% ethanol.
*Percentages of inhibition within the row followed by the same uppercase letter are not significantly different at $P < 0.05$ as determined by LSD test. Percentages of inhibition within the column followed by the same lowercase letter are not significantly different at $P < 0.05$ as determined by LSD test.

growth of *C. gloeosporioides* mycelium at 100 and 97.60%, respectively (Table 2); moreover, both of these plant extracts at 1.25% also completely prevented *C. gloeosporioides* spore germination (100%) (Table 3). However, the other crude leaf extracts at lower concentrations did not exhibit significant antifungal activity against *C. gloeosporioides* (Tables 2 and 3).

4. Discussion

In this study, 12 leaf extracts (obtained from 7 plant species) that were found to inhibit *C. gloeosporioides* mycelial growth very strongly at 5% were selected, then prepared at lower concentrations, and used for further evaluation against

C. gloeosporioides mycelial growth and spore germination (Tables 2 and 3). At 2.5%, the crude methanol extract of *P. sarmentosum* and chloroform extract of *M. cordifolia* were shown to inhibit *C. gloeosporioides* mycelial growth, and at 1.25%, both of these could also completely prevent *C. gloeosporioides* spore germination.

From previous reports, there are a variety of plant extracts that were used to control fungal anthracnose. For instance, crude methanol, chloroform, and acetone extracts of *Piper betle* leaves at the concentration of 10 μg/mL could inhibit the growth of *Colletotrichum capsici* (responsible for anthracnose disease in pepper) mycelium at 85.25, 78.53, and 73.58%, respectively [9]. Also, at the same concentration, crude methanol, chloroform, and acetone extracts of these *P. betle* leaves were found to prevent *C. capsici* spore germination at 80.93, 74.09, and 72.91%, respectively [9]. Moreover, the leaf extracts of *O. bacillicum* and *Allium sativum* exhibited 100% inhibition of *C. gloeosporioides* (responsible for anthracnose in para rubber) mycelial growth when applying at 50 and 100% w/v, respectively, and both of these extracts could completely suppress spore germination when applying as minimal as 10% w/v [12]. Furthermore, the ethanol extracts of *Ocimum gratissimum* and *Aframomum melegueta* leaves were shown to inhibit the growth of *Botryodiplodia theobromae* mycelium (causative agent of banana anthracnose) at 72.1 and 68.2%, respectively [13].

Other plant pathogenic fungi could also be inhibited by plant extracts. For example, the ethanol extracts of *O. gratissimum* and *A. melegueta* leaves were also reported to prevent *Fusarium oxysporum* and *Aspergillus niger* spore germination at over 65% [13]. In addition, *Rhizopus oryzae* spore germination and mycelia growth were found to be suppressed by the leaf extracts of *O. gratissimum* [14].

This study showed that *P. sarmentosum* and *M. cordifolia* leaves had significant antifungal activity. The studies of phytochemical characteristics showed that bioactive compounds in *Mentha* sp. are sitosterol and β-sitosteryl-β-D-glucoside, and in *Piper* sp. are lignans, steroids, neolignans, alkaloids, propenylphenols, terpenes, piperolides, chalcones, flavanones, flavones, and amides bearing isobutyl, pyrrolidine, dihydropyridine, and piperidine moieties, all of which could exhibit high antimicrobial and antifungal properties [15–17]. The levels of plant bioactive compounds with antifungal activity could be influenced by many factors which include the age of plant, harvesting time point, extraction solvent, and method of extraction [18].

In conclusion, this study shows that crude leaf extracts of *P. sarmentosum* have strong antifungal activities against *C. gloeosporioides*. This may suggest their potential for future formulation into products for controlling anthracnose diseases of mango and other fruits. More extensive study of their phytochemical characteristics and *in vivo* efficacy remains to be determined.

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Research Article

Effect of Crude Plant Extracts on Mushroom Mite, *Luciaphorus* sp. (Acari: Pygmephoridae)

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The use of plant extracts for controlling agricultural pests has become increasingly popular in the recent years. Mushroom mite, *Luciaphorus* sp., is a destructive pest of several mushroom species and has been reported to cause severe loss of mushroom cultivation in many settings. The efficacies of 23 rhizome and leaf extracts were evaluated against female adults of *Luciaphorus* sp. At 3 days after treatment, the rhizome extracts derived from *Curcuma xanthorrhiza* Roxb. and *Zingiber montanum* (Koenig) Link ex Dietr. were found to have very strong acaricidal activities, resulting in 100% mite mortality, followed by *Curcuma longa* Linn. (98.89%), *Zingiber zerumbet* (L.) Smith. (97.78%), *Kaempferia parviflora* Wall. Ex Baker (88.89%), and *Zingiber officinale* Roscoe. (84.44%). The leaf extracts of *Ocimum sanctum* Linn. and *Melissa officinalis* L. also resulted in 100% mite mortality 3 days after treatment, while the other leaf extracts induced mite mortality only below 70%. The results suggested that rhizome extracts of *C. xanthorrhiza* and *Z. montanum* and leaf extracts of *O. sanctum* and *M. officinalis* have a great potential for future development as natural acaricides for controlling *Luciaphorus* sp.

1. Introduction

Luciaphorus sp. (Acari: Pygmephoridae) is considered as one of the most destructive pests of mushroom cultivation in Thailand. This pygmephorid mite is responsible for the severe production losses of *Lentinus squarrosulus* (Mont.) Singer, *L. polychrous* Lev., *Auricularia auricula-judae* (Bull.:Fr.) Wettst. and *Flammulina velutipes* (Curt.:Fr.) Karst. mushrooms in the Northeast of Thailand [1]. Despite that, little is known about the effective measures for controlling this mite and routine horticultural hygiene is the only procedure to alleviate the problem. To make the situation worse, desperate mushroom growers in Thailand use a large amount of carbamate and organophosphate insecticides and even some harmful solvents to manage this mite; however, this results in very limited success [2].

As a consequence, this mite becomes rapidly resistant and more harmful miticides have to be applied. The use of toxic miticides raises the concerns because of their effects on environments, human safety, and nontarget organisms. Hence, the use of nontoxic natural products for controlling this agricultural pest has been proposed. There are several higher plants that are rich in natural substances, especially the secondary metabolites, such as terpenes, steroids, alkaloids, phenolics, and cardiac glycosides, and can be used as nonharmful, environmentally friendly agents for insect control. Indeed, the use of natural compounds derived from plant extracts has been suggested as alternative treatments for insect and mite controls due to their multiple modes of action, including repellence, feeding and oviposition deterrence, toxicity, and growth regulatory activity [3–6]. Moreover, plant-based pesticides are often found to contain a mixture of active substances which can delay or prevent resistance development [7]. Therefore, in this study, the acaricidal activities of 23 plant extracts were determined against the mushroom mite, *Luciaphorus* sp.

2. Materials and Methods

2.1. Mushroom and Mite Culture. Lentinus squarrosulus Mont. mushroom culture was obtained from the Mushroom Growers Society of Thailand. The mycelium was freshly sub cultured on 90 mm plastic Petri dish plates containing potato dextrose agar (PDA, Sigma) and grown at 25°C.

Luciaphorus sp. mites were collected from infested *L. squarrosulus* composts obtained from Rapeephan mushroom farm in Khon Kaen province in the Northeast of Thailand. A pair of male and female mites was maintained at 28°C using fresh *L. squarrosulus* spawn that was grown on sawdust and sorghum grains in a glass bottle. The offspring that were in-house bred inside this glass bottle were used for all the experiments.

