Islamic University – Gaza
Deanship of Graduate Studies
Faculty of Science
Biological Sciences Master Program
Botany & Mycology

الجامعة الإسلامية- غزة
عمادة الدراسات العليا
كلية العلوم
برنامج ماجستير العلوم الحياتية
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In Vitro Control of *Ascosphaera apis* Fungus by Some Plant Extracts

Prepared By

Ashraf A. F. El Shafai

Supervisors

Dr. Kamal J. El Nabris — **Ph.D. Marine Biology**

Dr. Abboud Y. El Kichaoui — **Ph.D. Botany**

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Dedication

This thesis is dedicated to my parents. My father who is always supporting me, through the years. To the spirit of my mother who paid the price for my rest and my success. To my wife and my children who are the closest to me and who have always given me a warmth love.

Declaration

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains material neither previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university of other institute, except where due a acknowledgment has been made in the text'.

Name	Signature	Date
Ashraf A. F. Al-Shafai	***Ashraf***	***February,2012***

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{وَأَوْحَى رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتاً وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ (٦٨) ثُمَّ كُلِي مِن كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلاً يَخْرُجُ مِن بُطُونِهَا شَرَابٌ مُّخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاء لِلنَّاسِ إِنَّ فِي ذَلِكَ لآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ(٦٩) } سورة النحل

Abstract

Chalkbrood is one of the most dangerous disease of the honeybees, *Apis mellifera*. It causes a significant reduction in brood and honey production thus, leading to an economic loss in apiculture. In present study, the pathogenic fungus was isolated from infected larvae in Northern Governorate- Palestine and identified based on morphological and cultural characters as *Ascosphaera apis*. Crude aqueous extracts of ten different plant species including *Punica granatum, Artemisia monosperma, A. absinthium, A. herba-alba, Cyperus rotundus, Callistemon viminalis, Cinnamomum zeylanicum, Tagetes patula, Annona squamosa* and *Psidium guajava* were evaluated *in vitro* for their antifungal activities against *A. apis*. Results showed that extract of *P. granatum* was the most active one among all. Other extracts vis., *P. guajava*, *C. zeylanicum*, and *C. viminalis* demonstrated a moderate activity with MICs of 1.25, 2.5 and 5% respectively. The remaining extracts of *A. monosperma*, *A. absinthium*, *A. herba-alba*, *T. patula*, *C. rotundus*, and *A. squamosa* were totally inactive against *A. apis*. For further evaluation of the antifungal properties of the different plant extracts, the radial mycelial growth inhibition test was also performed on agar media supplemented by plant extracts to achieve different concentrations of 5%, 10% and 20%. The results showed that the average radial mycelia growth of *A. apis* was significantly reduced by *P. granatum, P. guajava*, *C. viminalis* and *C. zeylanicum* ($P < 0.05$). Contrary to this, The remaining extracts of *A. monosperma*, *A. absinthium*, *A. herba-alba, C. rotundus, T. patula* and, *A. squamosa* were found to have weak antifungal activities at all concentrations tested.

In order to assess the antifungal effect of the *P. granatum* extract on *A. apis*, the growth of profile for the fungus was followed at extract free agar media after incubation at different concentrations of *P. granatum* extract.

The results found that the extract of *P. granatum* flower exhibits a fungistatic effect because it causes changes on the normal growth profile of *A. apis* at the different concentrations tested (5%, 10% and 20%).

Since the extract of *P. granatum* flower was proved to be the most effective *in vitro* against *A. apis*, its toxicity to worker bees was evaluated. The results of the toxicity test showed that, the flower extract of *P. granatum* was not lethal to adult workers at low concentrations tested since the a cumulative mortality percentages were 4.4%, 1.1 % and 3.6% at 0% 12.5% and 25% concentrations respectively.

In conclusion, results from these findings suggest that the aqueous extract of *P. granatum* flower may be used as natural antifungal agents to inhibit growth of *A. apis*. These findings however need to be progressed to field applications to evaluate the efficacy of the most active antifungal plant extract identified in this study against the causative agent of chalkbrood disease in an apiary system.

Keywords: MIC, Plant extracts, fungistatic, *Ascosphaera apis*, *Apis mellifera*, *Punica granatum, Artemisia monosperma, A. absinthium, A. herba-alba, Cyperus rotundus, Callistemon viminalis, Cinnamomum zeylanicum, Tagetes patula, Annona squamosa, Psidium guajava*

ملخص الدراسة

يعتبر فطر *Ascosphaera apis* المسبب الرئيسي لمرض الحضنة الطباشيري في نحل العسل، وغالبا ما يؤدي إلى خسارةٍ اقتصادية بسبب تأثيره السلبي على يرقاتِ النحل، مما يقلل عدد أفراد الطائفة فيقلل إنتاج العسل و الإنتاج النباتي المعتمد على التلقيح الخلطي. وحتى الآن، لا توجد إستراتيجية محددة لمكافحة هذا المرض معتمدة عالمياً. وبناء على ذلك، تهدف هذه الدراسة للبحث عن طريقة بديلة لمكافحة هذا الفطر باستخدام مستخلصات نباتية تتميز بتوفرها في المنطقة وبقلة تكلفتها و عدم تأثيرها السلبي على البيئة.

خلال هذه الدراسة تم عزل الفطر *A. apis* من يرقات نحل العسل الميتة والتي جمعت من خلايا مصابة في محافظة الشمال، قطاع غزة، فلسطين، وتم تحديد الفطر و التعرف عليه بواسطة المعايير المظهرية و المجهرية له و بواسطة اختبار التزاوج. و تم تقييم الفعالية لعشرة مستخلصات نباتية تشمل: الرمان و العاذر و الدمسيسة و الشيح و السعد و الفرشاة و القرفة و القطيفة و القشطة و الجوافة على نمو الفطر بواسطة اختبار التركيز الأدنى للتثبيط (MIC) و اختبار معدلات أنصاف أقطار النمو للغزل الفطري، كما دراست نوع التأثير لمستخلص زهرة الرمان على الفطر المذكور، وأجريت تجربة لدراسة السمية لمستخلص زهرة الرمان على شغالة نحل العسل.

ولقد أظهرت النتائج أن مستخلص زهرة الرمان كان الأكثر فاعلية ضد *A. apis* حيث كان التركيز الأدنى للتثبيط (MIC) له (0.625%). تلاه في التأثير كلا من مستخلص الجوافة و القرفة و الفرشاة حيث أظهرت تأثيرا متوسطاً بنتائج (MIC) 1.23% ، 2.5% و 5% على التوالي. أما تأثير المستخلصات الأخرى للعاذر و الدمسيسة و الشيح و القطيفة و السعد والقشطة لم تعطي أثرا فعال على الفطر المذكور في هذه الدراسة. وبالنسبة إلى دراسة معدلات أنصاف الأقطار للنمو الفطري، فقد أظهرت النتائج دلالات إحصائية ($P < 0.05$) لمستخلصات الرمان و الجوافة و الفرشاة و القرفة وكانت النتائج متفقة مع الاختبار الأول بينما كان تأثير المستخلصات الأخرى ضعيف على نمو الغزل الفطري لجميع التراكيز المستخدمة في الدراسة ولم تعطي أي دلالة إحصائية ($P < 0.05$). وأوجدت النتائج أن تأثير التراكيز المختلفة لمستخلص زهرة الرمان يعتبر تأثير كابح للفطر *A. apis* وليس تأثيراً قاتلاً له. وكما أظهرت الدراسة أن التراكيز المستخدمة لمستخلص زهرة الرمان لم يكن له تأثير مميت على شغالات نحل العسل، حيث كانت نسبة الوفيات التراكمية للشغالات 1.1% و 3.6% و 4.4% للتركيز 12.5% و 25% و 0% للمستخلص على التوالي .

وبناءً على النتائج، تم التوصل إلى أن مستخلص زهرة الرمان الذي يظهر نشاط قوي ضد فطر *A. apis* يمكن اعتباره كمضاد فطري لمرض الحضنة الطباشيري في نحل العسل، إلا أن ذلك يتطلب المزيد من الدراسات لإظهار ما إذا كانت المستخلصات المستخدمة يمكن استخدامها لعلاج أمراض نحل العسل لتقليل الآثار السمية على النحل وعلى جودة المنتجات النحلية.

الكلمات الرئيسية: *Ascosphaera apis* - MIC - مستخلصات نباتية - الكبح الفطري - نحل العسل - نبات الرمان- نبات العاذر - نبات الدمسيسة- نبات الشيح - نبات السعد - نبات الفرشاة – نبات القرفة – نبات القطيفة - نبات القشطة - نبات الجوافة.

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Abbreviations

A. mellifera	*Apis mellifera*
A. apis	*Ascosphaera apis*
^{o}C	degrees Celsius
cm	centimeter
g	gram
hr	hour
Min	Minute
mm	Millimeter
rpm	Round per minute
W/V	Weight to volume
V/V	Volume to volume
mL	milliliter
µL	micro liter
MIC	Minimal inhibition concentration
µm	micrometer
ppm	parts per million
PCR	Polymerase Chain Reaction
KGray	Kilo Gray
Cu(II)	cupper
iu	International unit
RH	Relative humidity
IC_{50}	half maximal inhibitory concentration
PDA	Potato Dextrose Agar
YE	Yeast Extract
psi	Per square inch
10X	Total magnifications of 100X
40X	Total magnifications of 400X
Mt	Metric tons

Chapter 1

General Introduction

1.1. Overview

Honey bees (*Apis mellifera* L.) are one of the most well-known, popular and economically beneficial insect on earth (Delaplane, 2010). They produce honey, beeswax, propolis, royal jelly and bee venom.

Honeybees, provide pollination services to many sexually reproducing agricultural crops and wild plants. The value of honeybees pollination to worldwide agriculture is about 215 billion dollars (Gallai *et al*., 2009).

Previous researches in the USA have valued crops that require pollination by honeybees at an estimated $24 billion annually, and the value of commercial bee pollination on contracts at around $10 billion annually (Gallai *et al*., 2009). These are huge figures by any standard and they show that bees are big business.

In 2004 estimated world production of honey was higher 1.38 million metric tons (mt) MT. Additionally, In the next year 2005, The world trade of honey reached the value of US$ 738 million (SADC Trade,2007).

Palestine, *'land flowing with milk and honey'* had approximately 46,020 honey bee colonies in 1994 (PNIC, 2000). The number of bee hives, was increased in Palestine to reach 65,921 bee hive in the year 2000 (Irjoob, 2007), and 66,733 bee hive in the year 2008 (PCBS, 2008). Honey production during year 2007-2008 was 223 mt (PCBS, 2008; FAO, 2012) and, this is worth about US$ 5 (FAO, 2012). Through the years 2007-2008, Gaza Strip has produced 46 tons of honey as follows: Northern Governorate, 12 tons; Gaza Governorate, 10 tons; Middle Governorate, 8 tons; Khan Yunis Governorate, 12 tons; and Rafah Governorate, 4 tons; (PCBS, 2008).

In recent years, the yield of honey was obviously decreased in Gaza Strip. This can be attributed to the continuous military attacks and the destruction of the orchards, agricultural crops and wild plants by the occupation army during Al-Aqsa Intifada. In 2011, the NGO

Development Center reported that of the total 70,000 dunums of agricultural land in Gaza Strip, an area of approximately 10,000 square dumums to the east of the Gaza city has been intentionally destroyed by military bulldozers and turned into barren and dissolute areas. Moreover, a total of 5,150 dunums of fruitful trees, 5,150 dunums of irrigation networks and 1,150 dunums of agricultural green houses were completely destroyed.

The wild plants were also demolished by inhabitants of the restricted overpopulated area of Gaza Strip. Uncontrolled and heavy use of pesticides in Gaza Strip (Safi *et al*., 2000) may also contribute to obstruction in the apiculture.

During the last years, several civil and governmental foundations concerned with beekeeping were established in Gaza Strip. They trained hundreds of Palestinian farmers and provide them with bee hives and equipments.

This beneficial insect however, may attacked during the different stages of its life cycle by a number of pathogens like bacterial, fungal, viral, and parasites (Delaplane, 2010). Some of these pathogens may affect adult bees, while others which are called as brood diseases affect the immature stages of the bees such as larva and pupae stages (Palmer-Jones, 1967).

Beekeepers in Gaza strip declared, that many of these diseases were reported in Gaza strip since 1980s (Lord, 1994).

Chalkbrood is a common fungal disease of the honeybee (*A. mellifera*) brood. It is caused by the spore forming fungi *Ascosphaera apis* (Maassen ex Claussen) Spiltoir and Olive. Chalkbrood is considered to be one of the most serious illness plaguing apiculture today and is nearly cosmopolitan distribution but with various degrees.

Infection of the larvae mainly occur when spores within the larval food, provided by adult worker bees, are ingested, but can be also occurred as a result of growth of the fungus through the cuticle. Since larvae are the most susceptible stage throughout bees' life cycle, infected colonies can be easily recognized by the presence of white and/or black mummified larvae. As *A. apis* is heterothallic fungi, diseased larvae are white if a single strain is successful in colonizing a larva (Christensen and Gilliam, 1983). On the other hand, diseased larvae are black due to the presence of spore producing fruiting bodies that are brown to black when mature (Anderson *et al*., 1998) and occurs when a diseased larva is simultaneously infected

with both + and – strains of *A. apis* that have mated (Flores *et al*., 1996; Gilliam *et al*., 1978; Moeller and Williams, 2006).

As a brood disease, the fungus germinates in the larval gut when they are 3 to 4 days old. Later, the larvae are died, and became dry, hard, shrunken and chalklike (Gilliam *et al*., 1978; Glinski and Buczek, 2003).

Although chalkbrood is not usually fatal to honeybee colonies it can cause considerable losses in both bee numbers and colony productivity (Bailey, 1963), thus causing high economic losses through reduced honey production. Losses attributed to chalkbrood have been estimated to vary from 5-37% of honey yields (Invernizzi *et al*., 2011; Puerta *et al*., 1999)

In occupied Palestine chalkbrood was recognized as the most serious infectious brood disease (Jakobsons, 2005), and the first case of it was reported in 1984 (Yacobson *et al*.,1991). In 2005, every apiary in the country became affected to some level by *A. apis* and about 50% of the hives have shown clinical signs of the disease (Aronstein *et al*., 2010). In 1991, the decline of honey production in occupied Palestine due to chalkbrood disease was estimated to be about 10%- 15% (Jakobsons, 2005).

The development of chalkbrood disease is usually connected with the prevalence of the infectious materials (fungal spores) in honeybee colonies and in the hive environment (Flores and Gutierrez, 1997).

Various studies indicated that many factors are involved in the infection and spread of the chalkbrood disease. These factors include; spore contaminated pollen, genetic of the queens, drifting of bees from infected colonies, spore levels in combs from previously infected colonies and spore contaminated food source such as water, nectar and honey. An upset of microbial equilibrium, caused by extensive use of antibacterial agents has been suspected to be a predisposing factor as well (Collison, 2009; Flores and Gutierrez, 1997; Witte, 2003).
Chalkbrood also appeared to be stress-related disease as infections have been noted when one or more of following conditions exist: excessive hive moisture, dry conditions, cool and wet weather, an increase in CO_2 levels, poor nutrition conditions, weak colonies, a failing queen,

poor hive management and other bee diseases such as Varroa and nosema(Aydin *et al*., 2006; Jakobsons, 2005; Witte, 2003; Gilliam *et al*., 1978)

At present, several strategies were suggested to control and manage the chalkbrood disease including; destruction of combs containing a large number of mummies, strengthening of badly diseased colonies by adding bees and brood, and requeening.

A common beekeeping practice to control this disease is to raise the acidity of hive environment by means of organic acids, e.g. formic, acetic, sorbic or citric acid (Milino, 2001). Other control strategies have focused on the utilization of chemotherapeutic agent to control fungal growth. Although some of these chemicals are thought to be promising for controlling fungal growth, no information are presently available on the successful use of chemical for the control of the disease with proven efficacy (Hornitzky, 2001).

In recent years, application of synthetic fungicides such as nystatin or clotrimazolum to manage the chalkbrood disease (Glinski and Ghmielewski, 1996) has been banned in western world because of the serious threat to human health due to their residual toxicity when these substances are transferred to honey (Bogdanov *et al*., 1999).

Despite the various strategies which were proposed so far to control or minimize the chalkbrood disease, there is still however, to the best of our knowledge, no efficient strategy to control this disease.

Considering the worldwide distribution of chalkbrood disease, the serious destruction to honeybee colonies, the lack of registered chemicals to fight it, and the ban of synthetic fungicides, it is necessary to search and develop an effective, efficient, feasible and safe alternative approach to control this disease.

Plants are factories of natural chemicals which provide the richest organic chemicals on earth (Grainge and Ahmaed, 1988). For centuries, the bark, leaves, flowers, fruits, and seeds of plants have been used in traditional medicine for treating common infections and diseases.
Plants, generally accumulate a variety of secondary metabolites which constitute an important source of natural products with biological activities (Khaing, 2011). These compounds may be involved in the defense of the plants against invading pathogens including insects, bacteria,

fungi, and viruses. So far, many different biologically active substances have been isolated from plant extracts (Bokhari, 2009).

The bioactive bases responsible for the antimicrobial property in plants include alkaloids, anthraquinones, saponins, flavonoids, isoflavonoids, cumarins, glycosides, terpens, tannins, phenolic and polyphenolic compounds (Ebana *et al*., 1993; Souza *et al*., 2005). Extracts isolated from several plants have been proved to have antifungal (Satish *et al.*, 2007), antibacterial (Saxena and Gomber, 2006), anti-inflammatory (Schinella *et al*., 2002), and antioxidant activities (El-Massry *et al*., 2002; Schinella *et al*., 2002).

Despite the fact that, extracts of plants have been extensively studied for antimicrobial activity, the antifungal activity of plant extracts that exhibit inhibition effect against *A. apis* are rarely investigated. To the best of our knowledge, the only study in this field was that of Chantawannakul *et. al.* (2005), wich has demonstrated an antifungal action of the aqueous extracts of some medicinal plants such as cinnamon and betel piper against *A. apis*.

Taking into consideration the importance and the safe of naturally occurring compounds found in most plants, the use of their extracts is still of great interest as possible antifungal agent fundamentally active against the chalkbrood disease.

Therefore, the purpose of the present study was to evaluate *in vitro* the antifungal activities of the crude extracts of ten plant species including *Punica granatum, Artemisia monosperma, A. absinthium, A.herba-alba, Cyperus rotundus, Callistemon viminalis, Cinnamomum zeylanicum, Tagetes patula, Annona squamosa,* and *Psidium guajava* against *A. apis*, the causative agent of chalkbrood disease in honeybee larva. The plants were mainly selected on base of their ethnomedicinal properties, where they showed antimicroial activity when tested against bacteria, fungi and yeasts, but less information about their antifungal activity against *A. apis* was found in our bibliographic searches. Furthermore, these plant species could be easily found in the studied area.

1.2 Significance of the Study

Chalkbrood is considered to be one of the most serious disease in honey bee. It can cause considerable losses in both bee numbers and colony productivity, thus causing high economic losses through reduced honey production. Despite the various strategies which were proposed to control the chalkbrood, there is still however, no efficient strategy to control this disease. It is wise therefore to find other means of controlling and managing this disease.

Plants are rich in a wide variety of secondary metabolites which have been found to have different biological activities. The antifungal activity of plant extracts that exhibit inhibition effect against *A. apis* however were rarely investigated.

Therefore, the main purpose of this study is to evaluate *in vitro* the antifungal activities of the crude extracts of ten plants against the pathogenic fungi, *A. apis*, the causative agent of chalkbrood disease in honeybee. The plants were mainly selected on base of their ethnomedicinal properties and their local availability. If proven to be effective, it could be used to help minimize the occurrence of the disease in honey bees, thus chalkbrood control could be stabilized. Furthermore, the result of the study will provide some baseline data, which may encourage researchers to conduct further research, and hopefully to formulate environmental friendly, plant based drugs for control of *A. apis* infections.

In the long-run this could also help resource-poor Palestinian farmers to improve colony productivity and thus farmers income will increase through sale of honey and other products, eventually, the Palestinian economy will improve. Also, The risks of drug residues in honey, wax and other honeybee products make the research involving possibilities of biological control important.

Information gained from this study will be made available to all honeybees' keepers, through Ministry of Agriculture, agricultural organization or some other means of dissemination.

1.3. Objectives of the study

1.3.1. General objective

- To evaluate the antifungal activity of the aqeuous crude extracts of different plant species against, *Ascosphaera apis*, the causative agent of chalkbrood disease in honeybees.

1.3.2. Specific objectives

- To isolate and identify the fungus *A. apis* from Gaza apiaries.
- To prepare aquous crude extract of ten plants species.
- To evaluate the antifungal activity of ten plant extracts against *A.apis*.
- To evaluate the toxicity of pomegranate extract on honeybees (*A. mellifera* workers) those responsible for feeding the colony member.

Chapter 2

Literature Review

2.1. Honeybees (*Apis mellifera* L.)

Honeybees (*Apis millifera* L.) are social insects belong to the order Hymenoptera, family *Apidae* which is characterized by the presence of a pollen basket or corbicula on the hind tibia (Winston, 1987). *A. millifera* is the most widely distributed species of other bee species, that adapted to a large area of earth's surface (Free, 1980). This eusocial insect forms colonies of thousands of individuals. The colony builds of several parallel wax combs. Each cell in the comb is hexagonal and could be used for rearing of brood, or for food storage (Free, 1980). The members of the colony are differentiated into three physically distinct castes: queen, worker and drone (Needham, 2010). The colony has only one reproductive female called a queen. She could lay nearly 2000 eggs per day during the peak of egg production season (Hooper T., 1997). The second major function of queen is producing pheromones (Cllison, 2004).

Drones "males" lack the body parts that harvest nectar or pollen to feed themselves. They also lack stinger and wax glands. They are only designed for mating with the bee queen (Winston, 1987).

Honeybee workers, are represent the majority of the colony with clustering density in brood area (Hooper, 1997). They are sexually undeveloped females and under normal conditions do not lay eggs. They have specialized structures, such as brood food glands, scent glands, wax glands, pollen baskets, and a venomous stinger which allow them to perform all labors of the hive including; cleaning, brood rearing, queen tending, receiving nectar, handling pollen, comb building, ventilation, guard duty, foraging (Cllison, 2004; Winston, 1987). They generate the heat that keep the temperature of the colony up to the required level (Hooper, 1997).

During life cycle, bees go through four stage; egg, larva, pupa that collectively called a brood and the latter stage is adult. The eggs are parthenogenetic, (Hooper, 1997). The queen lays both fertile eggs and haploid eggs. While the fertile eggs will develop into females, worker or queens, the haploid ones will develop into males (Winston, 1987). Moreover, nutrition plays an important role in caste development. Thus, the female larvae in order to become workers, they receive less jelly compared to the queen larvae (Cllison, 2004). During larval development, it pass through four molts before the cell has been sealed with a cap of wax by workers (Hooper, 1997). The Worker, queen, and drone cells are capped when larvae are approximately 6, 5.5 and 6.5 days old respectively. And new workers, queens, and drones emerged in approximately 12, 7.5, and 14.5 days respectively after their capping (Cllison, 2004).

Many, practices of modern beekeeping like; feeding , swarm prevention, gathering weak colonies together, adding frames of bees or brood to increase population of the new hive, queen introducing, exchange equipments between hives, and using second hand frames and equipments in an apiary can increase horizontal transmission of pathogens between individual bees and colonies (Fries and Camazine, 2001). It is known that, stress factors like transportation of bee hives for long distances looking for good forging affect the physiological parameters of bees (Huang *et al*., 2010). Also, there is an evidence that using some chemicals in beekeeping are hazardous to bees, and suppress their immune system (Johnson, 2010).

2.2. Chalkbrood disease

2.2.1. Description

Chalkbrood is a world wide brood disease of honeybee larvae caused by the fungus *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir, where the infected larvae die and are mummified by the fungal mycelial growth (Hale and Menapace, 1980). It affects worker, queen and drone brood (Witte, 2003). The larvae and prepupae are the target stages of chalkbrood with older larvae being the most susceptible while, eggs and pupae do not support the growth of *A. apis* (Benecke, 2003).

Many species of *Ascosphaera* have been identified, and all of these are found only in association with bees, either as a pathogen or as a saprophyte on the pollen stores in nests (James and Skinner, 2005). *A.aggregata* causes chalkbrood in alfalfa leafcutter bee *(Megachile rotundat)* (Huntzinger *et al*., 2008), *A. torchioi* affect the larvae of orchard mason bee (*Osmia lignaria propinqua*), (Torchio, 1992), and *A. apis* is the causative agent of chalkbrood in honeybees (Puerta *et al*., 1999).

A. apis rarely kills a colony, but the loss of larvae leads to a reduction in the adult bee population and so the production of honey and pollen and pollination efficiency (Puerta *et al*., 1994). In New Zealand, from 5% to 37% reduction in honey production was recorded as a result of chalkbrood disease (Goodwin, 2002). In Egypt it is reduce the clover honey and chinus honey (Zaghloul *et al*., 2005).

2.2.2. Morphology of *Ascosphaera apis* fungi

Chalkbrood disease can be easily identified by its gross symptoms. An affected larva becomes overgrown by fluffy, cottonlike mycelia and swells to the size of the brood cell. It is a heterothallic so, it produce spores only when mycelia of opposite mating types came into contact (Spiltoir and Olive, 1955). So, when the + and – thalli are present in a larva, spore cysts can form, and the resulting mummies appear either mottled (black on white) or completely black (Gilliam *et al*., 1978; Shimanuki and Knox, 2000).

In culture, *A. apis* exists as a dense grayish-white mycelium containing aerial, surface, and subsurface hyphae. Surface hyphae are 4-8 μm in diameter, smooth walled, dichotomously branched, septet and vegetative nuclei are very small (Anderson *et al*., 1998; Davis and Ward, 2003; Spiltoir and Olive, 1955). Spore cysts, (the fruit bodies) about 60 μm in diameter which, contain many smaller round bodies known as spore balls (average 12 μm in diameter) which contain spores (average 2.9 x 1.4 μm) (Anderson *et al*., 1998; Hornitzky, 2001).

2.2.3. Taxonomy of *Ascosphaera apis* fungi

According to the catalogue of life (2012), the accepte name of this fungus is *Ascosphaera apis* (Maasen ex Claussen) L.S. Olive & Spiltoir 1955.

The synonyms of it are, *Pericystis apis* Maasen 1916, *Pericystis apis* Maasen ex Claussen 1921, and *Ascosphaera apis* var. *apis* (Maasen ex Claussen) L.S. Olive & Spiltoir 1955. (Catalogue of Life, 2012)

This is the current accepted taxonomy.

Kingdom : Fungi

Phylum : Ascomycota

Class : Eurotiomycetes

Order : Ascosphaerales

Family : Ascosphaeraceae

Genus : *Ascosphaera*

Species: *Ascosphaera apis* var. *apis* (Maasen ex Claussen) L.S. Olive & Spiltoir 1955. (Catalogue of Life, 2012)

2.2.4. Life cycle of *Ascosphaera apis*

The life cycle of *A. apis* is not clearly defined in nature (Moeller and Williams, 2006). It has two types of mycelia, which may called male and female, they lack any important morphological differences between the two mycelial types when maintained separately, and no sex organs are produced under these circumstances (Gilliam *et al*., 1978).

When, the culture is paired, the female mycelium produces ascogonia, while the male produces no sex organs. Ascogonium is comprised of a trichogyne, a nutriocyte and a stalk-like base, where the nutriocyte performs the function of nourishing the developing ascogenous system, the trichogyne fuses with the male hypha, and the receptive papillum appears on the latter at the point of contact (Spiltoir and Olive, 1955). The contact with the male hypha take only 15 to 20 minutes (Moeller and Williams, 2006).

Following plasmogamy, a cylindrical mass of protoplasm passes from the trichogyne into the nutriocyte where it develops a thin wall around itself and proliferates to produce an

ascogenous system of crosiers and asci. The asci aggregated into balls, each ascus apparently produceing 8 ascospores and asporball will formed. So, the ascogonium function not only as a trichogyne but also as the female gametangium (Spiltoir and Olive, 1955). The spores of *A. apis* require a nearly anaerobic environment for germination, but the mycelium requires an aerobic environment for growth (Anderson *et al*., 1997; Bailey, 1963). The optimum temperature for growth is about 30ºC, and spores germinate best at 35ºC (Gilliam *et al*., 1978).

2.2.5. General epidemiology

Chalkbrood disease has been recognized since the early 1900s and extensively studied over the years (Morse and Calderone, 2000)**.** However, to place control strategies in perspective it is also important to understand the epidemiology of the disease.

The disease, probably occurs in all the countries of the Mediterranean and it was considered to be a primarily European disease, but in 1971 it became recognized as economically important in the USA**.** It can be assumed that the disease is distributed worldwide (Hale and Menapace, 1980). It has been detected in Argentina, Japan, the Philippines, central America and Mexico (Hornitzky, 2001).

The disease is prevalent in the entire temperate zone, spreading apparently from southeastern Europe westward, and the massive outbreaks have occurred since 1990 in Hungary, where infection rates of 90-100% are common in certain apiaries and the infected bee colonies were not able to produce enough honey, and on many occasions beekeepers found empty hives (Robert *et al*., 2000).

In Argentina, the appearance and expansion process of chalkbrood in honeybees was similar to other countries. By the end of 1978, it was observed in different apiaries, and this fungal disease occurs widely in temperate regions (Hornitzky, 2001).

Its first report, in Australia was in 1993 from south-east Queensland and by 1995 had spread to other regions of the country (Hornitzky, 2001). The first reference to this disease in South Africa is in 1969 but it was not until 1999 that it was positively identified (Swart, 2003). It was regarded as “the most widespread infectious honeybee disease” in Thailand and has led to economic loss in apiculture (Chantawannakul *et al*., 2005).

Chalkbrood was first identified in Occupied Palestine in the summer of 1984, where, mummified larvae were found in four apiaries "three in central and one in northern" (Sanford, 1991). It became epidemic in the area, in 1989 when 20% of colonies suffered serious damage. All apiaries were infested by 1990. Possible causes of this problem include stresses caused by a new strain of the causative fungus (Jakobsons, 2005; Sanford, 1991).

2.2.6. Pathogenesis and transmission

The sexually produced *A. apis* spores (ascospores) are the primary source of brood infection . The infection occurred both through ingestion of *A. apis* or by the growth of the fungus through the cuticle (Gilliam *et al*., 1978). *A. apis* can infect brood of any caste (workers, drones, or queens), but larvae are most susceptible at 3–4 days of age (Bailey, 1981; Gilliam *et al*., 1978). Adult bees are not susceptible to this pathogen but, they can however, play an important role to transmit the disease within the beehives (Puerta *et al*., 1999). Transmission of infectious stage of the pathogen between adult bees within the colony occurred by food sharing and the spores can be carried by foraging bees and passed onto larvae by nurse bees feeding them with contaminated food (Puerta *et al*., 1999).

Spores of *A.* a*pis* may remain infective for more than 15 years (Aronstein and Murray, 2010; Gilliam, 1986). Viable spores may remain in stored honey, pollen, pollen capsules, used hive components, used beekeeping tools and equipment and possibly in soil around infected apiaries (Witte, 2003). Beekeeper may transmit the pathogen between managed colonies by using of contaminated materials and equipments (Fries and Camazine, 2001). Spores consumed by the honey bee larvae germinate in the lumen of the gut, Infected larvae rapidly reduce food consumption, and then stop eating altogether (Theantana and Chantawannakul, 2008). Recently identified several enzymes produced by *A. apis*, especially protease and β-N-acetylglucosaminidase, that help the pathogen in penetration of the peritrophic membrane of the bee larval midgut. After penetrating the gut wall, the fungal mycelium grows inside of the body cavity, eventually breaking out through the posterior end of the larva (Theantana and Chantawannakul, 2008). Death occurs as a result of mechanical and enzymatic damage, disruption of haemolymph circulation and general toxicoses (Glinski and Buczek, 2003). *A. apis* vegetative growth extends from the posterior end to the anterior end of the larva. Later,

the fungal growth is mottled with brown or black spots, due to production of ascomata (Gilliam *et al*., 1978). Dead mummies are removed from brood cells by worker bees and deposited outside the hive (Bailey, 1981). So, the spreading of spores may be done by wind, from mummies carried to the exterior (Moeller and Williams, 2006). The spore spreading between colonies also, can occur through drifting and from robbing (Witte, 2003). Common forage sites and water sources act as a reservoir for spores (Gilliam *et al*., 1978). The spread of the pathogen may be done by wild bee species, science *A.apis* has been detected in other bee species like leafcutter bees (Huntzinger *et al*., 2008).

2.2.7. Factors affecting the growth of *A. apis*

The development of *A. apis* infection seems to be mainly dependent upon the physiological and environmental conditions of the larvae.

2.2.7.1. Humidity

Relative humidity play an important role in the disease development. Most authors assuming prevalence of the disease in damp regions or during periods of high humidity inside the colony (Flores *et al*., 1996). Liang *et al.* (2000) reported that, the RH below 80% inhibited spore germination of *A. apis*. Also, Flores *et al*., (1996) in their experiment, found that the high relative humidity 87% which could reached in a colony during the nectar flow period, induces a higher percentage of pupal mortality when compared to the same temperature with lower RH..

2.2.7.2. Temperature

Germination of *A.apis* spores is independent on temperature within the range of 25-40 ºc and, the stage of germ tube production is correlated with optimum temperature range of 31-35 ºc (Liang *et al*., 2000). Honeybees, *Apis spp*., controlled the brood nest temperature precisely within a range of 33 – 36 °C with central brood area temperature 35°C ± 0.5°C during the pupal period and environmentally induced temperature changes within the hive are compensated by individual honeybee workers via endothermic heat production or evaporation (Groh *et al*., 2004; Starks *et al*., 2000). Additionally, Liang *et al*. (2000) showed that the

optimum temperature for *A. apis* growth is 30°C, so the fungus is a highly specialized pathogen to live within honeybee larvae.

Chilling of brood following capping of brood cell can cause significant increase in infection and outbreaks of chalkbrood may happen (Gilliam *et al*., 1978).

2.2.7.3. pH

The pH value within the range of 5-7.8 did not affect the spore germination significantly, but acidic environment, pH < 5 reduced the enlargement and germ-tube production drastically (Liang *et al*., 2000).

2.2.7.4. Stresses on the honey bees colony

The larvae of honeybees are more susceptible to chalkbrood infection when colony is under stress, include poor nutrition, lack of ventilation, watery food, disease, and genetic predisposition. Also, stress plays an important role in the disease severity (Witte, 2003).

Other diseases, pathogens and predators can severely decrease colony population (Ball, 1997). There is an increase in the incidence of chalkbrood infection in honeybees colonies infested with *V. jacobsoni* (Medina and Mejia, 1999). Since, the body surface of mites can become contaminated with fungal spores and spread them (Ball, 1997). Also, colonies that is infected with chalkbrood were to be common in *nosema apis*, as a secondary infection (Aydin *et al*., 2006; Jakobsons, 2005).