2.2. Preparation of Plant Extracts. Leaves and rhizomes of 23 plants were collected locally from Mahasarakham province in the Northeast of Thailand (Table 1). Plant materials were cut into small pieces and dried in hot air oven at 45°C for 3 days.

The dried plants were separately ground into powders using a small grinder and stored at 4°C in polypropylene bags. For extraction, 100 g of each powdered plant materials and 300 mL of 80% ethyl alcohol were added into sterile 2L Erlenmeyer flask, and the flask was agitated at 100 rpm for 24 h. After filtering through a Buchner funnel and Whatman No. 1 filter paper, the extracts were concentrated under low pressure using rotary evaporator. The crude extracts were reconstituted to have the concentration of 20% (w/v) using 80% ethyl alcohol (v/v, in distilled water) and stored at 4°C in glass vials to be used as stock plant extracts. For the tests, these stock plant extracts were dissolved in distilled water containing 0.05% Tween 80 to have the concentration of 5% (w/v).

2.3. Bioassay. For evaluation of each plant extract, 100 adult female mites were transferred to a 50 mm Petri dish plate containing mushroom mycelial culture grown on PDA medium, and the plate was then sprayed with 500 μL of each plant extracts prepared at the concentration of 5% (w/v). The same volumes of the sterilized distilled water (DW) and 0.005% Omite (commercial miticide) were used as control groups. The experiments for each plant extracts were performed in triplicates. All plates were incubated in the growth chamber at 28°C and 85% RH in the dark. The mortality of mites was recorded every day for 5 days after application with plant extracts.

2.4. Statistical Analysis. Data on the percentages of mite mortality due to application with plant extracts were arcsine-transformed and subjected to analysis of variance using the general linear models procedure (SAS Institute, Cary, NC, USA). Significant differences between the treatments were determined using the LSD test at $P < 0.05$.

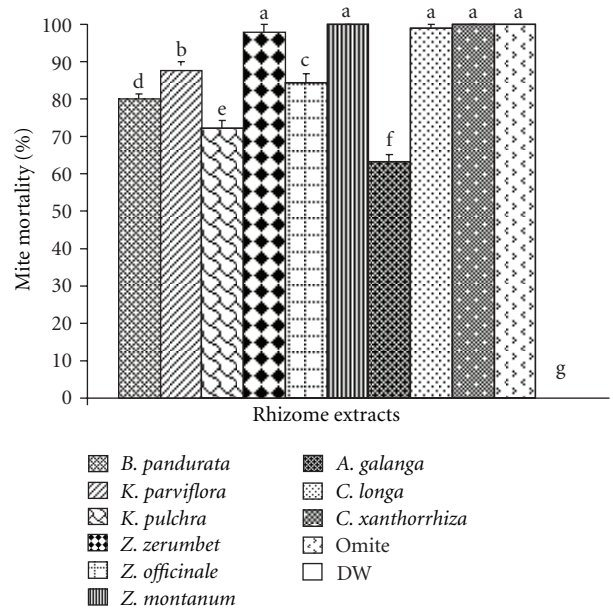

Figure 1: The mortality rates of adult female *Luciaphorus* sp. after being treated with 5% rhizome extracts at 3 days after application. Bars (mean ± SE) with same letter(s) are not significantly different as determined by LSD test at $P < 0.05$.

3. Results

3.1. Acaricidal Activities of Rhizome Extracts. In this study, all rhizome extracts were shown to have acaricidal activities against *Luciaphorus* sp., and the percentages of mite mortality increased progressively and reached the plateau after 3 days of applications (Figure 1). On day 3, the significantly high levels of mortality rates were caused by the rhizome extracts of *C. xanthorrhiza* (100%), *Z. montanum* (100%), *C. longa* (98.89%), and *Z. zerumbet* (97.78%), followed by *K. parviflora* (88.89%), *Z. officinale* (84.44%), *B. pandurata* (80.00%), *K. pulchra* (72.22%), and *A. galanga* (63.33%) (Figure 1). Interestingly, on day 1, *K. parviflora*, *Z. officinale, C. longa*, and *C. xanthorrhiza* extracts resulted in mortality rates at over 70% which were significantly higher than the other treatments (data not shown). However, on day 2, mite mortality rates in almost all treatments were over 70% with the exception of *A. galanga* (56.67%) and *K. pulchra* (67.78%) (data not shown).

3.2. Acaricidal Activity of Leaf Extracts. The levels of mite mortality after applications with leaf extracts also reached maximum on day 3 (Figure 2). On day 3, the leaf extracts of *O. sanctum* and *M. officinalis* resulted in maximum mortality (100%), but the other treatments were shown to result in mortality at levels below 70% (Figure 2). This was not unexpected because only the applications with the leaf extracts of *O. sanctum* and *M. officinalis* caused over 70% of mortality on day 1 (data not shown). Also, on day 2, mortality rates in all treatments increased and the leaf extracts of *O. sanctum* and *M. officinalis* still resulted in mite mortality at the levels significantly higher than the rest,

Table 1: Plants and their parts used for evaluation of acaricidal activities against *Luciaphorus* sp.

Scientific name	Family	Common name	Parts
Boesenbergia pandurata (Roxb) Schltr.	Zingiberaceae	Fingerroot	Rhizome
Kaempferia parviflora Wall. Ex Baker	Zingiberaceae	Belamcanda chinensis	Rhizome
Kaempferia pulchra (Ridl.) Ridl.	Zingiberaceae	Peacock ginger, resurrection lily	Rhizome
Zingiber zerumbet (L.) Smith.	Zingiberaceae	Wild ginger, Martinique ginger	Rhizome
Zingiber officinale Roscoe.	Zingiberaceae	Ginger	Rhizome
Zingiber montanum (Koenig) Link ex Dietr.	Zingiberaceae	Phlai, cassumunar	Rhizome
Alpinia galanga (L.) Swartz.	Zingiberaceae	Kha, galingale, galanga	Rhizome
Curcuma longa Linn.	Zingiberaceae	Turmeric	Rhizome
Curcuma xanthorrhiza Roxb.	Zingiberaceae	Curcuma	Rhizome
Cymbopogon citratus Stapf.	Gramineae	Takhrai, lemongrass	Leaf
Citrus hystrix DC.	Rutaceae	Leech lime	Leaf
Ocimum basilicum Linn.	Labiatae	Ho-ra-pa, sweet-basil, common basil	Leaf
Ocimum canum Linn.	Labiatae	Hairy basil	Leaf
Ocimum sanctum Linn.	Malvaceae	Holy basil, sacred basil	Leaf
Moringa oleifera Lam.	Moringaceae	Horse radish tree	Leaf
Annona squamosa Linn.	Annonaceae	Sugar apple	Leaf
Psidium guajava Linn.	Myrtaceae	Guava	Leaf
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Red river gum, Murray red gum, red gum	Leaf
Artocarpus heterophyllus Lam.	Moraceae	Jackfruit tree	Leaf
Piper sarmentosum Roxb. Ex Hunter.	Piperaceae	Cha-plu	Leaf
Murraya paniculata (L.) Jack.	Rutaceae	Orange jessamine, satin-wood,	Leaf
Melissa officinalis L.	Lamiaceae	Kitchen mint, marsh mint	Leaf
Cassia siamea (Lam.) Irwin et Barnaby	Fabaceae	Kassod tree, siamese senna, Thai copperpod, siamese cassia	Leaf

accounting for 97.78% and 94.44%, respectively (data not shown).

4. Discussion

Several plants have been found to contain bioactive compounds with a variety of biological actions against insects and mites, including repellent, antifeedant, anti-ovipositional, toxic, chemosterilant, and growth regulatory activities [4, 8]. Therefore, botanical insecticides have long been recommended as attractive alternatives to synthetic chemical insecticides for pest management because these chemicals pose little threat to the environment or to human health [9]. For example, the crude foliar extracts of five subfamilies of Australian Lamiaceae, including Ajugoideae, Scutellarioideae, Chloanthoideae, Viticoideae, and Nepetoideae, were found to have contact toxicity against the polyphagous mite (*Tetranychus urticae* Koch) [10]. This *T. urticae* could also be inhibited by the essential oil in crude foliar extract of sandalwood (*Santalum austrocaledonicum*), resulting in 87.2 ± 2.9% mortality and 89.3% reduction of the total number of eggs on leaf disks treated with this oil [11]. Piperoctadecalidine, which is the alkaloid isolated from *Piper longum* Linn., was also found to have activities against *T. urticae* at LD_{50} of 246 ppm [12]. Moreover, Aslan et al. [13] reported that essential oil vapours from *Satureja hortensis* Linn., *Ocimum basilicum* Linn, and *Thymus vulgaris* Linn. had potential against *T. urticae*, but the essential oil obtained from *S. hortensis* was the most effective at 1.563 μL/L air dose by causing 100% mortality of *T. urticae* after 4 days of treatment.

In recent years, many studies have also been conducted to investigate the activities of plant extracts or essential oils against carmine spider mite (*Tetranychus cinnabarinus* Boisd. Tunc) and Hawthorn red spider mite (*Tetranychus viennensis* Zacher). The chloroform extract of *Kochia scoparia* Linn. was shown to have rapid acaricidal activities against *T. urticae*, *T. cinnabarinus,* and *T. viennensis*, resulting in the highest mortality at 92.58, 88.88, and 84.47%, respectively, within 24 h after treatment [14]. Also, toxicity against *T. cinnabarinus* and *T. viennensis* could be quickly induced by the petroleum ether extract of *Juglans regia* Linn., resulting in mortality rates at 81.58 and 78.58%, respectively, within 24 h [7].

Furthermore, the complete 100% mortality of *T. cinnabarinus* was found to be induced by the essential oils of *Cuminum cyminum* Linn., *Pimpinella anisum* Linn., and *Origanum syriacum* var. *bevaii* (Holmes) as fumigants in greenhouse experiments [15]. This complete mortality could also be produced by using the acetone parallel extract of *Artemisia annua* Linn. leaves collected in July [16]. In addition, Zhang et al. [17] reported that benzene extracts derived from *C. longa* Linn. had LC_{50} against *T. cinnabarinus* at 99.3 ppm after 72 h. The high mortality rates of *T. cinnabarinus* could be induced by methanol extracts of *Gliricidia sepium* (Jacq) Kunth ex Steud. (100%) and *Lippia origanoides* Kunth (96.6%) when used at the concentration of 20% [18]. Additionally, Sertkaya et al. [8] evaluated the

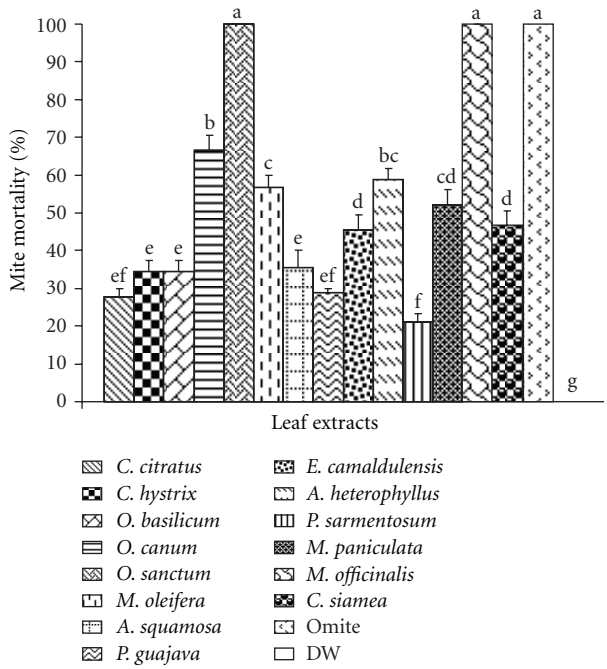

FIGURE 2: The mortality rates of adult female *Luciaphorus* sp. after being treated with 5% leaf extracts at 3 days after application. Bars (mean ± SE) with same letter(s) are not significantly different as determined by LSD test at $P < 0.05$.

efficacy of essential oils derived from medicinal plants against *T. cinnabarinus* and showed that thyme (*Thymbra spicata* Linn. subsp. *spicata*), oregano (*Origanum onites* Linn.), mint (*Mentha spicata* Linn.), and lavender (*Lavandula stoechas* Linn. subsp. *stoechas*) essential oils had LC_{50} values of 0.53, 0.69, 1.83, and 2.92 ppm, respectively. Moreover, the acetone extract of *Aloe vera* Linn. leaves was shown to have acaricidal activity against female *T. cinnabarinus* at 3 days after treatment with LC_{50} value of 90 ppm [6].

Other insect pests were also found to be inhibited by plant extracts. According to the results of Liu et al. [19], the ethanol extracts of *Eupatorium adenophorum* Spreng. (0.1% w/v) could cause mortality of citrus red mite (*Panonychus citri* (McGregor)) at 71.10 and 73.53% after 12 and 24 h, respectively. Also, the activities against *P. citri* of the ethanol extracts derived from *Boenninghausenia sessilicarpa* H. Lev., *Laggera pterodonta* (DC.) Benth., *Humulus scandens* (Lour) Merr., and *Rabdosia* were reported with LC_{50} values of 0.9241, 0.9827, 0.9905, and 1.0196 mg/mL, respectively [20]. In addition, applications with aqueous extracts of *Acorus calamus* Linn., *Xanthium strumarium* Linn., *Polygonum hydropiper* Linn., and *Clerodendron infortunatum* (Gaertn.) could lead to more than 50% mortality of *Oligonychus coffeae* (Nietner) [21]. Moreover, 3% methanolic extracts of *Ocimum tenuiflorum* Linn. and *Cassia alata* Linn. exhibited acaricidal activities against *Tetranychus neocaledonicus* Andre. and resulted in the mortality at 93.3 and 97.0%, respectively [22]. On the other hand, 3% aqueous extracts of *C. alata* and *O. tenuiflorum* could lead to mortality of *T. neocaledonicus* at 75% and 82.2%, respectively, after exposure for 3 days. In addition, the volatile oils of *Citrus reticulata* Blanco. and *C. longa* Linn. could cause mortality of *Sitophilus oryzae* Linn. as high as 100 and 90%, respectively [23]. The essential oils of *Ocimum basilicum* Linn., *Coriandrum sativum* Linn., *Eucalyptus globulus* Labill, *Mentha piperita* Linn. and *Satureja hortensis* Linn. were toxic against poultry red mite (*Dermanyssus gallinae* (De Geer)), and, when using the *in vitro* direct contact method, these essential oils at the dose of 0.6 mg/cm could result in mortality rates over 80% after 24 h of contract [24]. Furthermore, *Eucalyptus citriodora* Hook extract was found to be effective against *D. gallinae*, resulting in 85% mortality over a 24 h exposure period in contact toxicity tests [25].