In Gaza strip, beekeepers face many of these stress factors especially in summer season. During this period, a shortage of blooming plants, enforce beekeepers to feed bees a watery food (sugar serb) which will elevate the moisture inside the hive and increase the incedence of the disease.Furthermore, Gaza Strip (which is a part of the Mediterranean coast) has the highest annual mean relative humidity, increasing from winter to summer. The annual average relative humidity fluctuates between 65 % in the daytime and 85 % at night in the summer, and between 60 % and 80 % respectively in winter (Hadid 2002).

2.2.8. Diagnosis of chalkbrood disease

Chalkbrood disease can be easily identified by its symptoms. The affected larva becomes overgrown by fluffy, cottonlike mycelia and swells to the size of the brood cell. The mummies remain whitish if they are infected with only one strain of the fungus but will turn grey or black when infected with both strains of the fungus (Hornitzky, 2001).

In case of sever infection, mummies can be found at the entrances or on the bottom boards of the hive (Shimanuki and Knox, 2000).

2.2.8.1 Microscopic diagnosis

This is achieved by mounting some diseased material, "mummies which have turned grey or black" on a microscope slide. The presence of spore cysts is usually sufficient to make a diagnosis. These spore cysts, which are about 60 μm in diameter, contain smaller round bodies known as spore balls (average 12 μm in diameter) (Hornitzky, 2001).

2.2.8.2. Cultural diagnosis

In the presence of white mummies only, a suspension of diseased material must be plated on the agar medium to detect reproductive structures (Johnson *et al*., 2005).

A. apis grows luxuriantly on Potato Dextrose Agar fortified (PDA) with 4 g yeast extract per liter (Shimanuki and Knox, 2000). Also, Sabarouds Dextrose Agar (SDA) culture media could be used for inoculation of the pathogen (Davis and Ward, 2003). For identification purposes, a heat treatment of the inoculum is performed before plating to kill non-spore forming microbes that are found in chalkbrood mummies (Johnson *et al*., 2005). Fungal growth is typically visible on plates in 2–3 days. After 4–6 days of incubation, black specks of ascomata will appear on the mycelial lawn. When, strains of different mating types are inoculated onto plates a slight distance apart, ascomata will appear as characteristic black lines where the mycelia intersect (Christensen and Gilliam, 1983).

2.2.8.3. Molecular diagnosis

Usually, honey bee colonies contaminated with *A. apis* have no visible signs of the disease. Also, bees and hive products sometimes must be certified free of *A.apis* during

trading. Therefore, it is useful to have early detection techniques, such as biochemical assays or molecular diagnostics that will allow detection of the pathogen at sub-clinical levels (Gilliam and Lorenz, 1993). Recently, different molecular methods have been developed for identification of *Ascosphaera spp.* using DNA-based level.

A polymerase Chain Reaction (PCR) has been used for detection and identification of *A. apis* especially for rapid detection and identification. This can by performed by using primers specific for *A. apis* (Hornitzky, 2001). The PCR can detect co-infections of multiple *Ascosphaera* species in a single host. This diagnostic methods eliminate the need for culturing samples, and could be used to process a large number of field collected bee larvae (James and Skinner, 2005).

2.2.9. Treatment and control of chalk brood disease

Despite the broad range of research that has been conducted to develop chalkbrood control strategies, no specific control strategy has been universally accepted or adapted to beekeepers (Fassbinder-Orth and Rinderer, 2005). So far, there is no registered chemical treatment for the disease (Hornitzky, 2001). Maintain strong colonies and requeen with queens from hygienic stocks reduce the severity of the disease (McRory *et al*., 2007).

Over the past two decades, reports of chalkbrood resistant bee strains have been made. Most of these reports have focused on the hygienic behavior as the cause of resistance to chalkbrood (Fassbinder-Orth and Rinderer, 2005).

To control the disease, there are a number of techniques that can be used to minimize the effects of chalkbrood and a range of chemicals both artificial and natural that are reported to have activity against *A.apis* (Davis and Ward, 2003; Hornitzky, 2001).

2.2.9.1 Management techniques

It is recommended to replace some combs in the brood chamber every year with new drawn comb. This practice will help to reduce the level of spores (McRory *et al*., 2007). Strengthening badly diseased colonies by adding young adult bees, hatching brood, by feeding sugar syrup, not allowing bees to winter in too large brood chamber. Increasing the colony entrance for ventilation in humid periods is recommended (Hornitzky, 2001). Using clean

equipment, and avoiding transfer of combs between colonies to prohibit spore spreading (Flores *et al*., 2005). Gamma radiation treatment at 10 KGray could be used to sterilize hive material, and as a routine sanitation treatment of beekeeping equipments (Baggio *et al*., 2005). Methyl bromide gas has also been used to disinfect contaminated equipment (Sanford, 1987), but chemical residue was detected in wood and wax of the bee hives (Hornitzky, 2001).

2.2.9.2. Chemicals control

Many studies were carried out on the control of *A. apis* and treatment *in vivo* and *in vitro* conditions using chemical fungicides, organic acids, phytochemical products, and biological control reagents.

Liu. (1991) found that the benomyl fungicide in culture at a concentration of 1µg/ml slower mycelia growth of *A. apis* colony but, sporocysts were observed. While at a concentration 5µg/ml of benomyl the mycelia grew and the sporocyst formation was stopped.

In a laboratory study in Egypt, the fungicides (Galben C 46%, Radomil gold pluse WP 42.5% and Daconil 2787) at a recommended rates did not exert any effect on the mycelical growth of the fungus. In the same study, mycostatin, was effective at the rates of 50.000 and 100.000 IU (Mourad *et al*., 2005).

Huntzinger *et al*. (2008) tested four fungicides in the laboratory for efficacy against hyphal growth of *A. aggregata* cultures, and were tested for their effects on incidence of chalkbrood disease. Benlate, and Rovral 50 WP reduced the incidence of chalkbrood with minimal mortality on larval bees also reduce hyphal growth. While, Orbit was effective in reducing hyphal growth, but it did not reduce incidence of chalkbrood and was toxic to bee larvae. The fourth fungicide, Captan was not effective in reducing hyphal growth or chalkbrood incidence, and it was toxic to bee larvae.

Many trials were held to control the pathogen by using organic acids and natural products to minimize the hive products contamination by fungicides and synthetic chemical .

In a study, trichloroisocyanuric acid in water placed in the hive controlled chalkbrood. They found that the effect, was dependent on the number of spores and the degree of aggregation of spores in spore balls (Hornitzky, 2001).

Jendrejak and Kopernicky (1998) evaluated the efficiency of 85% formic acid, against ascosphaeriose. They found that the treatment was 100% in 1994, and 87.9% in 1997.

2.2.9.3. Control by natural products

Gochnaur *et al*. (1979) showed that, citral and geraniol inhibited the fungus *A. apis*. The vapors of 5 µL of citral or 10 µL of geraniol per culture dish prevented vegetative growth. The daily applications of 30 µL of citral per dish killed sporulated cultures within 48 hr. In their study, the vapors of a geranic and nerolic acid mixture, 2-heptanone, isopentyl acetate, octanoic acid, and citronella and melissa oils were less inhibitory effect than citral or geraniol. While, potassium sorbate, sodium propionate and tetracycline had no inhibitory effect when placed on the culture medium.

Calderone *et al.* (1994) found that cinnamon oil completely inhibited the growth of *A. apis* at 100 ppm for 168 hr. while, bay oil, citronellal, clove oil, origanum oil and thymol inhibited all growth at 1,000 ppm for 168 hr. They found also that, camphor inhibited all growth at 10,000 ppm for 168 hr, and alpha-terpinene inhibited all growth for 72 hr at 10,000 ppm.

Dellacasa *et al.* (2003) evaluated the fungicidal effect of eight oils in the vegetative cycle of the *A. apis*. They demonstrate that the oils of *Tessaria absinthioides*, *Aloysia gratissima*, *Heterotheca latifolia*, *Lippia juneliana*, *L. integrifolia* and *L. turbinata* exhibited varying levels of fungicidal activity, while the oils of *Baccharis coridifolia* and *Eupatorium patens* did not possess any activity.

Davis and Ward (2003) mentioned that Nepalese lemon grass oil, *Eucalyptus citrodora* oil, *Leptospermum petersonii* oil and *L. scoparium* oil were the most active antifungal test agents and were presented with a Minimal Fungicidal Concentration 250 ppm against *A. apis*.

Using, the disks of filter paper impregnated with *Heterothalamus alienus* oil around colonies, Ruffinengo *et al*. (2006) found that, the inhibition of *A. apis* growth was significantly by 51% compared to the control in the first experiment and by 31% in the second experiment .

Abou El-Enain *et al.* (2009) showed that, cinnamon, cloves, rose, thyme oils and propolis were efficacious in inhibition growth of *A.apis in vitro.* While, fennel, ginger, henna, onion and worm-wood oils had a little inhibition against *A. apis in vitro*. In the same study, amalaki,

fenugreek, violet oils and fennel honey plants didn't show any inhibition against the growth of the pathogen.

Chantawannakul *et al*. (2005) demonstrated that, the aqueous extracts of *Eugenia caryophyllum*, *Illicium verum*, *Cinnamomum cassia*, *Acorus calamus* and piper betel gave inhibitory effect when tested with isolates of *A. apis*. They also reported that, *Cinnamomum cassia* and piper betel at concentration range 1.5-3.5% (w/v) showed the best inhibitory effects on the fungal growth.

Bailac *et al.* (2006) showed that the essential oils of the genus *Lippia* produced the highest inhibition halo compared to other plants. They also found that the MIC value were 250-300 ppm for clove oil, 25-50 ppm for cinnamon oil, 150 ppm for thyme, 300 ppm for anise and 250ppm for fennel essential oil.

Mourad *et al*. (2005) reported that, the essential oils of ceder, clove, peppermint, parsley, black cumin, garden rocket, and ricin showed a great success in managing the chalkbrood disease. They were also, reported that the ceder oil at a concentration 4% showed a 100% reduction in mummies numbers in apiary studies.

Beside using synthetic chemicals and natural products to control and treat the disease, there are many studies applied and show the efficiency of biological control agents to control *A. apis* as:

Al-Ghamdi *et al.* (2004) used three species of *Trichoderma spp* as antifungal agents against *A. apis* and other fungi that were isolated from honey bee combs. They observed that the *Trichoderma asperillium* had the maximum inhibition activity against mycelial growth of *A. apis* . Also, Sabaté *et al*. (2009) found that surfactin produced from *Bacillus subtilis* species inhibit *Paenibacillus larvae* bacteria, but it failed to affect *A. apis*.

2.3. Plants of the study

All plant specimens were photographed as well as collected in the field and later brought to the laboratory except *Artemisia herba-alb*.

Scientific names, common names, classification and description of the plant species which used in our study are mentioned as follow:

2.3.1. *Artemisia monosperma* Delile (Figure 2.1)

English common name: Sand wormwood

Arabic common name: Ader or Ather

Classification

Kingdom: Plantea

Subkingdom: Tracheobionta

Superdivision: Spermatophyte

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae

Genus: *Artemisia*

Species: *Artemisia monosperma* Delile

Source (http://flora.huji.ac.il/browse.asp?)

Figure 2.1.: A photograph of *A. monosperma*

Description

It is, ascending perennial shrublet, 50-100 cm high. **Leaves** linear-oblanceolate, solitary or clustered entire or with linear lobes 3-7 cm long, sessile tapering to base. **Heads** very short peduncled to sub sessile in somewhat, sided numerous racemes emerging laterally from the stem apices, together forming an elongated compound inflorescence up to 40 cm long. (Al-Soqeer, 2010; http://flora.huji.ac.il/browse.asp?).

Biological Activity

The main metabolites *A. monosperma* are sesquiterpene lactones, coumarins, acetylenes. The group of sesquiterpene lactones exhibit the highest antibacterial activity and anti-inflammatory properties (Teixeira da Silva, 2004). *Artemisia* species, have an antioxidant activity, and their contents especially, when extracted by n-butanol has a significant increase in the rat liver cytosolic superoxide dismutase and catalase activities (Kim *et al*., 2003). Many components with a characteristic flavor at a low threshold, such as trans - ethyl cinnamate which present 20.8% of artemisia oil could be suitable for using as antioxidant and flavoring agent in food industry (El-Massry *et al*., 2002).

A. monosperma, is a fragrant plant grows widely in the Arabian deserts. It is the most important dwarf shrub of the sand vegetation in the coastal sands, the Western Negev, and North Sinai (http://flora.huji.ac.il/browse.asp?; Hijazi and Salhab, 2010). It is important plant, when the plant is dominated, sand mobility stops by its fast grown branches and the sand become enriched by airborne dust and by humus (http://flora.huji.ac.il/browse.asp?).

Despite being very valuable in sand stabilization, it has chemical compounds such as alkaloids and flavonoides that have been recognized as allelopathic agents (Assaeed, 2003). The leaves of the plant are taken in folk medicine by certain women for abortion induction (Hijazi and Salhab, 2010). In a phytochemical study of *A. monosperma* by Stavri *et al*. (2005) it has three compounds were characterized and evaluated for their ability to inhibit 12-lipoxygenase, which act as an antibacterial substance. As antifungal agent, *A. monosperma* extracts reduce the severity of some plant pathogenic fungi disease but not prevent it in all tested pathogens (Assaeed, 2003).

2.3.2. *Artemisia absinthium* L. (Figure 2.2)

English common name: Absinthe wormwood

Arabic common name : Damsisa

Classification

Kingdom: Plantea

Subkingdom: Tracheobionta

Superdivision: Spermatophyte

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae

Genus: *Artemisia*

Species: *Artemisia absinthium* L.(NRCS., 2011)

Figure 2.2: A photograph of *A. absinthium*

Description

Absinth wormwood is a nonnative, long-lived, fragrant, perennial herb that grows each year from a woody base. An individual plant has 20 or more stems each growing 0.4-1.5 m tall. The **leaves** are spirally arranged, greenish grey above and white below, covered with silvery white trichomes, the basal leaves are up to 25 cm long, bipinnate to tripinnate with long petioles, uppermost leaves are less divided, smaller, and may be imple or sessile. The **flowers** are pale yallow, tubular, and clustered in spherical bent- down heads. The **fruit** is a cypsela without a pappus. The well-developed **root** system consists of a taproot with shallow lateral branches extending in all directions (Maw *et al*., 1985; Selleck and Coupland, 1961).

Biological Activity

A. absinthium is used in Palestinian folk medicine as an antiparasitic, Diuretic, Stomach pains and antispasmodic (Jaradat, 2005). Similar uses have also been reported in other countries (Valdés *et al*., 2008). *A. absinthium* extracts are a promising alternative to the commercially available anthelmintics for the treatment of gastric intestinal nematodes of sheep (Tariq *et al*., 2009).

Dulger *et al*. (1999) have found that this plant revealed antimicrobial activity, but it had no antifungal effect against yeasts used in their study. Moreover, Its ethanolic extraction showed some antiprotozoal activity against *Trypanosoma b. brucei* and none effect against *Plasmadium falciprum and trypanosom cruzi* (Valdés *et al*., 2008).

Lopes-Lutz *et al*. (2008) found that *A. absinthium* oil was characterized by high amounts of myrcene (10.8%), trans-thujone (10.1%) and trans-sabinyl acetate (26.4%). They also, found that its oil is the most active oil against *Staphylococcus* strains. Many studies were used worm-wood plant in the honeybee pathogens control, Abou El-Enain *et a*l. (2009) demonstrate that, the oils of worm-wood had a little inhibition against *A. apis in vitro*. While, Pohorecka (2004) found that *A. absinthium* significantly inhibited the development of the *Nosema apis* in naturally and artificially infected worker bees. An antimicrobial assays carried out by Fuselli *et al*. (2008) showed that the oils of *A. absinthium*, had the highest inhibitory capacities against the bacterial strains of *Paenibacillus larvae* **.**

2.3.3 *Artemisia herba-alba* **Asso** (Figure 2.3)

English common name : White wormwood

Arabic common name: Shehk

Classification

Kingdom: Plantea

Subkingdom: Tracheobionta

Superdivision: Spermatophyte

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae

Genus: *Artemisia*

Species: *Artemisia herba-alba* Asso

Source (NRCS., 2011)

Figure 2.3.: A photograph *of A. herba-alba* source (http://flora.huji.ac.il/browse.asp?)

Description

Greenish-silver perennial herb grows 20-40 cm in height, it is a chamaeophyte (the buds giving rise to new growth each year are borne close to the ground). The **stems** are rigid and erect. The grey **leaves** of sterile shoots are petiolate, ovate to orbicular in outline whereas leaves of flowering stems are much smaller. The **flowering** heads are sessile, oblong and tapering at base. The plants flower from September to December. Plants are oblong and tapering at base (Proksch, 2002).

Biological Activity

The genus *A. herba-alba* is a medicinal and aromatic plant that grows wild in arid areas of the Mediterranean basin, extending into northwestern Himalayas (Mohamed *et al*., 2010; Salido *et al*., 2004).

A. herba-alba is abounds over large areas in the Negav and Judean deserts. It is widely used by the local inhabitants, mainly for the treatment of gastric disturbances such as diarrhea, abdominal cramps and for healing external wounds (Feuerstein *et al*., 1986).