In this study, the rhizome extracts of *C. xanthorrhiza* and *Z. montanum* and the leaf extracts of *O. sanctum* and *M. officinalis* at the dose of 5% (w/v) were found to be highly effective against female adults of *Luciaphorus* sp. The results revealed that the rhizome extracts were likely to have more potent acaricidal activities than those derived from leaves. The acaricidal activities of plant extracts against *Luciaphorus* sp. mites have been previously described. The essential oils derived from lemon grass (*Cymbopogon citratus* Stapf.) and citronella grass (*Cymbopogon nardus* Rendle) were shown to be effective against *Luciaphorus perniciosus* Rack., and the median effective concentration (EC_{50}) was 18.15 and 19.66 ppm, respectively [26]. In addition, the essential oils of *Litsea cubeba* Pers. were effective against *L. perniciosus* by contact and fumigation methods with LD_{50} values equivalent to 0.932 and 0.166 ppm, respectively [27].

In conclusion, the results in this study suggest the possibility of developing plant extracts derived from the rhizomes of *C. xanthorrhiza* and *Z. montanum* and the leaves of *O. sanctum* and *M. officinalis* for controlling *Luciaphorus* mites. The effective concentration and mode of action of these plant extracts against *Luciaphorus* sp. remain to be determined for the future development of highly potent products to be used in the real settings.

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Research Article

Chemical Constituents and Combined Larvicidal Effects of Selected Essential Oils against *Anopheles cracens* (Diptera: Culicidae)

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A preliminary study on larvicidal activity against laboratory-colonized *Anopheles cracens* mosquitos revealed that five of ten plant oils at concentration of 100 ppm showed 95–100% larval mortality. The essential oils of five plants, including *Piper sarmentosum, Foeniculum vulgare, Curcuma longa, Myristica fragrans*, and *Zanthoxylum piperitum,* were then selected for chemical analysis, dose-response larvicidal experiments, and combination-based bioassays. Chemical compositions analyzed by gas chromatography coupled to mass spectrometry demonstrated that the main component in the oil derived from *P. sarmentosum, F. vulgare, C. longa, M. fragrans, and Z. piperitum* was croweacin (71.01%), anethole (63.00%), ar-turmerone (30.19%), safrole (46.60%), and 1,8-cineole (21.27%), respectively. For larvicidal bioassay, all five essential oils exerted promising efficacy in a dose-dependent manner and different performances on *A. cracens* after 24 hours of exposure. The strongest larvicidal potential was established from *P. sarmentosum*, followed by *F. vulgare, C. longa, M. fragrans*, and *Z. piperitum*, with LC_{50} values of 16.03, 32.77, 33.61, 40.00, and 63.17 ppm, respectively. Binary mixtures between *P. sarmentosum*, the most effective oil, and the others at the highest ratio were proved to be highly efficacious with a cotoxicity coefficient value greater than 100, indicating synergistic activity. Results of mixed formulations of different essential oils generating synergistic effects may prove helpful in developing effective, economical, and ecofriendly larvicides, as favorable alternatives for mosquito management.

1. Introduction

Presently, the risk of contracting arthropod-borne diseases has increased due to the climate change and intensifying globalization [1]. Malaria, a life-threatening disease transmitted by mosquitoes, is continuing to be a major public health problem causing death and illness in children and adults around the world, especially in tropical countries. About 3.3 billion people—half of the world's population—are at risk of malaria. Every year, this leads to about 250 million malaria cases and nearly one million deaths [2]. Malaria control requires an integrated approach, including prompt treatment with effective antimalarials and prevention, primarily based on vector control. However, an inappropriate use of antimalarial drugs in the past century contributed to the increasing and widespread drug-resistant malarial parasites in the endemic areas, leading to rising rates of sickness and death. Therefore, mosquito management has played an essential role in the substantial reduction of malaria. The control of mosquito at the larval stage is necessary and efficient in the integrated approach to mosquito management. Mosquito adulticides, although effective, are often

applied only as a temporary solution to disease outbreaks for transiently minimizing adult populations. Furthermore, in recent years, control of adult mosquitoes has become increasingly difficult because of insecticide resistance and behavioral changes such as the avoidance of mosquito vectors to residual insecticides [3–5]. It is easier and more efficient to control the delicate larvae that are relatively immobile and more concentrated, having not yet left their aquatic breeding sites [6, 7]. Moreover, there has been increasing documentation of resistance of larval populations of anopheline mosquitoes, malaria vectors, to one or more of the main groups of conventional synthetic insecticides, that is, organochlorines, organophosphates, carbamates, and pyrethroids [8–14]. One of the most promising ways of minimizing development of insecticide resistance and reducing negative impacts to human and other living organisms and the environment is applying nonchemical materials, that is, biopesticides that do not confer cross-resistance to current insecticides and are naturally biodegradable into nontoxic [15–18].

Insecticides of botanical origin are attractive alternatives because they contained rich sources and various bioactive compounds, many of which are selective and have little or no harmful effect on nontarget organisms and the environment [19, 20]. Furthermore, the complex and variable mixtures of bioactive constituents with different modes of action may lessen the chance of resistance in mosquito populations [21]. Recently, essential oils have received considerable attention as a potentially useful bioactive insecticide, with their low mammalian toxicity and rapid degradability in the environment [22]. Larvicidal activities have been demonstrated in many plant oils such as neem, basil, cinnamon, citronella, camphor, eucalyptus, lemon, and pine [15, 23–26]. Combined formulations of different essential oils, which have more active substances than individuals, have also been investigated as larvicides, and some mixtures were found to be more effective than neem (*Azadirachta indica*) extract [27, 28]. Neem and neem-based products have been widely acknowledged and currently available as the prominent biopesticides because of their pesticidal potential with larvicidal and growth regulating activity. Nevertheless, if they are used indiscriminately, they may induce resistance in the pests and can be rendered ineffective within a few years [29]. Thus, the finding of new botanical pesticides, particular combinations of two or more toxicants with different mechanisms of action, is the need of the hour. However, a lot more work has been done on the coupled effects of synthetic-synthetic pesticides than plant-synthetic and plant-plant pesticide combinations [30]. Furthermore, most studies on the combined insecticidal efficacy of phytochemical-mixed formulations have been conducted on agricultural pests rather than pests of medical importance [31]. The present study was undertaken, therefore, to investigate the chemical composition and larvicidal efficacy of indigenous plant-derived essential oils and their combinations against *A. cracens*; a potential vector of malaria, with the aim of developing essential oil-mixed larvicides as supplementary and complementary measures for the management of malaria vectors.

2. Materials and Methods

2.1. Plant Materials. Ten plant species belonging to six families, Cyperaceae, Myristicaceae, Piperaceae, Rutacea, Umbelliferae, and Zingiberaceae, which mostly consist of botanicals with promising bioactivity against mosquitoes [31, 32], were selected for screening larvicidal activity against *A. cracens*. The plant materials (Table 1) were collected from natural habitats or commercially obtained from medicinal herb suppliers in Chiang Mai province. The herbarium specimen of each plant was identified and authenticated by botanists and plant taxonomists from the Department of Biology, Faculty of Science and the Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Thailand. The voucher specimens were numbered and deposited at the Department of Parasitology, Faculty of Medicine, Chiang Mai University.

2.2. Extraction of the Essential Oils. The plant materials utilized for extracting the essential oil were shade-dried at the environmental temperature (27–36°C) and then separately ground by an electrical blender. Dried and coarsely ground plants were extracted individually by steam distillation at 100°C for at least 3 hours to obtain the ethereal oil. The oil layer was separated from the aqueous phase, filtrated and dried over anhydrous sodium sulfate (Na_2So_4) to remove traces of moisture. Physical characteristics of the oil were recorded and the percentage yield was averaged over three experiments and calculated according to dry weight of the plant materials. The resulting essential oils were subsequently stored in an amber-colored bottle under refrigeration (4°C) until analysis for chemical compositions and larvicidal activity.

2.3. Mosquito Colony Handling. The colony of *A. cracens* [33], formerly *A. dirus* (species B), was obtained originally from the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. The free-mating populations of this mosquito had been established for more than 2 decades in the insectary of Department of Parasitology, Faculty of Medicine, Chiang Mai University [34]. The mosquito colony was maintained continually without exposure to any pathogens and insecticides under a constant laboratory condition at temperature of 27 ± 2°C and 70–80% relative humidity under a photoperiod of 12 : 12 hours (light/dark). Adults were incessantly provided with 10% sucrose and 5% multivitamin syrup solution in a small bottle with a cotton wick. Rats were supplied as a blood source for egg production of adult females. Eggs were collected and kept in plastic cups lining with moistened filter paper. Larvae were reared in plastic trays on the meal of powdered fish food. Freshly molted larvae (L_4) of *A. cracens* taken from the mass culture were available continuously for the mosquito larvicidal experiments.

2.4. Preliminary Screening for Larvicidal Activity of Essential Oils. Preliminary screening of essential oils derived from various parts of ten plants was carried out at the high concentration, 100 ppm, to check for larvicidal activity. Essential

Table 1: Physical characteristics and percentage yields (% Yield) of essential oils derived from ten plant species.

Family and botanical name (reference number)	English name	Part used	Physical characteristics			% Yield
			Color	Odor	Density (g/mL)	
Cyperaceae						
Cyperus rotundus Linn. (PARA-CY-001/1)	Nut grass	Tuber	Golden yellow	Nut grass-like	0.95	0.42
Myristicaceae						
Myristica fragrans Houtt. (PARA-MY-001/1)	Nutmeg	Mace	Light yellow	Nutmeg-like	0.96	3.41
Piperaceae						
Piper nigrum Linn. (PARA-PI-004/1)	Black pepper	Fruit	Clear	Pepper-like	0.90	0.39
Piper longum Linn. (PARA-PI-001/5)	Long pepper	Fruit	Light yellow	Pepper-like	0.87	0.64
Piper sarmentosum Roxb. (PARA-PI-003/2)	Wild betel	Leaf and stem	Brown	Pepper-like	0.91	0.31
Rutacea						
Zanthoxylum piperitum DC. (PARA-ZA-002/4)	Japanese Prickly Ash	Fruit	Pale yellow	Orange-like	0.74	0.34
Umbelliferae						
Coriandrum sativum Linn. (PARA-CO-002/2)	Coriander	Fruit	Pale yellow	Bug-like	0.86	0.97
Foeniculum vulgare Mill. (PARA-FO-001/3)	Fennel	Fruit	Pale yellow	Anise-like	0.89	0.57
Zingiberaceae						
Amomum uliginosum Koenig (PARA-AM-002/2)	Cardamom	Rhizome	Light yellow	Camphor-like	0.92	0.95
Curcuma longa Linn. (PARA-CU-005/1)	Turmeric	Rhizome	Pale yellow	Ginger-like	0.81	0.56

oil was individually dissolved in a nontoxic emulsifying agent, dimethylsulphoxide (DMSO). Groups of 25 early 4th instar larvae of *A. cracens* were selected and then exposed to the test concentration containing 249 mL of distilled water and 1 mL of essential oil-DMSO solution. Bioassays were set up according to a slightly modified version of the standard WHO larval susceptibility test methods [35] under the similar conditions used for rearing. Four replicates were maintained for the individual oil along with the concurrent control and untreated groups. A control group received DMSO-distilled water, while the untreated one was maintained in distilled water only. Mortalities of treated larvae were determined after an exposure period of 24 hours. The larvae were considered dead if they were unable to move or respond when stimulated by probing with a blunt dissecting needle. Moribund larvae were those incapable of rising to the surface of the water or showing a characteristic diving reaction when the water was disturbed. The moribund and dead larvae in each test were combined and expressed as percentage mortalities, which were corrected for control mortality using Abbott's formula [36].

2.5. Dose-Response Bioassay. Based on the initially larvicidal screening results, the promising oils, which produced 95–100% larval mortalities, were subjected to a dose-mortality response bioassay. Plant oil-DMSO solutions were prepared into different concentrations with distilled water in the range of 10 to 80 ppm, depending on the plant species. The dose response bioassays were carried out as in the screening protocol previously described. Tests were conducted using four batches of 25 larvae with the final total number of 100 larvae for each concentration. Every bioassay was replicated four times with mosquitoes from different rearing batches. The percentage mortality was reported from the average of four replicates.

2.6. Essential Oil-Mixed Formulation Experiment. Combinations comprising various mixing ratios of pairs of the most effective and the other oils established from the dose-response experiments were evaluated against *A. cracens*, as previously done, to determine whether these mixtures increase larvicidal efficacy compared with the constituted oil

Table 2: Chemical constituents of essential oils derived from five plants.

No.	Constituent	RT	Percentage composition (%)				
			P. sarmentosum	*F. vulgare*	*C. longa*	*M. fragrans*	*Z. piperitum*
1	α-Thujene	7.12				0.67	
2	α-Pinene	7.27				0.98	1.40
3	Sabinene	8.13				14.25	6.13
4	β-Pinene	8.20				0.52	
5	β-Myrcene	8.48				1.07	3.08
6	Phellandrene	8.76				0.81	
7	α-Terpinene	8.99				1.11	
8	p-Cymene	9.15			0.87	1.52	4.42
9	α-Limonene	9.23		2.07			12.03
10	β-Terpinene	9.24				16.13	
11	1,8-Cineole	9.29			0.91	0.66	21.27
12	γ-Terpinene	9.79				2.66	
13	p-Mentha-1,4-diene	9.97				0.72	
14	α-Terpinolene	10.33				0.65	
15	Fenchone	10.35		8.90			
16	Linalool	10.52				0.67	6.10
17	Thujene	10.92					0.83
18	1-Terpinen-4-ol	11.85				6.56	4.74
19	2-Allyltoluene	11.97					0.86
20	Cryptone	12.01					3.15
21	α-Terpineol	12.06				0.57	5.48
22	Estragole	12.16		5.70			1.54
23	Cuminal	12.83					0.68
24	3-Carene	12.98					2.96
25	4-Anisaldehyde	13.04		16.29			
26	Piperitone	13.05					7.31
27	Anethole	13.50		63.00			
28	Safrole	13.56				46.60	
29	Limonene	14.36					8.50
30	Geraniol	14.76					1.21
31	α-Copaene	14.78	3.77				
32	p-Acetonylanisole	14.84		1.16			
33	β-Elemene	14.98	0.70				
34	Methyleugenol	15.06				2.80	
35	β-Caryophyllene	15.40	7.38		1.58		
36	α-Humulene	15.84	0.80				
37	γ-Muurolene	16.09	0.48				
38	α-Curcumene	16.12			9.53		
39	d-Germacrene	16.19	1.22				
40	β-Selinene	16.26	1.56				
41	Zingiberene	16.27			3.93		
42	α-Selinene	16.37	1.56				
43	β-Bisabolene	16.44			2.25		
44	α-Amorphene	16.58					0.70
45	β-Sesquiphellandrene	16.64			8.55		
46	Croweacin	16.67	71.01				
47	Elemicin	16.96	2.47			1.03	
48	Farnesol	17.07	0.44				

Table 2: Continued.