Its essential oil showed a weak antifungal activity against *Penicillium digitatum*, *Phytophthora citrophthora*, *Geotrichum citri-aurantii*, and *Potrytis cinerea* compared to other plants used in the same study (Bouchra *et al*., 2003). In addition, Tantaoui-Elaraki and Errifi (1994) found a synergistic action of its essential oil to the mycelium growth of *Zygorrhynchus* sp. and *Aspergillus niger* isolates, when associated with sodium chloride or fatty acid.

Mohamed *et al*. (2010) in their review mentioned that, the oil of *A. herba-alba* showed a very strong action against *Candida* and *Microsporum*. Also, the flower heads of *A. herba-alba* had an inhibitory effect on aflatoxin production of a toxigenic strain of *Aspergillus flavus* .

The antifungal activity of *A. herba-alba* was found to be associated with two major volatile compounds isolated from the fresh leaves of the plant. Carvone and piperitone purified compounds that had antifungal activity estimated to be 5 μg/ml and 2 μg/ml against *Penicillium citrinum*, and 7 μg/ml and 1.5 μg/ml against *Mucora rouxii*, respectively (Saleh *et al*., 2006).

2.3.4. *Callistemon viminalis* Sol. ex Gaertn (Figure 2.4)

English common name: Bottle brush

Arabic common name: Forshah

Classification

Kingdom: Plantea

Subkingdom: Tracheobionta

Superdivision: Spermatophyte

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Myrtales

Family: Myrtaceae

Genus: *Callistemon*

Species: *Callistemon viminalis* Sol. ex Gaertn

Source (NRCS., 2011)

Figure 2.4.: A photograph of *C. viminalis*

Description

Shrub or small tree to 8 m high, bark dark, furrowed. **Branches** generally pendent. **Leaves** linear to narrow-elliptic with one side straighter than the other, 3–7 cm long, 3–7 mm wide, apex acute, lateral veins obscure. **Spikes** 4–10 cm long, 30–60 mm diameter, filaments fused into a ring at the base and shed as a unit, bright red. **Capsules** 5–6 mm in diameter, cup-shaped with orifice wide. Flowering during spring to early summer, also sporadically throughout year (Spencer and Lumley, 1991).

Biological Activity

Callistemon species are used for forestry, essential oil production, farm windbreak plantings, degraded-land reclamation, and ornamental horticulture among other applications (Spencer and Lumley, 1991). *C. viminalis* is native to New South Wales, Australia (Maiden, 1999). In Palestine, we can found it be grown as garden, street trees or ornamental plants for their decorative flowers.

In traditional Chinese Medicine, *Callistemon* species, especially *C. viminalis*, are used for the treatment of hemorrhoids (Islam *et al*., 2010).
Many phytochemical compounds from this genus were identified like C-methyl flavonoids, triterpenoids and phloroglucinol derivatives (Wollenweber *et al*., 2000). Besides, some medicinal properties like antimicrobial, anti-staphylococcal, antithrombin, repellent and nematicidal activities as well as larvicidal and pupicidal values have been reported for the genus (Islam *et al*., 2010; Sangwan *et al*., 1990; Saxena and Gomber, 2006).

The ethanolic extraction of *C. citrinus*, were the most active extract against rice seed borne fungus under laboratory and in irrigated crop system, also it increase the emergence and farm production of some rice varieties (Nguefack *et al*., 2007). The ethanolic, aqueous and methanolic crude extracts from the leaves of *C. viminalis* showed antibacterial and antifungal activity against both the Gram-positive (*Bacillus cereus, Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus*, and *Streptococcus pyogenes*), Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella enteritidis,* and *Shigella sonnei*) and all the three crude extracts were effective at inhibiting growth of the fungus *Candida albicans* (Delahaye *et al*., 2009). The major components of *C. viminalis* leaves oil are, 1,8-Cineole which presents (61.7%), *α*-pinene (24.2%) and menthyl acetate (5.3%) (Srivastava *et al*., 2003).

2.3.5. *Punica granatum* L. (Figure 2.5)

English common name : Pomegranate

Arabic common name: Rom'man

Classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Myrtales

Family: Punicaceae

Genus: *Punica*

Species: *Punica granatum* L.

Source (NRCS., 2011)

Figure 2.5.: A photograph of *P. granatum*

Biological Activity

The pomegranate, is an ancient, mystical, and highly distinctive fruit. It is the predominant member of Punicaceae family. It is native from the Himalayas in northern India to Iran but, it has been cultivated and naturalized since ancient times over the Mediterranean region. It is also found in India and more arid regions of Southeast Asia, the East Indies, and tropical Africa (Jurenka, 2008).

P. granatum therapeutic properties are wide-ranging and include treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, arthritis, and obesity (Jurenka,

2008). In Iran it is considered as an important medicinal plant where its flowers are used as astringent, hemostatic, antibacterial, antifungal, antiviral and as a remedy for cut wound, bronchitis, diarrhea, digestive problems, dermal infected wounds and diabetes (Pirbalouti *et al*., 2010).

Some studies have demonstrated that various pomegranate extracts (juice, seed oil, peel) potently inhibit prostate cancer, and inhibit tumor growth (Albrecht *et al*., 2004). In a study that *P*. *granatum* flower extracted in ethanol was capable of promoting wound healing activity in Winstar Rats (Pirbalouti *et al*., 2010).

As anti microbial agent, the aqueous decoction of pomegranate was showing the highest inhibition of *Candida albicans* when compared with the other extracts of the study (Pai *et al*., 2010). Moreover, McCarrell *et al*. (2008) found that the combinations of pomegranate rind extracts with Cu(II) ions enhanced antimicrobial effects against *E. coli*, *Ps. aeruginosa* and *P. mirabilis* and moderate activity is observed against *S. aureus* .

The ethanol pomegranate extracts showed a high antimicrobial activity against *Bacillus licheniformis* (Phattayakorn and Wanchaitanawong, 2009). Also, its gel controlled the bacteria and yeasts responsible for oral infections such as caries, periodontal disease and stomatitis (Souza *et al*., 2006). The antifungal activity of Its aqueous extracts has a significant antifungal activity against *Aspergillus* species (Satish *et al*., 2007).

2.3.6. *Tagetes patula* L. (Figure 2.6)

English ommon name: *French marigold*

Arabic common name: Katefa

Classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae

Genus: *Tagetes*

Species:*Tagetes patula* L.

Source (NRCS., 2011)

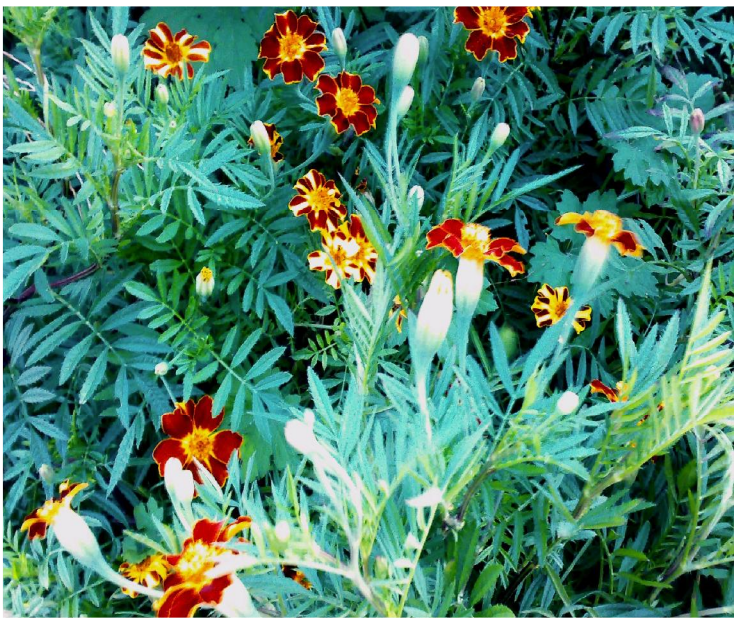


Figure 2.6.: A photograph of *T. patula* L

Description

Annual plant, upright, 30-90cm hight. The **leaves** are green, oblong in shape and pinnately compound, with dentate margin, arranged in opposite or sub opposite position and the blade length is less than 5 cm. The **flower**, is a tubular flower head with, showy, orange, yellow or bicolor. The **fruit** is a black achene (Gilman, 1999).

Biological Activity

T. patula, grows in gardens for ornaments and is used for the preparation of high grade perfumes in France (Chadha, 1976). The carotenoid pigments from *Tagetes* are useful in food coloring (Vasudevan *et al*., 1997). Due to its antimicrobial activities, the plant is used to treat several dermatological disorders (Vasudevan *et al*., 1997).

Bioactive extracts of different *Tagetes* parts exhibit nematocidal, fungicidal and insecticidal activity, and the nematocidal activity of roots is attributed to thienyls, while the biocidal components of the essential oil from flowers and leaves are terpenoids (Vasudevan *et al*., 1997).

González and Marioli (2010) in their study found that the decoctions, the essential oils and the waters remaining after hydro-distillation of *T. patula* showed biological activity as growth inhibitor against all the *Paenibacillus larvae* strains.

2.3.7. *Cyperus rotundus* L. (Figure 2.7)

English common name: Purple nut sedge

Arabic common name: Alci'ed

Classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Liliopsida

Subclass: Commenlinidae

Order: Cyperales

Family: Cyperaceae

Genus: *Cyperus* L.

Species:*Cyperus rotundus* L.

Source (NRCS., 2011)

Figure 2.7.: A photograph of *C. rotundus* L

Description

Perennial weed with slender, scaly creeping rhizomes, bulbous at the base and arising singly from the tubers which are about 1-3 cm long. **The stems** grow to about 25 cm tall and the **leaves** are linear, dark green and grooved on the upper surface. **Inflorescences** are small, with 2-4 bracts, consisting of tiny flowers with a red-brown husk. **The tubers** are externally blackish in colour and reddish white inside, with a characteristic odour (Lawal and Oyed, 2009).

Biological Activity

Purple nut sedge grows naturally in tropical, subtropical and temperate regions, and it is widely distributed in the Mediterranean basin areas (Kilani *et al*., 2005). This plant, is a major problem in cultivated lands in almost every warm region of the world (Urgisd,1969). Also, it is widespread in Palestine (Abou Auda, 2010).

The root powder of *C. rotundus* is used as cosmetics to reduce acne, and for washing hairs to remove dandruff in India (Patil and Biradar, 2011). There are a number of pharmacological and biological activities including, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antioxidant, antimalarial, anti-inflammatory, antipyretic and analgesic activities have been reported for this plant (Raut and Gaikward, 2006).

The phytochemical investigation of *C. rotundus* rhizome have revealed the presence of polyphenol, flavonol glycoside, alkaloid, saponins, sesquiterpenoids and essential oil (Nagulendran *et al*., 2007). Where, the essential oil from its tubers was characterized by a high content of sesquiterpenes and cyperene (30.9%) being the major (Kilani *et al*., 2005).
The antibacterial activity of its tubers oil, showed more important activity against Gram-positive bacteria specially *Staphylococcus aureus* than Gram-negative bacteria (Kilani *et al*., 2005). *C. rotundus* inhibits cariogenic properties of *Streptococcus mutans* (Yu *et al*., 2007). Also, the rhizomal ethanolic extract has oxidative activities *in vitro* (Nagulendran *et al*., 2007).

In many studies on the chemical composition of essential oils of *C. rotundus* from around the world, α-cyperone, cyperene, cyperotundone and β-selinene were the major compounds identified in higher concentrations, along with other constituents such as, α-copaene, valerenal, caryophyllene oxide, patchoulenyl acetate and sugeonyl acetate (Lawal and Oyed, 2009).

2.3.8. *Cinnamomum zeylanicum* Garcin ex Blume (Figure 2.8)

English common name: Cinnamon

Arabic common name: Kerfa

Classifications

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Magnoliidae

Order: Laurales

Family: Lauraceae

Genus: *Cinnamomum*

Species: *Cinnamomum zeylanium* Garcin ex Blume

Source (NRCS., 2011)

Figure 2.8.: A photograph of *C. zeylanicum*

Description

evergreen small **trees**, up to 10 m tall. **Bark** black-brown, inner bark with cinnamic aldehyde flavor. Young **branchlets** gray, somewhat tetragonous, white-maculate. **Buds** sericeous-puberulent. **Leaves** usually opposite; petiole ca. 2 cm, glabrous; leaf blade greenish white abaxially, green and shiny adaxially, ovate or ovate-lanceolate, 11-16 x 4.5-5.5 cm, leathery or subleathery, glabrous on both surfaces, triplinerved, midrib and lateral veins elevated on both surfaces, transverse veins and veinlets reticulate, conspicuously foveolate abaxially, base acute,

margin entire, apex acuminate. **Panicle** axillary or terminal, 10-12 cm; peduncle and rachis sericeous-puberulent. **Flowers** yellow, ca. 6 mm. Perianth tube obconical; perianth lobes 6, oblong, subequal, gray puberulent outside. Fertile stamens 9; filaments hairy near base, those of 3rd whorl each with 2 glands, others glandless; anthers 4-celled; cells of 1st and 2nd whorls introrse but those of 3rd whorl extrorse. Ovary ovoid, 10-15 mm, glabrous; style short; stigma discoid. **Fruit** ovoid, 10-15 mm, black when mature; perianth cup in fruit cupuliform, dilated, dentate, teeth truncate or acute at apex (Flora of China online).

Biological Activity

The genus *Cinnmomum* consist of 250 species of trees and shrubs distributed in south east Asia, China, and Australia. *Cinnamomum zeylanicum*, true Cinnamon, is a native plant of Sri Lanka and South India, where Siri Lanka is the major cinnamon producing country in the world (Leela, 2008). Cinnamon has traditionally used to treat toothache and fight bad breath and its regular use is believed to used to alleviate indigestion, stomach cramps, intestinal spasms, nausea, and flatulence, and to improve the appetite, and treat diarrhea (Debjit *et al*., 2011).

Its bark is used as foods and drinks flavour, as a component of perfumes and in many pharmaceutical preparations. *C. zeylanicum*, provides various types of oils depending on the used part of the plant. The major component of the leaf oil being ugenol (about 70% of the total volatiles), but cinnamaldehde is the major component of stem bark (Senanayake *et al*., 1978). Its dried bark contains 59.5% carbohydrates , 20.3% fibers, 9.9% moisture, 4.6% protein 2.2% fate, 3.5% total ash and other minerals and vitamins. It was founded that, the chemical composition is varies depending on the geographical origin of the species (Leela, 2008). Cinnamon, has been reported to have remarkable pharmacological effects and many modern medical studies have been carried out on cinnamon. Quale *et al*. (1996) reported that, in *in vitro* study, *C. zeylanicum* has activity against fluconazole resistant and susceptible *Candida*, and in a clinical trials, three of five patients had improvement of their oral candidiasis. Bailac *et al*. (2006) showed that, cinnamon oil presented a good antimicrobial activity against strains of *Paenibacillus larvae* the causative agent of American foulbrood disease in honeybee, and against *A. apis*. In another study, Cinnamon oil has potential to

control American Foulbrood, where, the treated honeybee hives with cinnamon oil showed a lesser incidence of infected larvae than the control group (Gende *et al*., 2009).

In a study, the total phenolics content of dried cinnamon's fruits was found to be the highest in water extract compared to other extraction solvents, and the study indicated that the water extract is a good source of antioxidant and antimutagenic (Jayaprakasha *et al*., 2006).

2.3.9. *Annona squamosa* L.(Figure 2.9.)

English common name : Sugar apple

Arabic common name: Kishta

Classifications

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Magnoliidae

Order: Magnoliales

Family: Annonaceae

Genus: *Annona* L.

Species: *Annona squamosa* L.

Source (NRCS., 2011)

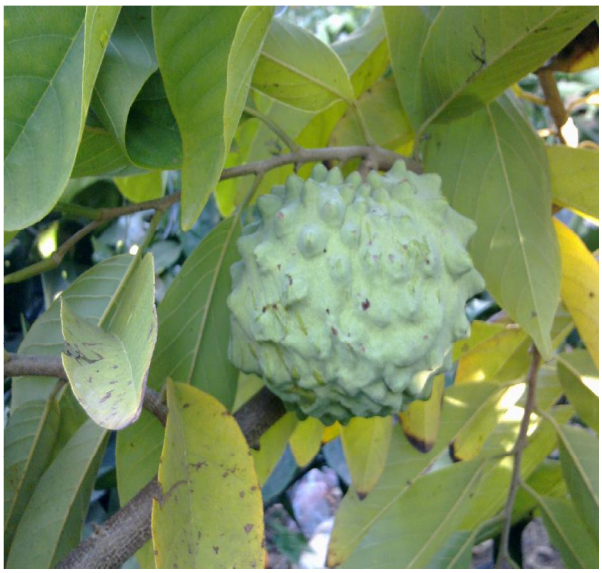


Figure 2.9.: A photograph of *A. squamosa*

Description

A shrub cultivated for ornament, with sympdial growth. **leaves** are alternate, ellipitic , chartaceous with acute apex and base, the margins are entire and the **petiole** is inflated. The

flower is chasmogamous with absent epicalyx with three fleshy green sepals and three light yellow lanceolate petals. The **fruit** is yellowish green, syncarpous, oval in shape and rough skin. It is atropical fruit plant (Folorunso and Olorode, 2006).

Biological Activity

The *genus* has approximatlely 119 speecies of which seven in addition to a hybrid one are grown for domestic and commercial use (Folorunso and Olorode, 2006). The plant has many traditionally uses. It is used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, haemorrhage, antibacterial infection, dysuria, fever, and ulcer (Soni *et al*., 2011). Its leaves extract has an antihyperglycaemic effect in induced diabetic rats (Kaleem *et al*., 2008).