No.	Constituent	RT	Percentage composition (%)				
			P. sarmentosum	*F. vulgare*	*C. longa*	*M. fragrans*	*Z. piperitum*
49	Caryophyllene oxide	17.46					1.45
50	Aromadendrene	17.47	0.77				
51	α-Cedrene	17.71			0.75		
52	γ-Gurjunene	18.24	0.61				
53	β-Maaliene	18.26	0.52				
54	ar-Turmerone	18.32			30.19		
55	Tumerone	18.36			19.02		
56	Brevifolin	18.42					6.15
57	Curlone	18.73			13.30		
	Total identified		93.29	97.12	90.88	99.98	99.99

RT: Retention time (min).

alone. The combined action of essential oils individually in the oil-mixed formulation was decided on the basis of LC_{50} value of each oil and cotoxicity coefficient (CTC) of mixtures.

2.7. GC/MS Analysis of the Effective Plant Oils. GC/MS analysis was carried out to identify the chemical constituents of the effective plant oils. Essential oils demonstrating highly larvicidal activity against *A. cracens* were subjected to analysis by using an Agilent 7890 GC system 5975 MSD, performing under the following conditions: carrier gas helium (1.0 mL/min), diluter dichloromethane (1/10, v/v), and injector temperatures 250°C using a capillary column (HP5MS 30 m × 0.25 mm, ID × 0.25 μm film thickness). The sample (0.5 μL) was injected neat with a split ratio of 250 : 1. The initial oven temperature was 50°C (hold 4 min) with a 10°C/min dynamic ramp to 250°C. Identification of oil constituents was made by comparison of mass spectra of each peak with those of authentic samples in a mass spectra Wiley 8N08 GC/MS library. Relative percentage amount of the identified compound was computed from a total ion chromatogram (TIC).

2.8. Data Management and Statistical Analysis. In all cases where deaths had occurred in the control experiment, the mortality data was corrected by Abbott's formula [36] and then determined by computerized probit analysis (Harvard Programming; Hg1, 2). Larvicidal activity was reported as LC_{50}, LC_{95}, and LC_{99} values along with corresponding 95% confidence intervals (CI), representing the concentrations that induced 50, 95, and 99% mortality, respectively. Values were considered to be significantly different ($P \leq 0.05$) if CI were nonoverlapping. A cotoxicity coefficient (CTC) for mixed formulation experiments, which is based on the lethal concentration and the proportion of each oil component in the mixture, was used to determine their responses: similar, synergism, and antagonism. When CTC of a mixture is 100, it indicates the probability of similar (additive) action. If the mixture gives a CTC greater than 100, it indicates a synergistic action. On the other hand, when a mixture gives a CTC less than 100, it is considered antagonism [37–39]. If a mixture (M) formulation of two oils (A and B), and both components have LC_{50}, then the following formulas are used (A serving as standard):

$$\begin{aligned}
&\text{Toxicity index (TI) of A} = 100,\\
&\text{Toxicity index (TI) of B} = \frac{LC_{50}\text{ of A}}{LC_{50}\text{ of B}} \times 100,\\
&\text{Actual TI of M} = \frac{LC_{50}\text{ of A}}{LC_{50}\text{ of M}} \times 100,\\
&\text{Theoretical TI of M} = \text{TI of A} \times \%\text{ of A in M}\\
&\qquad\qquad + \text{TI of B} \times \%\text{ of B in M},\\
&\text{Cotoxicity coefficient (CTC)}\\
&\qquad\qquad = \frac{\text{Actual TI of M}}{\text{Theoretical TI of M}} \times 100.
\end{aligned} \tag{1}$$

If one component of the mixture alone (e.g., B) causes low mortality at all doses (<20%), then CTC of the mixture was calculated by the formula:

$$\text{Cotoxicity coefficient} = \frac{LC_{50}\text{ of A alone}}{LC_{50}\text{ of A in the mixture}} \times 100. \tag{2}$$

3. Results and Discussion

Steam distillation of ten medicinal plants yielded from 0.31 to 3.41% (v/w) essential oils according to dry weight (Table 1). The highest oil content was found in *M. fragrans* (3.41%), followed by *C. sativum* (0.97%), *A. uliginosum* (0.95%), *P. longum* (0.64%), *F. vulgare* (0.57%), *C. longa* (0.56%), *C. rotundus* (0.42%), *P. nigrum* (0.39%), *Z. piperitum* (0.34%), and *P. sarmentosum* (0.31%). The physical and organoleptic properties of these oils presented in Table 1 demonstrate the slight differences in appearance, color, odor, and density. These volatile oils had a characteristic smell and were clear, yellow, and brown liquids that were less dense than water.

In the larvicidal screening experiment, of the essential oils initially tested at a concentration of 100 ppm, the oils

Table 3: Larvicidal activity of plant-derived essential oils against the 4th instar larvae of *A. cracens*.

Concentration of plant oil (ppm)	% Mortality (mean ± SE)	Larvicidal activity (95% CI, ppm)			Slope values ± SE
		LC_{50}	LC_{95}	LC_{99}	
Piper sarmentosum					
12.7	9.25 ± 3.30				
14.6	23.50 ± 1.29				
16.4	53.75 ± 5.44	16.03 (15.51–16.54)	20.64 (20.01–21.86)	22.91 (22.12–24.66)	14.9920 ± 0.5669
18.2	79.50 ± 2.65				
20.0	94.50 ± 3.11				
Foeniculum vulgare					
22.3	6.50 ± 1.73				
26.7	12.00 ± 4.08				
31.2	41.00 ± 11.83	32.77 (31.44–34.11)	46.56 (44.84–49.83)	53.86 (51.67–58.61)	10.7846 ± 0.3708
35.6	64.75 ± 6.65				
40.1	82.00 ± 3.74				
44.5	94.25 ± 4.99				
Curcuma longa					
20.3	12.50 ± 2.08				
24.3	17.50 ± 3.00				
28.4	23.50 ± 2.65				
32.4	31.00 ± 4.40	33.61 (29.43–39.15)	56.49 (59.66–82.13)	70.04 (79.34–112.48)	7.2941 ± 0.2698
36.5	47.25 ± 5.44				
40.5	83.75 ± 0.96				
44.6	90.50 ± 1.29				
Myristica fragrans					
28.8	10.00 ± 1.15				
33.6	17.75 ± 2.50				
38.4	34.75 ± 4.19	40.00 (37.33–43.32)	56.56 (55.76–67.70)	65.28 (65.35–81.90)	10.9335 ± 0.4652
43.2	63.75 ± 3.86				
48.0	85.75 ± 3.09				
Zanthoxylum piperitum					
51.8	11.75 ± 1.71				
55.5	29.50 ± 5.26				
59.2	35.25 ± 9.22				
62.9	43.50 ± 2.08	63.17 (61.90–64.50)	85.01 (82.59–89.33)	96.13 (92.67–102.67)	12.7574 ± 0.5292
66.6	61.00 ± 4.32				
70.3	73.50 ± 2.08				
74.0	83.00 ± 3.77				

derived from five plants, including *P. nigrum*, *A. uliginosum*, *C. sativum*, *P. longum*, and *C. rotundus* produced no or low larval mortality of 0, 4, 8, 36, and 52%, respectively. No larval mortality was observed in the control and untreated groups. The other oils, including *P. sarmentosum*, *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum* demonstrated promising efficacy with larval mortality of 100, 100, 100, 100, and 96%, respectively. These five plants were then selected for further experiments, including chemical analysis, dose-response larvicidal experiments, and combination-based bioassays for quantifying their toxicity.