The composition of leaves extract of *A. squamosa* in different solvents contains alkaloids, flavonoids, glycosides, phenols, saponins, carbohydrates, sterols and tannins (Soni *et al*., 2011). Also, it contain a group of long-chain fatty acid derivatives called, Annonaceous acetogenins, which has interesting and potent biological activities, including cytotoxic, *in vivo* antitumor, antimalarial, parasiticidal, and pesticidal effects (Johnson *et al*., 1999). In other study, the seed extract showed a remarkable antimicrobial and cytotoxic activities (Rahman *et al*., 2005). A hydroxy ketones "11-hydroxy-16-hentriacontanone" isolated from the leaf cuticular wax of *A. squamosa* was reported for its antifungal potential (Shanker *et al*., 2007). While, Frias and Kozusny- Andreanid (2009) in their *in vitro* evaluation of the antifungal activity of plant extracts, the *A. squamosa* show any effect against the dermatophyte, *Trichophyton mentagrophytes*.

2.3.10. *Psidium guajava* L. (Figure 2.10.)

English common name : Apple guava

Arabic common name: Guava

Classifications

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Myrtales

Family: Myrtaceae

Genus: *Psidium* L.

Species:*Psidium guajava* L.

Source (NRCS., 2011)

Figure 2.10.: A phototograph of *P. guajava*

Description

Evergreen shrub or small tree to 9 m tall, with scaly greenish-brown bark and young branches. **Leaves** opposite, simple, short stalked, entire, oval to oblong-elliptic, to 15 cm long. **Flowers** white, fragrant, to 4 cm wide, borne singly or a few together at leaf axils, many stamens. **Fruit** an oval or pear-shaped berry, 3-10 cm long, yellow at maturity, with yellow or dark pink flesh, seeds numerous (Ellshoff *et al*., 1995).

Biological Activity

P. guajava is native to tropical America, probably from southern Mexico south to South America, but its distribution greatly extended through cultivation areas (Ellshoff *et al*., 1995).

P. guajava leaf has a long history of folk medicinal worldwide. It is used as a cough sedative, an anti-diarrheic, in the management of hypertension, obesity and in the control of diabetes mellitus (Metwally *et al*., 2010). The aqueous leaf extract has analgestic and anti inflammatory properties (Ojewole, 2006). Also, it has a good hepatoprotective activity (Roy *et al*., 2006).

The chemical constituents of guava is differ according to the used part. Chen *et al*., (2006) identified its major constituents like, α-pinene, 1,8-cineole, β-caryophllene, nerolidol, globulol, C6 aldehdes, C6 alcohols, ethyle hexanoate, (Z)-3-hexenyl acetate. While, Joseph and Priya (2011) reported that, its leaves contain fixed oil and volatile oil and, the essential oil is rich in cineol, tannins, triterpenes, flovanoids, resin, eugenol, mallic acid.

The guava essential oil has many pharmaceutical effects, some of antimicrobial activity has been done by different researchers; Joseph and Priya (2011) reported the essential oil from the leaves of guava exhibited inhibitory effect against *Bacillus cereus, Enterobacter aerogenes* and *Pseudomonas fluorescens*. Additionally, Sacchetti *et al*. (2005) reported that the oil showed a strong resistance against the pathogenic yeast *Yarrowia lipolytica*. Gnan and Demello (1999) reported a complete inhibition of growth of *S. aureus, S. epidermidis* and *S. typhimurium* caused by aqueous guava leaf extract. And, Nair and Chanda (2007) reported the antibacterial activity of *P.guajava* extract was more clear against gram-positive bacterial and fungal strains and a moderate activity was shown against the gram-negative bacterial strains that were studied.

Chapter 3

Materials and methods

3.1 Materials

3.1.1 Equipments

#	Item	Manufacturer
1.	Autoclave	N-Bioteck- Korea
2.	Digital balance 0.0001g	AE-Adam- UK
3.	Micropipette 20-200 µL	Jencons Scientific- USA
4.	Laminar flow cabinet	N-Bioteck- Korea
5.	Refrigerator	J.P.Selecta- Spain
6.	Biological incubator	N-Bioteck- Korea
7.	Compound microscope	LW-Scientific- USA
8.	Water bath	N-Bioteck- Korea
9.	Hotplate with stirrer	Heidolph- Germany
10.	Centrifuge 6000rpm	LW-Scientific- USA
11.	Digital camera Omni	LW-Scientific- USA
12.	Neubauer counting chamber	Boeco- Germany
13.	Condenser	

3.1.2 Chemicals, regents and disposables

	Item	Manufacturer
1.	Potato dextrose agar	Liofilchem- Italy
2.	Yeast extract	Himedia- India
3.	Lactophenol blue stain	Himedia- India
4.	Commercial sucrose	Local market
5.	Ethanol alcohol	Furtaram- Occupied Palestine
6.	Tween 80	Himedia- India
7.	Petri dish plates 90mm	Miniplast- Occupied Palestine
8.	Plastic box Sterile plastic cups	Meheco Corp- China
9.	Inculcating needle	Himedia- India
10.	Microscopic slide	China
11.	Polyethylene bags	Local market
12.	Cheesecloth gauze	ZMS- Gaza
13.	Centrifuge tubes	Himedia- India
14.	0.45 µm filterpaper	PALL-USA
15.	Parafilm tape	Pechiny Plastic- USA
16.	Honeybees worker rearing cage	
17.	Honeybee hive	
18.	Glass ware	

3.2 Methods

3.2.1. Culture medium

Potato Dextrose Agar enriched with 0.4% yeast extract (PDA-0.4 YE) was used as fungal growth medium during this study. The medium was sterilized in autoclave at 15 psi and 121 ºC for 20 minutes and then left to cool to 50ºC in water bath in order to maintain the agar in a molten state. While molten, the medium was inoculated with 100 iu/ml of penicillin G and 100 µg/ml of streptomycin to inhibit the growth of both Gram positive and Gram negative bacteria.The media were poured in Petri dish plates (90 × 15 mm) inside the laminar flow cabinet, previously sterilized for 30 minutes by ultraviolet light. After solidification of the culture medium, the plates were placed in plastic box to prevent drying out and stored in refrigerator at 4ºC until use (Davis and ward, 2003).

3.2.2. Fungus isolation and cultivation techniques

Larvae showing characteristic symptoms of chalkbrood disease (white mummified larvae) were collected from naturally infected hives from a local apiary at Beit Lahia city, Northern Governorate, Palestine. This was done for isolation and identification of *A. apis* to be used throughout the study. The mummies were carefully collected in sterile plastic cups and transported to biology department laboratories where they stored at 4ºC until cultivation.

The isolation and cultivation technique followed here was previously described by Davis *et al. (2003*). Mummified larvae were dabbed onto the agar surface at various points around the PDA-0.4 YE plate. The plates were incubated at 30^{o}C in a biological incubator and monitored daily for emergence of culture.

When colonies appeared, those with white to pale buff color, and have matted mycelia were picked out from the surface of the plates and transferred to fresh media. These colonies were sub-cultured to purity and stored in PDA-0.4 YE media at 30 °C.

3.2.2.1 Fungal identification

Purified cultures were examined macroscopically and microscopically in order to identify the strains on the basis of their morphological traits, mycelium growth, colony texture, ascospore production and other growth characteristics of the fungi.

3.2.2.2. Microscopic examination

Microscopic examination for the purpose of identification was carried out by transferring a portion of the mycelium to clean microscopic glass slides. For better analysis of morphological characteristics, the slides were stained with Lactophenol blue solution (0.05g Aniline blue, 25g phenol, 25ml Lactic acid, 50ml Glycerol) to enhance contrast. The prepared slides were examined microscopically at 40× magnification (Anderson D.L *et al*., 1997; Chorbiński and Rypuła, 2003), for the presence of ascospore which is characteristic feature of *A. apis*

3.2.2.3. Cross mating experiments

- **Principle**

A cross mating test is an *in vitro* experiment employed to recognize fungal species based on the biological species concept. In biological species recognition, groups of mating compatible individuals are regarded as the same species (Taylor *et al*., 2000).

Since, *A. apis* belongs to the heterothallic Ascomycota, and their spores are readily produced when two strains of each mating type are plated together (Aronstein and Murray, 2010; Gilliam, 1986; Moeller and Williams, 2006).

- **Method**

Two mycelia from two different isolates were aseptically transferred to fresh PDA+0.4% Y plates. They were placed opposite to each other at approximately 1 cm from the edge of the Petri plate. Plates were incubated at 30ºC. After 8 days, the plates were examined for the presence of ascomata. Successful crosses result in a ridge of mature ascomata containing asci

with ascospores being formed at the line of contact. Ascomata are removed using fine forceps and mounted in a drop of sterilized water and crushed under a cover slip.

3.3. Plant material

The Plants investigated in this study are listed in Table 3.1. They were selected according to their antimicrobial properties reported in the literature (Dulger *et al.* 1999; Assaeed, 2003; Delahaye *et al*., 2009; Pirbalouti *et al*., 2010; González and Marioli, 2010;). Ten different plant species belong to different plant families were freshly collected or purchased dried (e.g. White wormwood and Cinnamon) at local market (Al-Zawya market, Gaza city). The plants were identified according to the criteria stipulated by international committee for botanical nomenclature; Natural Resources Conservation Services, United State, Department of Agriculture.

3.3.1. Plant material preparation

The selected parts of different plants as shown in table 3.1 were shade dried at room temperature (20-25ºC) for 7 days. Dried materials were then ground into powder by hand and packaged into polyethylene bags until used.

Table 3. 1: Checklist of the plant species utilized to detect for antifungal activities.

Scientific name		English Common name	Family	Habit	Used parts
1-	*Artemisia monosperma*	Sand wormwood	Asteraceae	Shrublet	Leaves and buds
2-	*Artemisia absinthium*	Absinth wormwood	Asteraceae	Herb	Whole plant
3-	*Artemisia herba-alba*	White Sage	Asteraceae	Herb	whole plant
4-	*Callistemon viminalis*	Bottlebrushes	Myrtaceae	Tree	Leaves
5-	*Punica granatum*	Pomegranate	Punicaceae	Tree	Fallen flowers
6-	*Tagetes patula*	French Marigold	Asteraceae	Herb	whole plant
7-	*Cyperus rotundus*	Purple nut sedge	Cyperaceae	Herb	Tubers
8-	*Cinnamomum zeylanium*	Cinnamon	Lauraceae	Tree	Inner bark
9-	*Annona squamosa*	Sugar apple	Annonaceae	Tree	Leaves
10-	*Psidium guajava*	Guava	Myrtaceae	Tree	Leaves

3.3.2. Preparation of plant extracts

Plant powders (20 g) were extracted by soaking overnight in 80 ml distilled water i.e. 20% (W/V). The mixture was boiled for thirty minutes using a reflux distillatory system and filtered through a piece of cheesecloth gauze. The filtrates were centrifuged at 3000 rpm for 5 min to remove plant debris. The supernatants were sterilized by filtration through sterilized, disposable filter unit with a pore size of 0.45µm into sterile cups. All extracts were preserved

at 4ºC for further investigation. This preparation is designated as 100% crude plant extract and used to examine their effects on *A. apis*.

3.4. Determination of the Minimum Inhibitory Concentration (MIC)

- **Principle**

Crude extracts of the different plants were screened in order to test their antifungal activity, using the Minimum Inhibitory Concentration (MIC) method on PDA+0.4 y media as described by Liu *et* al. (1991).

It was determined by mixing the different plant extracts with agar to attain different concentrations of the extracts and spreading spore suspension on the surface. The lowest concentration of plant extract that inhibit visible growth of fungi on the agar plate after 8 days of incubation was considered as the minimal inhibitory concentration (Davis and Ward, 2003; Hornitzky, 2001).

- **Method**

3.4.1. Preparation of spore suspension

Fifteen and twenty days before the experiment, the fungal pathogen *A. apis* was cultivated on PDA+0.4 y media plates in order to produce viable spores in sufficient numbers to allow a range of tests to be conducted on spores of the same age and life history. Spore suspension was obtained by washing the ascospores that formed on the surface of plates with 5-10ml of 0.01% sterile Tween 80. The suspension was collected in a sterile 100ml Erlenmeyer flask and loosened by shaking with sterile glass beads for 2 hr.

The density of spore suspension was adjusted with 0.01% sterile Tween 80 to correspond to a final concentration of approximately 50×10^6 spore/mL. The number of spores was quantified using a Neubauer counting chamber and a compound microscope (400×).

3.4.2. Preparation of plant extracts media

Plant extracts were separately incorporated into molten PDA+0.4 Y media to obtain final concentrations of 0.625, 1.25, 2.5, 5, 10 and 20%. The work was carried out while the agar is molten "soft" and held in water bath preadjusted at 48^{o}C in order to avoid agar solidification.

The media was moderately agitated to ensure even distribution of the extracts through the agar material and then immediately poured onto plates in equal quantities.

3. 4. 3. Inoculation and incubation of plates

After solidification, equal volumes (100 μL) of spore suspension containing approximately 5×10^{5} spores of *A. apis* was inoculated at the surface of agar plates. The suspension was spread evenly over the surfaces of the agar using sterile, L-shape glass rod. All plates were incubated at 30^{o}C for 8 days.

Plates were visually inspected for the presence of growth of *A. apis* colonies. The lowest concentration at which no visible growth of *A. apis* was observed is considered as the MIC of that extract. Control plates free of any extract were also prepared. All assay experiments were held twice with quadruple.

3.5. Evaluation of antifungal activity of plant extracts by radial growth method

In the second series of experiments, the antifungal activity of plant extracts was evaluated by the radial growth method. This technique is based on following up the mycelial growth of fungi, placed in the center of the Petri dishes containing culture medium supplemented with different concentrations of plant extract (Grover and Moore, 1962).

- **Method**

Three different concentrations of plant extracts of 5, 10, 20%, (V/V) were prepared in molten PDA+0.4 Y media which had been held in a water bath at 48^{o}C (Joseph *et al*., 2008), as described above.

The media thus supplemented were poured in Petri dishes of 90 mm at a rate of 15 ml per dish. After solidification, wells of 5 mm diameter were made in the center of agar medium with a sterile glass pipette. The control samples were PDA+0.4 Y medium without plant extract. Two perpendicular lines passing by the center of the dish and intersecting directly above the center of the hollow were drawn on the cover of the Petri dish.

3.5.1. Preparation of the inoculum

Stock cultures of *A. apis* were prepared 4 days before the start of the experiment on PDA+0.4 Y at 30°C. Five mm diameter agar discs covered with the fungus mycelium were cut out from the stock plates and used as inocula for the bioassay. The discs were cut out with a Pasteur pipette from places equally distant from the centre of the stock culture. The aim of these procedures was to obtain agar discs covered with fungus microculture containing elements of mycelium comparable with regard to their quality and quantity. The discs were aseptically transferred by the tip of a pasture pipette and placed in the central well of the dish. Plates were sealed with parafilm and incubated in a dark at 30°C.

The radial mycelial growth for all treatments and control were measured by averaging the length of the four radii (mm) of the mycelium ring from the center of the inoculum disc to the outer growing edge of the mycelia along the two perpendicular lines that had been previously drawn on the cover of each plate. Measurements were determined every 24 hr for 4 days.

The percentage of inhibition of fungi growth was also calculated with respect to the control using the following formula (Nwachukwu and Umechuruba 2001)

$$\text{Inhibition Percentage (\%)} = \frac{rc - rt}{rc} \times 100$$

Where;

rc = Average radius of the fungi grown in the control (mm).

rt = Average radius of the fungi grown in the extract (mm)

There were four replicates for each concentration and seven replicates for the control. Plants showing best antifungal activity against *A. apis* were chosen for further analysis.

3.5.2. Statistical Analysis and calculations

Antifungal activities of plant extracts, expressed in terms of radial growth using descriptive statistics, the mean and standard deviation of radial growth of the fungus.

The effect of the different concentrations of plant extracts on fungal growth was analyzed by one-way analysis of variance (one-way ANOVA) and the significance of the differences between means was determined by using the Tukey honest significant difference (HSD) test at 5% level. Statistical analyses of the data were performed with SPSS 13.0 statistical software for Windows.

3.6. Assessment the nature of antifungal effect of *P. granatum* extracts

The aim of this test was to assess the nature of antifungall effect of *P. granatum* extract on the growth of *A. apis*. *P. granatum* was selected for this analysis because it showed the lowest MIC and the highest percentage of inhibition against *A. apis*.

- **Methods**

The test was performed as follows: *A. apis* was first cultivated on PDA+0.4 Y media inoculated with *P. granatum* extract to final concentrations of 5, 10, and 20%. Later, 5 mm diameter, agar discs, colonized by the fungus *A. apis*, were cut from the three treatments and transferred into fresh PDA+0.4 Y media plates free from the extract.

The plates were incubated at 30°C and the radial mycelium growth was monitored and measured every day for 5 days. Mycelium disks obtained from 5-days old, cultures of *A. apis* were served as inocula in the control plates. The antifungal effect of the of the three different concentrations of *P. granatum* extract over time were assessed by plotting growth profile curves of *A. apis* and compared with the normal growth profile of the control. Thus, any abnormal growth of mycelium during the incubation period can be determined.

3.7. Evaluation of the toxicity of *P. granatum* extract on *A. mellifera*

Since worker bees are responsible upon feeding and delivering any substance to larvae, it is important to determine the toxicity of pomegranate extract on worker bees.

P. granatum extract was selected because the results of previous experiments showed that it exhibited *in vitro* the highest activity against *A. apis*, therefore, it could be considered as promising candidate to control the chalkbrood disease.