Results of phytochemical analysis of the essential oils with promising larvicidal activity are displayed in Table 2. A total of 57 compounds were identified from five essential oils, including *P. sarmentosum*, *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum*, representing 90.88–99.99% of the oil obtained. The oil derived from leaf and stem of *P. sarmentosum* contained 14 identified compounds, amounting to 93.29% of the whole oil with croweacin (71.01%) as the chief constituent, together with minor amounts of β-caryophyllene (7.38%), α-copaene (3.77%), and elemicin (2.47%). In the fruit oil of *F. vulgare*, 6 compounds

Table 4: Larvicidal activity and cotoxicity coefficient (CTC) of five essential oils and *P. sarmentosum*-combined oil formulations against the 4th instar larvae of *A. cracens*.

Essential oil	Combination of essential oil	LC_{50} (95% CI, ppm)	Slope values ± SE	Cotoxicity coefficient (CTC)	Effect
P. sarmentosum (P)	P 100%	16.03 (15.51–16.54)	14.9920 ± 0.5669	—	—
F. vulgare (F)	F 100%	32.77 (31.44–34.11)	10.7846 ± 0.3708	—	—
C. longa (C)	C 100%	33.61 (29.43–39.15)	7.2941 ± 0.2698	—	—
M. fragrans (M)	M 100%	40.00 (37.33–43.32)	10.9335 ± 0.4652	—	—
Z. piperitum (Z)	Z 100%	63.17 (61.90–64.50)	12.7574 ± 0.5292	—	—
P + F	P 25% : F 75%	28.60 (28.37–28.83)	17.0907 ± 0.8318	90.8595	Antagonism
	P 50% : F 50%	27.29 (26.09–28.43)	15.0440 ± 0.6262	78.8890	Antagonism
	P 75% : F 25%	18.32 (17.65–18.99)	9.1834 ± 0.4084	100.3105	Synergism
P + C	P 25% : C 75%	27.10 (25.04–28.80)	6.8012 ± 0.2937	97.3354	Antagonism
	P 50% : C 50%	22.08 (21.51–22.61)	14.9567 ± 0.5742	98.3108	Antagonism
	P 75% : C 25%	16.81 (16.59–17.03)	10.8101 ± 0.4357	109.7055	Synergism
P + M	P 25% : M 75%	35.72 (33.51–37.74)	9.7333 ± 0.4288	81.5108	Antagonism
	P 50% : M 50%	28.51 (27.48–29.67)	14.7402 ± 0.7607	80.2797	Antagonism
	P 75% : M 25%	18.18 (17.69–18.64)	12.4666 ± 0.4585	103.7110	Synergism
P + Z	P 25% : Z 75%	41.40 (40.45–42.19)	32.5982 ± 1.4760	87.9356	Antagonism
	P 50% : Z 50%	29.40 (28.33–30.59)	19.9294 ± 0.7629	86.9765	Antagonism
	P 75% : Z 25%	17.99 (16.31–20.45)	6.8213 ± 0.3053	109.5410	Synergism

were identified, representing 97.12% of the oils obtained. Compounds in this oil comprised mostly anethole (63.00%), followed by 4-anisaldehyde (16.29%), with minor contents of fenchone (8.90%), estragole (5.70%), and α-limonene (2.07%). For *C. longa* rhizome oil, 11 compounds were identified, corresponding to 90.88% of the total oil. The major components were ar-turmerone (30.19%), tumerone (19.02%), and curlone (13.30%), whereas α-curcumene (9.53%) and β-sesquiphellandrene (8.55%) were seen as minor constituents. The mace oil of *M. fragrans* demonstrated the presence of 19 compounds, accounting for 99.98% of the whole oil with safrole (46.60%) as the principal constituents, followed by β-terpinene (16.13%), sabinene (14.25%), and 1-terpinen-4-ol (6.56%). Twenty one compounds constituting 99.99% of all the volatile compositions were characterized from *Z. piperitum* fruit oil. The main chemical compounds identified were 1,8-cineole (21.27%) and α-limonene (12.03%), followed by minor quantities of limonene (8.50%), piperitone (7.31%), brevifolin (6.15%), sabinene (6.13%), and linalool (6.10%).

In the dose-response larvicidal assessment, all the oils examined exhibited a promising larvicidal efficacy on larvae of *A. cracens* with dose dependent and different performances among plant species. The strongest larvicidal potential was established from *P. sarmentosum*, followed by *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum*, with LC_{50} values of 16.03, 32.77, 33.61, 40.00, and 63.17 ppm, respectively (Table 3). Although bioactivity of the essential oil results from interaction among structural components, particularly the major constituents, the other compounds, even trace elements, can also have a vital function; this is due to coupled effects, additive action between chemical classes and synergy or antagonism [40, 41]. Further investigations of comparative toxicity of chemical constituents derived from these plants, either individually or in selected blends, are necessary for identifying components contributing to the observed larvicidal action. Bekele and Hassanali [42] investigated the lethal toxicity of major components derived from essential oils of *Ocimum kilimandscharicum* (camphor, limonene, 4-terpeneol, 1,8-cineole, camphene, and *t*-caryophyllene) and *Ocimum kenyense* (methyl chavicol, ethyl isovalerate, α-humulene, 1,8-cineole, and isoeugenol) against two postharvest insect pests, *Sitophilus zeamais* and *Rhyzopertha dominica*. They discovered that a major compound of *O. kilimandscharicum* was largely responsible for the toxic effect against *R. dominica*. However, the results with the other treatments indicated that the toxic action of the essential oils was due to the combined effects of different components, either with or without significant individual toxic action of their own against the insects. Some of these compounds such as 1,8-cineole, limonene, and humulene are presented in the plant oils tested in this study and also found in other plants with biological activity against various insect species [43–45].

Generally, individual botanical insecticides are slow acting, time consuming, and active only at high concentration, which makes them impractical and uneconomical for field application [27, 46]. Phytochemical-combined formulations, which not only improve activity, but also decrease the needed dose, are therefore considered very advantageous in vector control program. The importance of proper selection of plant extracts as synergists in mixed formulations with different botanicals is being increasingly recognized in mosquito management [30]. Mixtures of more than one insecticide