Thus, the objective of this experiment was to assess the oral toxicity of *P. granatum* extract on adult honeybee workers to evaluate the potential of this extract as safe effective treatment for chalkbrood disease in the colony.

3.7. 1. Principle of the test

Adult worker honeybees were fed on a range of *P. granatum* extract concentrations and the mortality was determined daily over 96 hr. Mortality was recorded and compared to that of control.

- **Methods**

3.7. 2. Collection and housing of bees

An average of 350±20 adult worker bees, obtained from adequately fed, healthy and disease-free colonies, were brushed without age differentiation from brood combs into each cage (140 × 140 × 70 mm) with a nylon mesh on the walls for air circulation (Figure 3.1.).

Every cage was equipped with an artificial bee feeder made of 9 × 3 cm plastic cylinder fixed on the floor at the corner of the cage.

Figure 3.1. Cages used in evaluation the effect of *P. granatum* extract on honeybees (140 × 140 × 70 mm dimension)

3.7. 3. Feeding conditions

A four different concentrations of pomegranate extract 12.5, 25, 50 and 100% were prepared in distilled water. Sugar (sucrose) was dissolved in each concentration to make 50% solution (50% w/v sucrose in pomegranate extract solution). The control was composed from 50% sucrose solution only.

Before the assays, the bees were starved for 2 hr to ensure maximum uptake of the test substances during the experiment. Feeders were then filled with 10 ml of the target concentrations and bees were allowed to feed. After 24 hr of starting, feeders were removed and the amount of the syrup consumed by bees in each cage was determined.Bees were then fed on 50% (w/v), freshly prepared sugar syrup *ad libitum* only.

During the assay, all cages were placed on the upper space inside their own hive in order to keep bees exposed to queen pheromone which is necessary for normal living condition.

3.7. 4. Bees mortality

The experiment was extended for four days, and the numbers of dead bees were recorded daily. Immobile bees, or those that fallen down at the floor of the cage and showed no response to mechanical stimuli were scored as dead.

In order to determine the initial number of bees used, at the end of the experiment, bees were killed by immersing the cages in 70% ethyl alcohol solution and then counted. Results were expressed as percentage of dead bees as a function of time. Thus, the daily percentages of dead bees were determined. The experiment was repeated two times and the average of measurements were considered. Mortality was checked daily, and dead bees were counted.

Chapter 4

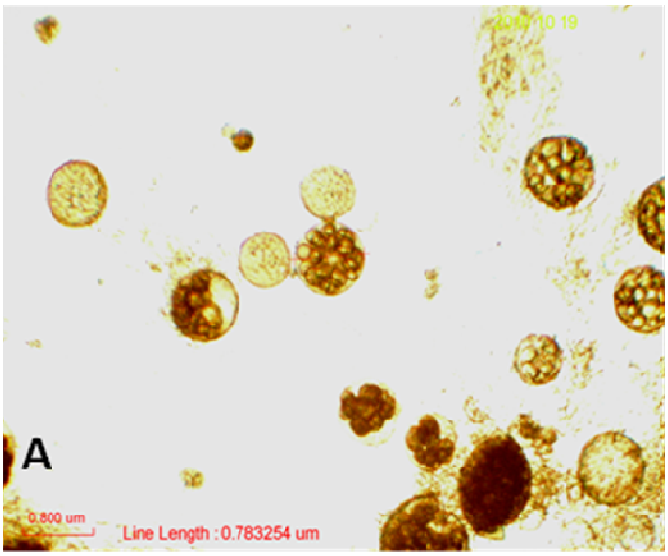
The Result

4.1. Morphological characteristics

The isolated fungus was identified on basis of cultural and morphological characteristics as *A. apis* illustrated in figure 4.1. and 4.2. and described in Table 4.1.

Table 4.1.: Cultural and morphological characteristics of *A. apis*

Colony parts	Shape	Septation	Color
Colony	Cotton like, with areal hayphae, after more than 6 dayes old a black areas appears.	absent	grayish-white mycelium.
Mycelium	hyphae are 4 to 8 microns wide, and vegetative nuclei are very small	present	aerial, surface, and subsurface hyphae
Ascospore diameter Figure 4.1.(A)	60 µm	-	formed due to the + and – thalli conjugation
Ascocyst diameter Figure 4.1. (B)	12 µm	-	-
Spore size Figure 4.2.	2.9 × 1.4 µm	-	-



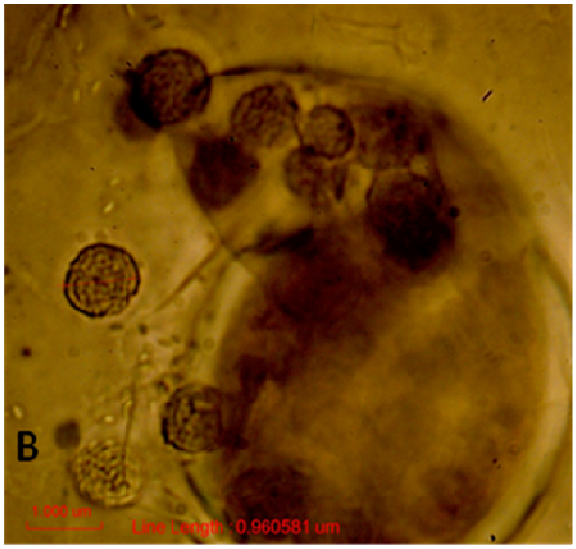


Figure 4.1. (A) Images of mature ascoma have a dark-brown appearance, The transparency of the ascoma walls allows observation of a number of small spherical shaped asci at 10×. (B) Ascospores out of ruptured ascoma at 40×.

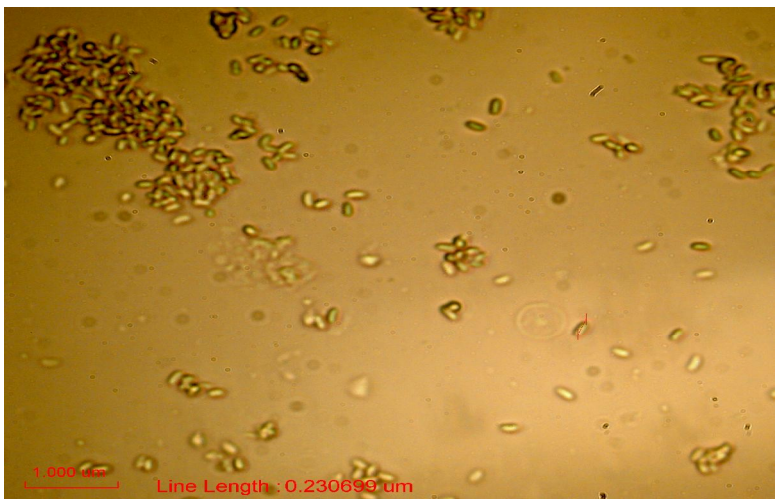


Figure 4.2. Isolated spores of *A. Apis* at 40 ×.

The fungal mating test were performed and the formation of ascospore were clear in the conjugation line in the middle of the plate (Figure 4.3).

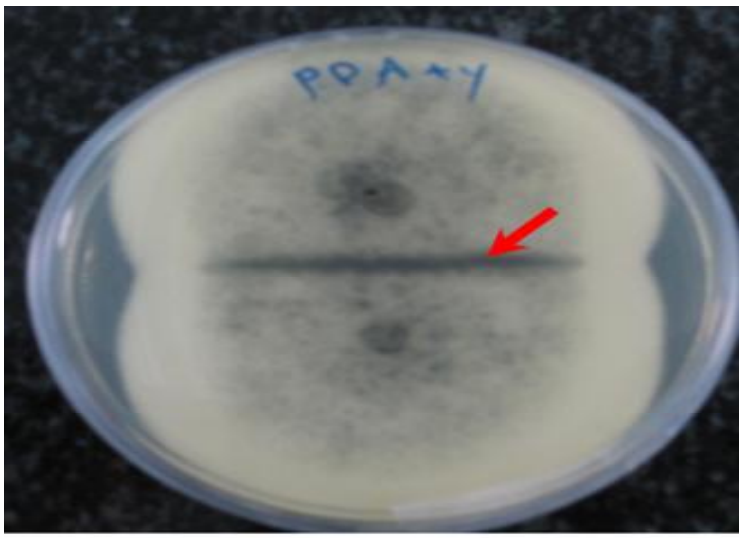


Figure 4.3. Cultural colony of *A. apis*, the red arrow pointed to the ascospores formed due to congregation of (+) and (-) types of the two separated fungal colony.

4.2. Minimum Inhibitory Concentrations (MICs) of the various plant extract

The Minimal Inhibition Concentrations of the different extracts after 8 days against *A. apis* are outlined in Table 4.2.

Table 4.2.: The Minimum Inhibitory Concentration (MIC) values of the extracts against *A. apis* after 8 days incubation

	Plant extract	MIC %
1.	*Artemisia monosperma*	> 20
2.	*Artemisia absinthium*	> 20
3.	*Artemisia herba - alba*	> 20
4.	*Tagetes patula*	> 20
5.	*Punica granatum*	0.625
6.	*Cyperus rotundus*	> 20
7.	*Callistemon viminalis*	5
8.	*Cinnamomum zeylanicum*	2.5
9.	*Annona squamosa*	> 20
10.	*Psidium guajava*	1.25

Among the tested plant extracts, *P. granatum* was the most active extract against *A. apis* with MIC of 0.625%. Other extracts *P. guajava*, C. zeylanicum, and *C. viminalis* exhibited moderate MIC against *A. apis* with MICs of 1.25, 2.5 and 5% respectively. The remaining extracts *A. monosperma*, *A. absinthium*, *A. herba- alba*, *T. patula*, *C. rotundus*, and *A. squamosa* were ineffectual against *A. apis* in this *in vitro* test system, where *A. apis* was not inhibited by the highest concentration (20%) used in this study. The photographs below

(figure 4.4 and 4.5) are typical growth presentations of *A. apis* in the *in vitro* system employed in this investigation.

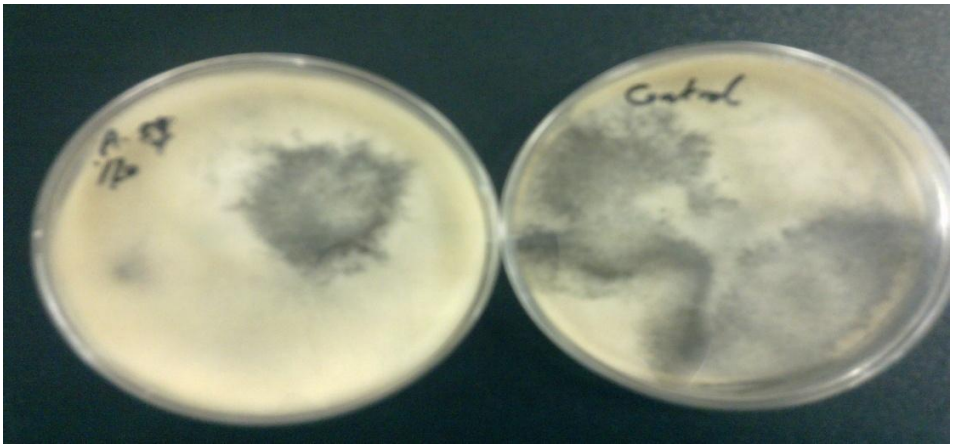


Figure 4.4. A pictorial presentation of the inhibitory effect of *A. squamosa* MIC compared with control after 8 days of incubation.

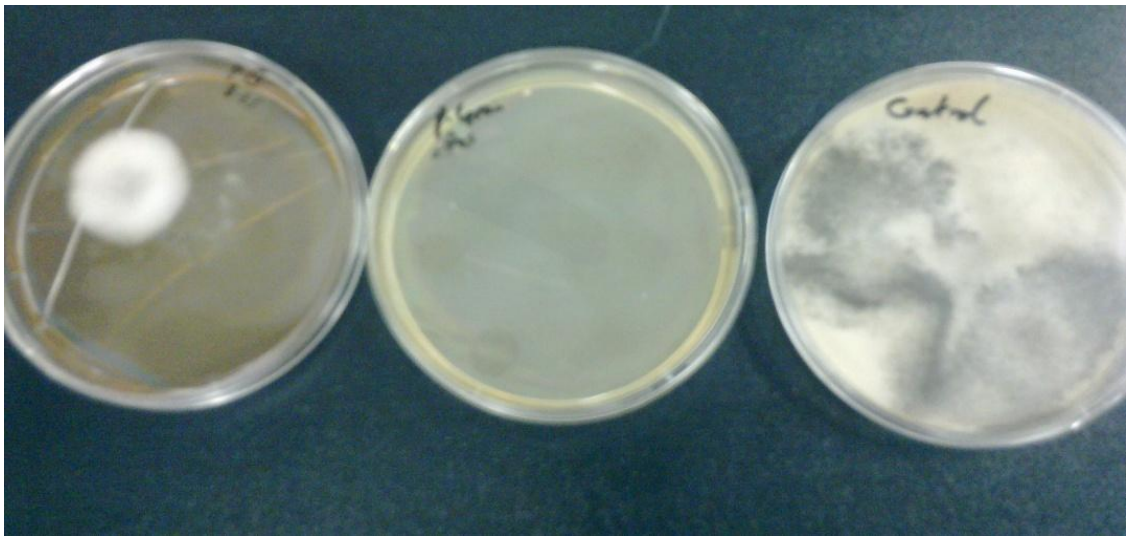


Figure 4.5. A pictorial presentation of the inhibitory effect of *P. granatum* MIC compared to the control after 8 days of incubation

4.3. Antifungal activity of plant extracts

The different plant extracts exhibited different degrees of activity against the mycelium growth of *A. Apis* ranged from inhibitory to stimulatory.

Among all plant extracts tested against *A. apis*, only *P. granatum*, *P. guajava*, *C. zeylanicum, and C. viminalis* proved to be effective.

Similar to the results of MIC test, the most active antifungal extract in this experiment was that of *P. granatum*, where the lowest radial growth was obtained from the plates containing that extract at the 4th day, with average radial growth of 8.4±0.5, 9.5±1.3 and 6.2±1.1 mm at 5, 10 and 20% concentrations respectively as compared with 32.6±1.5 mm of untreated control (Table 4.3.).

Table 4.3.: The effect of *P. granatum* extract over time on the radial growth of *A. apis* where 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2^{a}	5.0±0.9^{a}	4.6±1.0^{a}	4.3±0.5^{a}
2nd	14.9±1.6^{a}	7.3±0.6^{b}	6.8±1.8^{b}	5.4±0.4^{b}
3rd	24.8±1.9^{a}	7.6±0.9^{b}	9.0±1.1^{b}	6.0±0.9^{b}
4th	32.6±1.5^{a}	8.4±0.5^{b}	9.5±1.3^{b}	6.2±1.1^{c}

The value with a different letter in the same row is significantly different (P<0.05)

The activity of the flower extract of *P. granatum* against *A. Apis* was followed by *P. guajava* (Table 4.4.). The average radial mycelia growth of *A. apis* was significantly reduced (P < 0.05) by this extract at 2nd, 3rd, and 4th days of incubation at the different concentrations tested when compared with cultures grown on untreated media.

Table 4.4.: The effects of *P. guajava* plant extracts over time on the radial growth of *A. apis* . Where 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	5.1±0.4[a]	5.5±0.6[a]	3.2±0.4[b]
2nd	14.9±1.6[a]	11.2±0.9[b]	10.6±0.8[b]	8.3±1.0[c]
3rd	24.8±1.9[a]	16.8±1.2[b]	16.6±0.7[c]	11.7±0.5[d]
4th	32.6±1.5[a]	21.5±1.3[b]	20.8±1.0[c]	15.1±1.1[d]

The value with a different letter in the same row is significantly different (P<0.05)

The effect of the different concentrations 5, 10, 20% of *C. viminalis* extract are presented in Table 4.5. The radial mycelia growth of *A. apis* was significantly reduced at the 3rd day at 20% and at 4th day at 10% and 20% concentrations ($P < 0.05$).

Table 4.5: The effects of *C. viminalis* plant extracts over time on the radial growth of *A. apis* . Where, 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	7.6±0.7[b]	8.2±0.4[b]	7.6±0.3[b]
2nd	14.9±1.6[a]	15.8±1.7[a]	13.8±0.4[a]	13.6±0.3[a]
3rd	24.8±1.9[a]	26.7±1.7[a]	24.4±1.0[a]	20.9±0.6[b]
4th	32.6±1.5[a]	34.7±0.8[a]	30.3±0.3[b]	25.3±1.4[c]

The value with a different letter in the same row is significantly different (P<0.05)

According to the results presented in Table 4.6., the average radial mycelia growth of *A. apis* at 2nd, 3rd, and 4th days of incubation was significantly reduced ($P < 0.05$) at the

different concentrations of *C. zeylanicum* extract tested when compared with cultures grown on untreated media.

Table 4.6.: The effects of *C. zeylanicum* plant extracts over time on the radial growth of *A. apis* . Where, 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	5.6±0.7[a]	3.9±0.5[a]	3.5±0.6[b]
2nd	14.9±1.6[a]	10.8±0.9[b]	8.3±0.6[b]	6.2±2.0[b]
3rd	24.8±1.9[a]	19.0±0.5[b]	14.9±0.8[c]	11.7±0.3[d]
4th	32.6±1.5[a]	26.6±0.6[b]	20.9±0.8[c]	17.1±3.2[d]

The value with a different letter in the same row is significantly different ($P<0.05$)

In contrast to the fore mentioned plants, the extract of *A. squamosa* showed stimulatory effect on the mycelium growth of *A. apis* at all concentrations tested through the four incubation days (Table 4.7.).