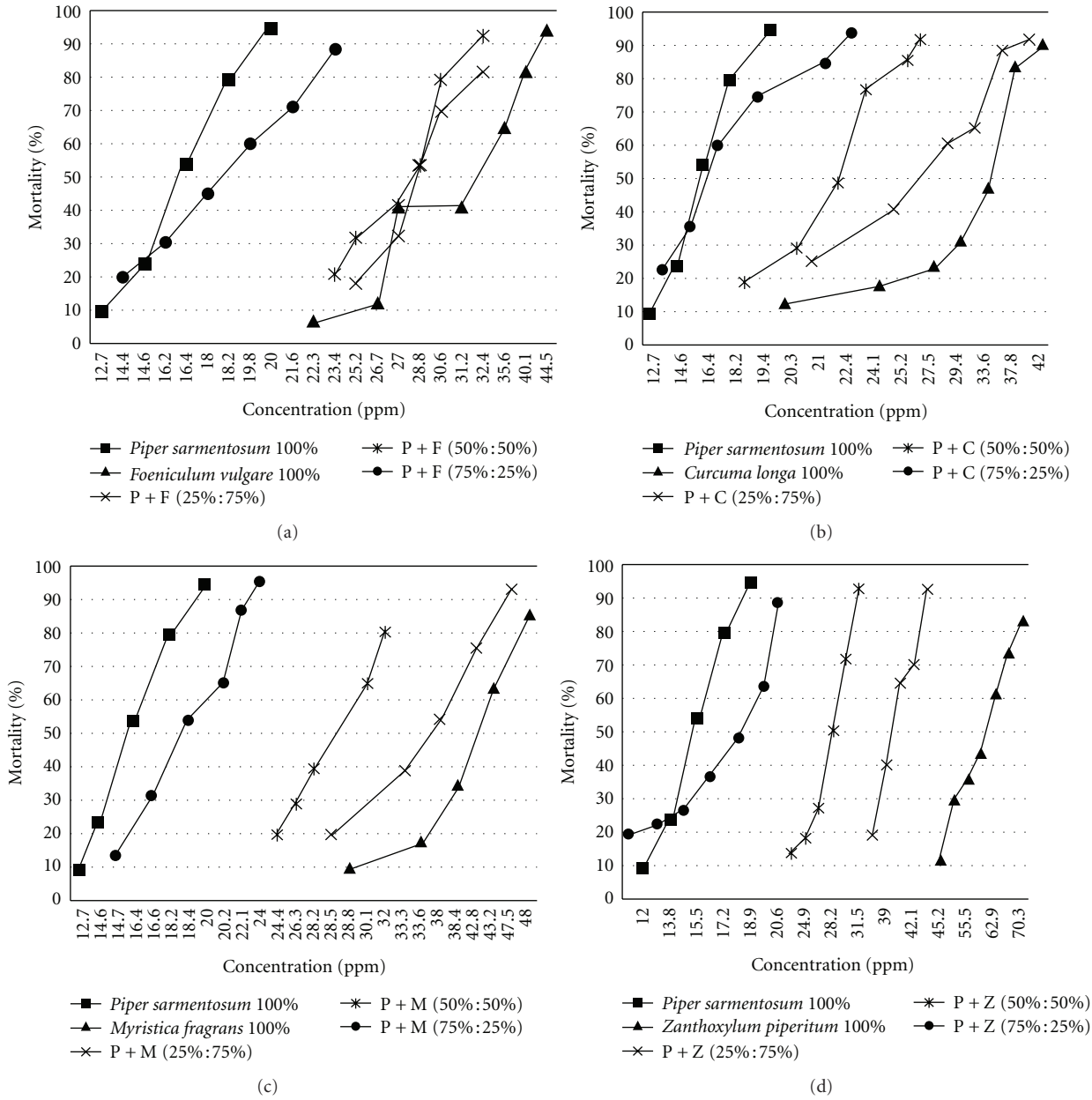

Figure 1: Larvicidal activity of combined formulations between *P. sarmentosum* (P) oil and the other plant oils: (a) *F. vulgare* (F), (b) *C. longa* (C), (c) *M. fragrans* (M), and (d) *Z. piperitum* (Z) against the 4th instar larvae of *A. cracens*.

with different modes of actions are proving to be effective and recommended for integrated resistance management in some insect pests [47–50]. In this study, comparative evaluation of the larvicidal efficacy of combinations between *P. sarmentosum*, the most efficient oil, and the others was carried out and the results are demonstrated in Figure 1 and Table 4. It was found that the addition of *P. sarmentosum* oil to the other individual oils affected the larvicidal activity, leading to increasing mortality of *A. cracens* larvae in all trials. The binary mixtures of oils of *P. sarmentosum* and the others, including *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum* at the ratios of 25% : 75%, 50% : 50%, and 75% : 25% showed remarkably reduced LC_{50} values, ranging from 18.32–28.60, 16.81–27.10, 18.18–35.72, and 17.99–41.40 ppm, respectively. The cotoxicity coefficient (CTC) determined from these LC_{50} values were ranged from 78.8890–100.3105, 97.3354–109.7055, 80.2797–103.7110, and 86.9765–109.5410, respectively. The combined effect of *P. sarmentosum* and the other oils at the highest ratio (75% : 25%) possessed synergistic activity with a value CTC (relative to LC_{50}) greater than 100. However, all mixtures at the lower ratios (25% : 75% and 50% : 50%) exhibited antagonistic action with a CTC value lower than 100.

In the present study, combinations of *P. sarmentosum* and the other oils exhibited better larvicidal activity than most independent oils. Although the effect at the lower ratios

(25% : 75% and 50% : 50%) was relatively moderate, the larvicidal activity was significantly improved when the mixtures (75% : 25% ratio) contained higher amount of *P. sarmentosum*. Of special interest is in the case of *C. longa*, *Z. piperitum*, and *M. fragrans* oils, which have lower larvicidal efficacy than that of *F. vulgare*; addition of *P. sarmentosum* in these three oils at the highest ratio (75% : 25%) gave a mixture that is more active (LC_{50} = 16.81, 17.99, and 18.18 ppm, resp.) than that of *P. sarmentosum-F. vulgare* mixed formulation (LC_{50} =18.32 ppm). From these findings, it was suggested that combinations between *P. sarmentosum* and the other oils in the appropriate varieties and proportions are beneficial in enhancing larvicidal toxicity toward anopheline mosquitoes. In addition, in the case of *Z. piperitum* oil (2.71 USD/mL), which is approximately three times more expensive than *P. sarmentosum* oil (0.94 USD/mL), combined formulations of these two oils provided not only better efficacy but also lower cost. The synergistic larvicidal activity of combinations between two plant extracts, *Hyptis suaveolens* and *Lantana camara*, was previously reported by Tanprasit [28]. It was revealed that the mixture of *H. suaveolens* and *L. camara* (LC_{50} = 14.04%) possessed significantly higher larvicidal activity against *Aedes aegypti* than those of the individual substances, *H. suaveolens* (LC_{50} = 20.24%) and *L. camara* (LC_{50} = 74.44%). The individual and combined efficacy of *Annona squamosa* and *Pongamia glabra* extracts against three mosquito vectors, *Culex quinquefasciatus*, *Anopheles stephensi*, and *A. aegypti*, compared to that of *A. indica* was investigated by George and Vincent [27]. It was found that *P. glabra* has a greater larvicidal effect than that of *A. squamosa*, and all of their combined formulations exhibited significantly greater effect than those of independent extracts. Furthermore, the most effective mixture of these plant extracts (LC_{50} = 28.804 ppm) was found to be more effective than the prominent biopesticide, *A. indica* (neem) extract (LC_{50} = 45.120 ppm). Singha et al. [51] reported the synergistic effect of *Croton caudatus* (fruit) and *Tiliacora acuminate* (flower) extracts against filarial vector, *C. quinquefasciatus*. The combined formulation of *C. caudatus* and *T. acuminate* exhibited good bioactive potentiality against *C. quinquefasciatus* larvae due to synergism of plant extracts. These findings correspond to those of this study, which presents an insight into the high possibility of developing new mosquitocides from combinations of different essential oils or phytochemicals, generating synergism. Remarkably better performance of *P. sarmentosum* in the essential oil-mixed formulation experiment herein suggests that it may have good potential to be an alternative synergist in efficient mixtures of control agents. This performance may achieve satisfactory levels of efficacy, economic benefit, and ecological friendliness and minimize the development of resistance in the vector population.

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