Table 4.7: The effects of *A. squamosa* plant extract over time on the radial growth of *A. apis* . Where, 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	6.3±0.1[a]	6.5±1.3[a]	6.3±0.7[a]
2nd	14.9±1.6[a]	18.2±0.1[b]	18.0±0.9[b]	18.1±0.7[b]
3rd	24.8±1.9[a]	27.3±1.7[a]	27.1±4.1[a]	28.3±0.9[a]
4th	32.6±1.5[a]	35.3±1.1[a]	34.8±7.9[a]	36.4±3.6[a]

The value with a different letter in the same row is significantly different ($P<0.05$)

Simillar to *A. squamosa***,** the extract of *Artemisia monosperma* also shows stimulatory effect on the mycelium growth of the fungi at all concentrations tested during the four days of incubation. The stimulatory effect was increased at lower concentrations (Table 4.8.).

Table 4.8.: The effects of *A. monosperma* plant extracts over time on the radial growth of *A. apis* . Where, 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	7.5±0.5[b]	6.8±0.4[b]	6.1±0.3[a]
2nd	14.9±1.6[a]	17.9±0.6[b]	16.7±1.2[a]	15.3±0.9[c]
3rd	24.8±1.9[a]	29.1±0.8[b]	28.0±1.2[c]	24.3±2.0[d]
4th	32.6±1.5[a]	37.4±1.5[b]	37.4±0.6[b]	34.6±3.7[a]

The value with a different letter in the same row is significantly different ($P<0.05$)

A weak stimulatory effect on the mycelium growth of the fungi at all concentrations tested of *A. absinthium* extract during the incubation period was observed in Table 4.9.

Table 4.9: The effects of *A. absinthium* plant extracts over time on the radial growth of *A. apis*. where, 20, 10 and 5 represent extract that was diluted; 0 = control

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	7.2±0.6[b]	7.4±0.4[b]	7.0±0.6[b]
2nd	14.9±1.6[a]	15.6±0.5[a]	15.4±0.7[a]	15.9±0.3[a]
3rd	24.8±1.9[a]	25.1±2.1[a]	25.4±1.4[a]	25.3±0.2[a]
4th	32.6±1.5[a]	33.3±1.5[a]	33.2±0.9[a]	32.8±0.9[a]

The value with a different letter in the same row is significantly different ($P<0.05$)

Like other species of *Artemisia spp.,* the stimulatory effect of *A. herba-alba* on the mycelium growth of the *A. apis* at all concentrations tested across the four incubation days is clear. The stimulation was found to be associated with the increase of the concentration of the extract (Table 4.10.).

Table 4.10.: The effects of *A. herba-alba* plant extracts over time on the radial growth of *A. apes*. Where, 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	7.6±1.2[b]	7.8±0.7[b]	8.0±0.9[b]
2nd	14.9±1.6[a]	17.3±1.1[b]	17.3±0.7[b]	17.8±0.8[b]
3rd	24.8±1.9[a]	28.1±1.2[b]	28.2±0.5[b]	28.9±0.9[b]
4th	32.6±1.5[a]	36.1±0.9[b]	36.9±1.1[b]	38.6±0.8[c]

The value with a different letter in the same row is significantly different ($P<0.05$)

The extract of *C. rotundus* showed stimulatory effect on the mycelium growth at all concentrations tested throuh the four days of incubation. The maximum mycelium growth was

recorded by this plant extract at 5%, which was 20.1% increase over the control at the 4th day (Table 4.11.).

Table 4.11.: The effects of *C. rotundus* plant extracts over time on the radial growth of *A. apes*. Where, 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	8.0±1.3[b]	7.3±0.9[b]	7.8±0.7[b]
2nd	14.9±1.6[a]	17.4±1.4[a]	16.2±1.0[a]	17.3±0.4[a]
3rd	24.8±1.9[a]	29.6±1.5[b]	28.3±0.9[b]	28.0±0.5[b]
4th	32.6±1.5[a]	39.2±1.4[b]	38.3±1.4[b]	35.9±1.3[b]

The value with a different letter in the same row is significantly different ($P<0.05$)

T. patula extract, shows a stimulatory effect on the mycelium growth at all concentrations tested across the four incubation days (Table 4.12.). Although the average radial growth of *T. patula* at 20% after 4 days incubation was less than that of the control, but this difference was not significant ($P = 0.942$).

Table 4.12: The effects *T. patula* plant extracts over time on the radial growth of *A. apis*. Where, 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2[a]	6.8±0.4[a]	6.8±0.9[a]	6.8±0.4[a]
2nd	14.9±1.6[a]	16.7±0.4[a]	16.3±1.2[a]	15.4±0.8[a]
3rd	24.8±1.9[a]	21.3±0.6[b]	22.5±0.9[c]	26.2±1.0[d]
4th	32.6±1.5[a]	35.6±0.4[a]	33.7±2.8[a]	32.0±0.7[a]

The value with a different letter in the same row is significantly different ($P<0.05$)

The results of calculation of percentage of inhibition are presented in Table (4.13). These results are in accordance with that of the average radial growth. *P. granatum* extract had the highest percentages of inhibition followed by *Psidium, Cinnamomum,* and *Callistimon* (Figure 4.6.).

Results indicated that, the inhibition percentage increased with increase in exposure time. The maximum inhibition percentage observed after 4 days of incubation at 20%. In some treatments however, such as *Tagetes* and *Cinamon*, the maximum inhibition percentage was attained at the 3rd day. The maximum inhibition percentages observed after 4 days of incubation were at 20% for *Punica, Psidium, Cinnamomum,* and *Callistimon* with 81.1, 53.7, 47.5 and 22.4% respectively.

Mycelium growth was inhibited by *Punica*, *Psidium* and *Cinnamomum* extracts at all concentrations. Inhibition by *Callistimon* extract however, was observed at concentrations of 10% and 20% only.

Tagetes extract slightly slowed (1.9%) the growth of fungus after four days incubation at 20%, while other concentrations proved to have slight stimulatory effect.

Results also indicated that, the inhibition percentage increased with increase in conentration. Extracts of 20% concentration was more efficacious and show high inhibition percentages than other concentrations. For example 20% concentration of *Punica, Psidium* and *Cinnamomum* extracts showed 14.7, 36.4 and 31.0% respectively even after the 1st day of exposure time.

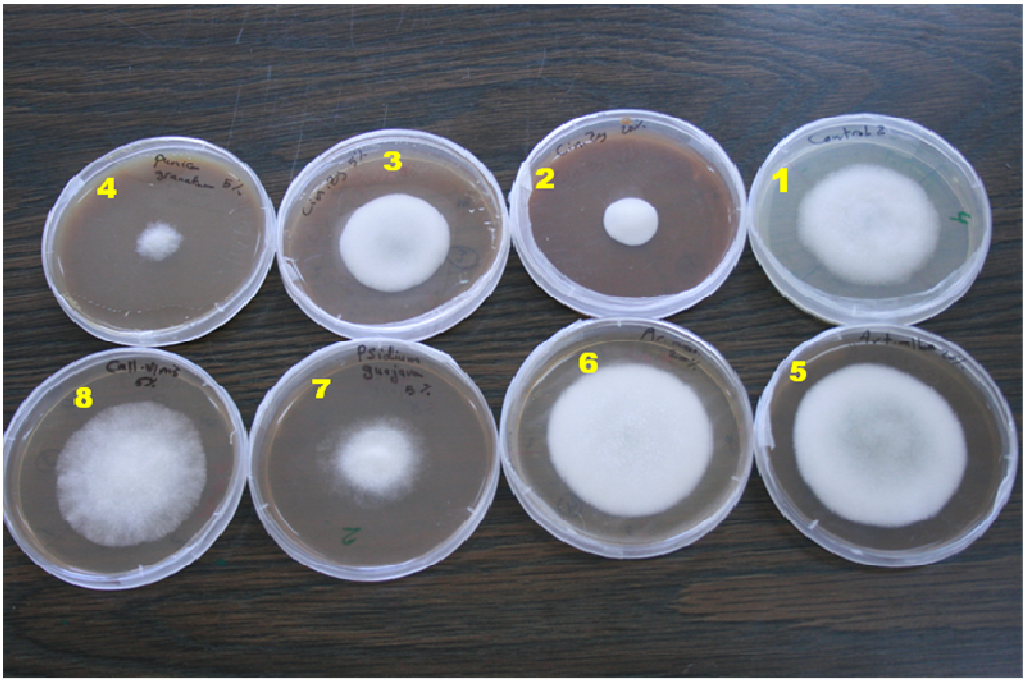


Figure 4.6.: Mycillium growth of *A. apis* on differnet plant extracts and control after 3 days of incubation. (1) Control (2) *C. zeylanicum* 20% (3) *C. zeylanicum* 5% (4) *P. granatum* 5% (5) *A. Alba* 20% (6) *A. monosperma* 20% (7) *P. Guajava* 5% (8) *C. viminalis* 5%.

Table 4.13.: Percentage of radial growth inhibition of *A. apis* during four days of incubation on agar media treated with plant extracts.

	Percentage of inhibition (%)											
	First day			Second day			Third day			Fourth day		
Plant extract	**5%**	**10%**	**20%**	**5%**	**10%**	**20%**	**5%**	**10%**	**20%**	**5%**	**10%**	**20%**
A. monosp.	-0.5	-35.5	-22.9	-20.2	-12.3	-2.6	-17.5	-12.9	+2.2	-14.8	-14.8	-6.0
T. patula	-37.2	-35.5	-37.2	-12.1	-9.9	-3.7	+14.0	+9.3	-5.5	-9.1	-3.2	+1.9
A. absinthium	-44.3	-49.3	-40.5	-5.1	-3.4	-6.8	-1.1	-2.3	-2.1	-2.2	-1.8	-0.4
P.granatum	-0.4	+8.4	+14.7	+50.8	+54.6	+63.6	+69.2	+63.7	+75.8	+74.3	+70.9	+81.1
A. alba	-51.8	-56.8	-60.6	-16.5	-16.0	-19.8	-13.2	-13.7	-16.5	-10.8	-13.3	-18.5
C.rotundus	-60.6	-45.5	-55.6	-17.2	-8.7	-16.0	-19.3	-13.9	-12.9	-20.1	-17.3	-10.1
C. viminalis	-53.0	-64.3	-53.0	-6.4	+7.1	+8.3	-7.6	+1.4	+15.8	-6.4	+7.2	+22.4
C.zeylanicum	-11.6	+22.2	+31.0	+27.7	+44.5	+58.4	+23.4	+40.0	+52.9	+18.3	+36.0	+47.5
A. squamosa	-27.1	-30.5	-25.4	-22.2	-21.1	-21.5	-9.9	-9.2	-13.9	-8.1	-6.8	-11.6
P. guajava	-1.6	-10.4	+36.4	+24.7	+28.5	+43.9	+32.2	+32.9	+52.9	+34.1	+36.4	+53.7

+ : growth inhibition.

- : no growth inhibition.

4.4. The nature of antifungal effect of *P. granatum* extracts

The average radial mycelial growth of *A. apis* when subcultured from treated plates into extract free media with respect to time of incubation are presented in (Figure 4.7.)

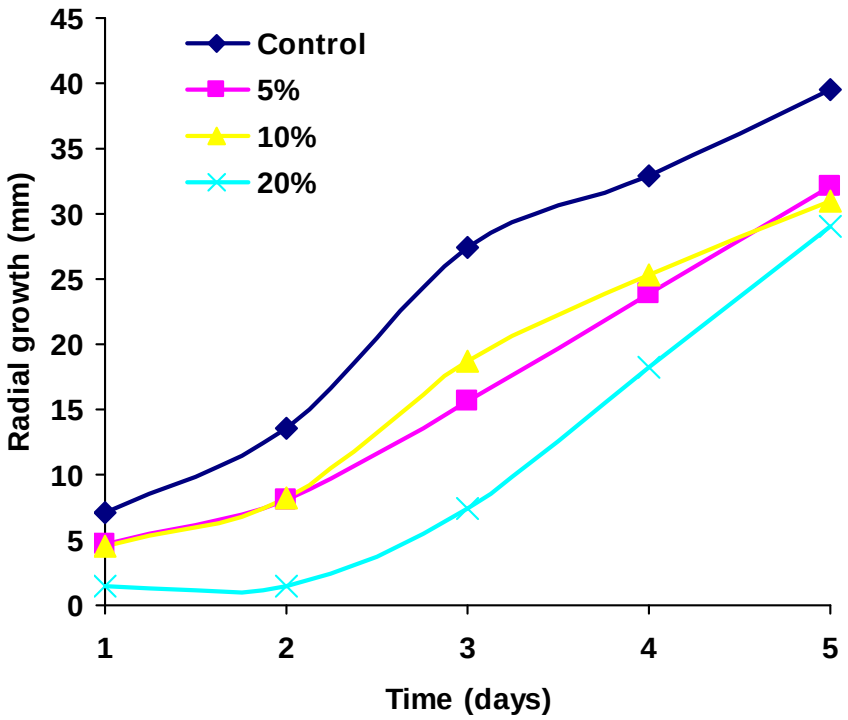


Figure 4.7. : Growth profile for *A. apis* on PDA with 0%, 5%, 10% and 20% concentrations of *P. granatum* flower extract.

The plotted data shows an initial slow in growth rates of *A. apis* obtained from the different concentrations during the first two days, followed by gradual increase with time up to the 5th day of incubation.

The statistical analysis showed a significant difference in the average radial growth of *A. apis* over time due to the effect of the different concentrations of plant extracts (P <0.05). Such difference between the three tested concentrations was gradually decreased over the time. After five days of incubation no significant difference was detected between plates

subcultured with mycelium discs from 5% and 10% ($P = 0.527$) and between 10% and 20% ($P = 0.096$). This difference however, was still significant between the different concentrations and control ($P < 0.05$).

4.5. Toxicity of *P. granatum* extract on *A. mellifera*

The results of the effect of different concentrations of *P. granatum* extract on worker bees though 96 hr period are presented in (Figure . 4.8.)

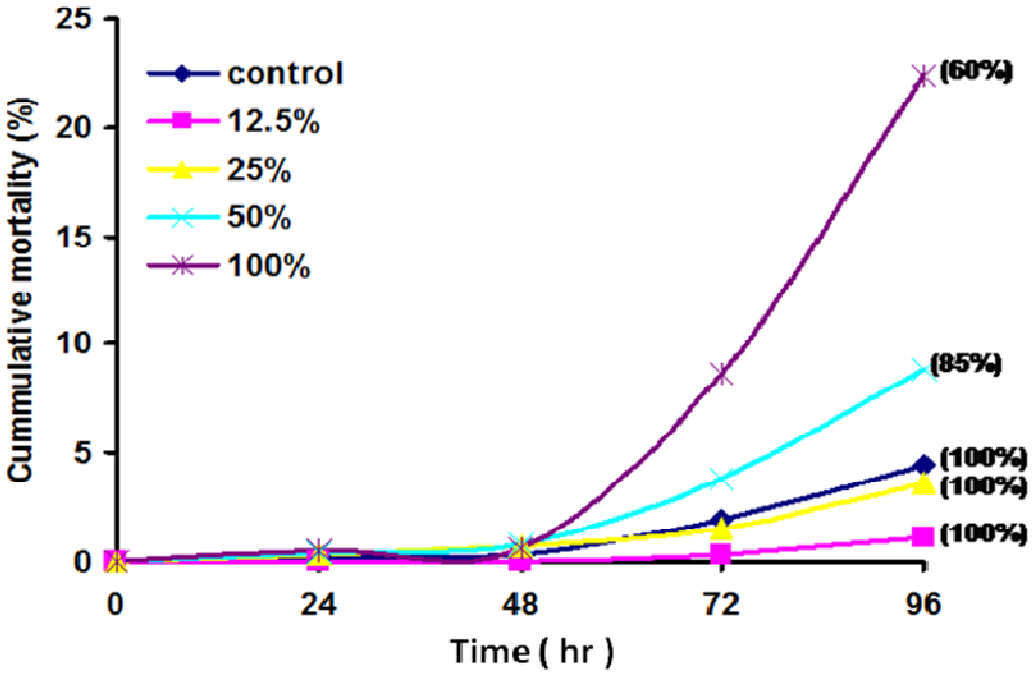


Figure 4.8. Cumulative mortality curves (%) of bees during 4 days period after feeding on four different concentrations of *P. granatum* extract as compared to control. Values between brackets are the percentages of the amounts of *P. granatum* extract-sucrose mixture consumed by bees in each treatment.

Similar to other toxicity tests, mortality of bees in this study appeared to have direct relationship with time and concentrations of tested materials.

The cumulative mortality of bees was ranged 0.0 - 0.47, 0.0 – 0.8 and 0.3– 8.6 after 24, 48 and 72 hr respectively.

The highest percentage of cumulative mortality was recorded after 96 hr and ranged from 1.1% to 22.4% at the different treatments, while the highest percentage of mortality (22.4%) was recorded at the highest concentrations tested (100%) after 96 hr of the test.

Although 1.1 % and 3.6% of mortality was recorded at 12.5% and 25% concentrations respectively from the beginning of the treatments to 96 hr, they still however, lower than that of the control group (4.4%). At the same time, the percentage of mortality at 50% concentration (8.5%) was higher than that of the control (4.4%).

The consumed quantity of *P. granatum* extract administered in sugar syrup decreased as the concentration of the extract increases. This is especially clear at 50% and 100% concentrations, where the percentages of consumed quantity were 85% and 60% respectively, in comparison with other treatments and control. The decrease of consumption was also found to be directly correlated with bees' mortality (Figure 4.7.).

Chapter 5

Discussion

The lack of any effective control agents for chalkbrood has led to increase in the investigation of alternative control methods and strategies. Plants are important source of potentially useful structures for the development of new control agents. Many reports are available on the antiviral, antibacterial, antihelmintic, antimolluscal and anti-inflammatory properties of plant extracts. Some of these reports were also carried out to discover the natural products that inhibit the fungus *A. apis* which is considered one of the pathogenic fungi in honeybees colonies (Abou El-Enain *et al*., 2009; Calderone *et al*., 1994; Chantawannakul *et al*., 2005; Dellacasa *et al*., 2003)

5.1. Identification of *Ascosphaera apis*

The *Ascosphaera* genus is usually found in the environment associated with populations of social and solitary bees (Chorbiński and Rypuła, 2003). The cultures we had were isolated and identified from larval mummies obtained from chalkbrood infected colonies. The morphological properties of the isolated colonies are in general agreement with previous reports (Chorbiński and Rypuła, 2003). Specifically, the ascospores growth was visible on 6 – 8 days after inoculation. Sizes of spores, ascospore and ascocyst diameters of the *A apis* agreed well with previously reported measurements (Chorbiński and Rypuła, 2003; Gilliam *et al*., 1978). Moreover, the mycelium was white, compact and aerial with septated haypha (Chorbiński and Rypuła, 2003). Since it is a heterothallic fungi, mating tests have been used to diagnose species as *A. apis* (Christensen and Gilliam, 1983). When cultures were paired with two types, a black line of ascoma was observed due to congregation between (+) and (-) types of the two separated fungal colony. This behavior of the fungus was previously confirmed by Spiltoir and Olive (1955).

The results of the present study confirm the presence of chalkbrood disease in honeybees' population of Gaza Strip.

5.2. Plant extract antifungal activity

As mentioned earlier, ten different plants belonging to six families (Table 3.1.) were collected and extracted with water and their antifungal activities were screened against *A. apis*. Crude extracts were obtained through the extracting action of the appropriate dry plant material by using reflux distillatory system and the active compounds are thus contained in the solvent used.

In the present study, two *in vitro* assay techniques; the MIC and the radial growth inhibition methods were used as currently these are the most common methods for assessing the antifungal activities of natural compounds. A bioassay that examines an extract's ability to inhibit fungal spores (MIC) would be a good screening test because spores are the most accessible stage for interrupting the disease cycle. Through such tests however, one cannot distinguish the actual effects of plant extracts wthether it is sporicidal or just inhibit germination, accordingly, the radial growth inhibition assay was necessary.

5.2.1. Plant extracts demonstrated antifungal activity

Four plant extracts were exhibited antifungal activity against *A.apis* in the two tests. The antifungal properties were found to be best in flowers of the *P. granatum* with MIC of 0.625%, followed by *P. guajava* (1.25%), *C. zeylanium* (2.5%) and *C. viminalis* (5%). The MIC of the remaining plants; *A. absinthium*, *A. monosperma*, *A. herba-alba*, *T. patula*, *C. rotundus* and *A. squamosa* didn't exhibit any antifungal activity against *A. apis* even at the highest concentrations tested (20%). On the other hand, the results of radial inhibition method of *A. Apis* were ranged from inhibitory to stimulatory effect.

5.2.1.1. *Punica granatum*

Several studies showed an antifungal activity of various plant extracts or pure compounds from the different parts of the *P. granatum* such as bark, leaves, seeds, peels and fruit (Jayaprakasha *et al*., 2006), but as far as it could be ascertained, the flowers of this plant have been rarely investigated for that purposes.

The current study found that the aqueous crude extract of *P. granatum* flower had the highest inhibition on fungal growth with a mean diameter of 6.2 mm at the concentration of 20% at the 4^{th} day compared to 32.6 mm of the control (Table 4.3.).

The obtained results were in agreement with Satish *et al.* (2007) who found similar effect of the aqueous extracts of *P. granatum* against *Aspergillus sp.* The fungitoxic properties of *P. granatum* have also been confirmed by Jassim (1998) who reported an inhibitory effect of *P. granatum* extract against *Penicillium citrinum*, *P. patulum* and *Aspergillus ochraceous*.

Pai *et al*. (2010) evaluated *in vitro* the antifungal efficacy of aqueous decoctions of *P. granatum, Acacia nilotica, Cuminum cyminum* and *Foeniculum vulgare* on *Candida albicans*. Similar to the present study, regarding the activities of the different plant extracts, the peel extract of *P. granatum* showed the highest antifungal property.

Water, ether and methanolic extracts (80%) of pomegranate peel have been evaluated against *Aspergillus niger, Candida utilis* and *Saccharomyces cerevisae*. The methanolic extract was found to be the most effective against the tested microorganisms and the results were explained by the presence of substantial concentration of phenolics and flavonoids in methanolic extracts as opposed to either water or ether extracts (Al-Zoreky, 2009).

In their evaluation of the antifungal and antibacterial activities of different parts (peel, seed, juice and whole fruit) of *P. granatum* fruit extracts on selected fungi and bacteria, Dahham *et al*. (2010) found that the peel extract has shown the highest antimicrobial activity compared to other extracts. Among the selected fungal and bacterial cultures, the highest antifungal activity was recorded against *Aspergillus niger* and among bacteria high activity against *Staphylococcus aureus* was recorded.

Due to their antifungal properties, *P. granatum* extracts have been used in different commercial antifungal preparation such as fungicide (Jia and Zia, 1998) and antifungal gel against *Candida albicans* (Vasconcelos, 2003).

The presence of phytochemicals such as phenols, tannins, flavonoids, and alkaloids in the pomegranate as major active constituents may be responsible for the biological activities (Aviram and Rosenblat, 2006). Phytochemical analysis of the flower extract of *P. granatum*

has revealed the presence of polyphenolic compounds such as ellagic acid, 3,3,4- tri-O-methylellagic acid, ethyl brevifolincarboxylate, urolic and maslinic acids and daucosterol (Wang *et al*., 2010). These polyphenolic compounds were known to be responsible for antimicrobial activity (Pirbalouti *et al*., 2010). Furthermore, *P. granatum* is known for its contents of tannins (Kansoh *et al*., 2001). In recent studies, it was confirmed that *P. granatum* peel has an antifungal peptide called Pomegranin (Guo *et al*., 2009) and triterpenes (Hui *et al*., 2007). These compounds may be present in *P. granatum* flowers and may play an important role in the antifungal activity.

5.2.1.2. *Psidium guajava*

In the present study, *P. guajava* leaf was found to be the second most active crude aqueous extract tested against *A. apis* (Table 4.2.). The average radial mycelia growth of *A. apis* was significantly reduced ($P < 0.05$) as shown in table 4.4. Leaf extracts of *P. guajava* have been found to have antimicrobial activity against several bacteria, fungi, viruses and parasites suggesting wide antimicrobial activity (Gutierrez *et al*., 2008). Similar to the current study, the aqueous extracts of the leaves were found to be effective inhibitors of growth spore formation, and enterotoxin production of *Clostridium perfringens* (Garcia *et al*., 2002). The bark tincture showed fungicidal activity at different concentrations but exhibited only fungistatic effect on *C. albicans* (Dutta and Das, 2000). The aqueous extract of guava leaf also showed good activity against different bacterial species, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Gnan and Demello, 1999b; Penecilla and Magno, 2011).

Preliminary phytochemical reports on *P. guajava* have revealed the presence of a number of active compounds such as alkaloid, saponin, tannin, phenol, flavonoid and steroid in the water extract of guava leaves (Doughari *et al*., 2007; Romasi *et al*., 2011). The antifungal compound mainly found in *P. guajava* were tannins, phlobatannins, saponins, terpenoids, alkaloids and poly phenols (Pandey and Shweta, 2011). The leaves of *P. guajava* were also found to contain volatile oil rich in cineol, tannins, triterpenes, resin, eugenol, mallic acid, that has some of antimicrobial activity (Joseph and Priya, 2011).

In previous work however, aqueous extracts of guava leaves had demonstrated the lowest activity against *Penicillium digitatum* and *Aspergillus fumigatus* compared to the methanol and acetone extracts. The difference observed in the activities of the various extracts was explained by the varying degrees of solubility in the different solvents (Doughari *et al*., 2007). In the same view, the water extracts did not inhibit all the microbes tested (*E.coli, S. aureus, B. cereus, Penicillium*). The results were attributed to the high heating temperature and the long heating time employed, which may negatively affect the active compound and the amount of the volatile component of the extract (Romasi *et al*., 2011). Doughari *et al*. (2007) however indicated that *P. guajava* extract is stable at high temperatures and its activity didn't affect even at high temperature of 100 °C.

5.2.1.3. *Cinnamomum zeylanicum*

C. zeylanicum exhibited a moderate MIC of 2.5% (Table 4.2). This effect is similar to the findings of Bailac *et al*. (2006) who found MIC of *C. zeylanicum* oil against *A. apis* very low copered withother plants. These finding have been also supported by Calderone *et al*. (1994) who found a complete inhibition of the growth of *A. apis* by cinnamon oil at 100 ppm for 168 hr.

The results also showed a significant reduction ($P < 0.05$) in the average radial mycelia growth of *A. apis* by *C. zeylanicum* extracts at 2^{nd}, 3^{rd}, and 4^{th} days of incubation at the different concentrations tested compared to untreated control. It also possessed an inhibition rate of 27.7% -58.4% at 3^{rd} day of incubation at different concentrations (Table 4.6.). In agreement with the result of the present study, Abou El-Enain *et al*.(2009) have reported that, *C. zeylanicum* oil was the most active against *A. apis* and produced an inhibition rate of 74.4%. Cinnamon extract was found to have an inhibitory effect against aflatoxin producing molds (Bullerman *et al*., 1977). In an *in vitro* study, *C. zeylanicum* was also found to be active against both fluconazole resistant and susceptible *Candida* (Quale *et al*., 1996). The antifungal activity of cinnamon may be attributed to its content of different active compounds like Cinnamaldehyde and Eugenol which represent the major active ingredients (Bullerman *et al*., 1977). The presence of these active components (Cinnamaldehyde and Eugenol) were confirmed elsewhere and found to be inhibitory to *P. larvae* bacteria (Gene *et al*., 2008).

5.2.1.4. *Callistemon viminalis*

In the present study, *C. viminalis* exhibited low activity against *A. apis* where the MIC was 5% (Table 4.2) and the radial mycelia growth were significantly reduced ($P < 0.05$) at the 3rd day at 20% and at 4th day at 10% and 20% only (Table 4.5). Similar results have been reported by Delahaye *et al.* (2009) they found that, the aqueous crude extract of *C. viminalis* had a lower MIC than other solvents. In a recent study carried out with another species of *Callistemon* (*C. rigidus*), the stem extract showed a moderate activity against seed-borne pathogenic fungi (Pawar, 2011).

The antimicrobial activity of the plant was attributed to the phytochemical compounds (Oyedeji *et al*., 2009). Several bioactive compounds such as alkaloids, flavonoids, and phenols have been separated from the polar extracts of different parts of *C.viminalis* (Delahaye *et al*., 2009). The plant leaves oil was found to contain 1,8-Cineole, α-pinene and menthyl acetate compounds which have been reported to possess antimicrobial activity (Srivastava *et al*., 2003).

Four of the 10 plants extracts exhibited different degrees of antifungal activity. The results indicated that, the antifungal activity of the active extracts was enhanced by increase in the concentration of the extracts and the exposure period which is in agreement with the results reported by (Banso *et al*., 1999).

The exhibition of antifungal activity of some plant extracts is a promising outcome because it is an indication that there is possibility of development of new antifungal agents from these plants that would be very effective against chalkbrood disease.

Useful antimicrobial phytochemicals are: phenolics and polyphenols (such as simple phenols and phenolic acids, quinones, flavones, flavonoids, and flavonols. tannins, coumarins); terpenoids and essential oils; alkaloids; lectins and polypeptides; in addition to other compounds. The mechanisms thought to be responsible for these phytochemicals against microorganisims vary and depend on these compounds (Aly and Bafiel, 2008). Their mechanism of actions may include enzyme inhibition by the oxidized compounds, and act as a source of stable free radical are often leading to inactivation of the protein and loss of function. They may also have the ability to complex with extracellular and soluble proteins

and to complex with cell walls and disrupt microbial membranes (Ali, 1999), some have ability to intercalate with DNA, formation of ion channels in the microbial membrane, competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (Cowan, 1999b).

5.2.2. Plants didn't demonstrate antifungal activity

Six plant extracts (e.g., *A. monosperma*, *A. absinthium*, *A. herba-alba*, *T. patula*, *C. rotundus*, and *A. squamosa*) had no effect on fungal growth, or even, sometimes promoted mycelial growth. Spore germination and mycelia growth were observed even at the highest concentration (20%) used in this study. Therefore, the aqueous extracts of these plants do not have antifungal properties at least against *A. apis*.

5.2.2.1. *Artemisia monosperma*

The poor performance of some potential fungicidal extracts used in the present study, such as *A. monosperma*, as shown in table 4.8, was verified by Abou El-Enain *et al*. (2009), who found that the oils of *A. monosperma* were not so efficient at inhibiting *A. apis in vitro*. Similar observations have been also reported by Assaeed. (2003) who, demonstrate that, *A. monosperma* extracts could reduce the severity of some plant pathogenic fungi, but not prevent it.

5.2.2.2. *Artemisia absinthium*

Similar to the results of the present study, Dulger *et al*. (1999) found that *A*. *absinthium* oil couldn't exhibit any antifungal effect against yeasts. Moreover, Valdes *et al*. (2008) confirmed the lack of antifungal activity of *A. absinthium*, which has high IC_{50} value for *C. albicans*.

In another study, the petroleum ether and ethanol extracts of *A. absinthium* exhibited negative antimicrobial activities against different microorganisms viz., *Staphylococcus aureus* ATCC 65538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 8739, *Klebsiella pneumonia* ATCC 4352, *Pseudomonas aeruginosa* ATCC 1539, *Salmonella typhi, Shigella flexneri, Proteus mirabilis* and *Candida albicans* ATCC 10231 (Uzuna *et al*., 2004)

5.2.2.3. *Artemisia herba-alba*

The weakness of antimicrobial activity of *A. herba-alba* extracts has been demonstrated by Seddik *et al*. (2010) who found that the antibacterial activity of *A. herba-alba* aqueous and ethyl acetate extracts was ranged from weak to no effect against selected bacterial strains. Furthermore, Bouchra *et al*. (2003) evaluated the essential oil of *A. herba-alba* as weak antifungal agent against *Penicillium digitatum*, *Phytophthora citrophthora*, *Geotrichum citri-aurantii*, and *Potrytis cinerea*. Similarly, Mohamed *et al*. (2010) reported that, the aqueous extract of *A. herba-alba* possess only a relatively weak antibacterial activity and no inhibitory activity against the yeast *Saccharomyces cerevisiae*.

5.2.2.4. *Tagetes patula*

The extract of *T. patula* did not show any effect on the mycelium growth of *A.apis in vitro* at all tested concentrations. These results are in agree with several reports. Larran *et al*. (2001) found that the species *T. Minuta* oil did not have any effect against different strains of *A.apis*. Additionally, Frias and Kozusny- Andreanid. (2009) recommended not to use *T. minuta* extract against dermatophyte fungi, because it did not have any effect on these fungi. On the other hand, Eguaras *et al*. (2005) found that the essential oil of *T.minuta* shows a moderate inhibition effect compared with other essential oils, and the oil inhibited mycelial growth of *A. apis* above concentrations of 200 ppm only. This inhibition may be due to the difference of the extraction methods, where, the essential oil concentration in our extract may be lower than that used in the previous study. Moreover, González and Marioli. (2010) found that the decoction of *T.minuta* inhibit the growth of *Paenibacillus larvae* bacteria, this is may be due to the variety of the plant and to the type of studied organism.

5.2.2.5. *Cyperus rotundus*

In this study it is obvious that, the extract of *C. rotundus* rhizomes had no effect against *A.apis* (Table 4.11). This is similar to many observations. Parekh and Chanda. (2006) reported that, the aqueous extract of *C.rotundous* were inactive against clinically important bacteria and candida. Sharma and Singh (2011) indicted that the ethanol extract of *C. rotundous* was ineffective against fungi.

5.2.2.6. *Annona squamosa*

In the present study, the extract of *A. squamosa* not only didn't inhibit *A. apis* but also it has some stimulatory effect on mycillium growth (Table 4.7). These results agree well with other studies. Frias and Kozusny- Andreanid. (2009) found that *A. squamosa* did not have any antifungal effect against dermatophytes, and the authors didn't recommend to use it against the studied pathogens.

In spite of the observed lack of activity of the aqueous extracts of some plants against *A. apis*, the present investigation does not claim that these plants do not have antimicrobial or biological activities. Several studies have indicated various degrees of activities of these plants against different microorganisms including fungi.

In controlling honeybee bacterial diseases *in vitro*, Fuselli *et al.* (2008) showed that the oils of *A. absinthium*, had the highest inhibitory activity against different bacterial strains of *P. larvae*. Also, *A. herba-alba* was found to be the most active plant against *Mycoplasma* (Al-Momani *et al.*, 2007). Although it was ineffective against fungi, the ethanol extract of *C.rotundous* exhibited significant activity against the tested bacteria (Sharma and Singh, 2011). Furthermore, many phytochemicals were isolated from *A. squamosa* and were identified as antimicrobial (Rahman *et al*., 2005), and antifungal agents (Shanker *et al*., 2007).

Accordingly, the negative inhibition effects of some plant extracts observed in the present study may be attributed to the denaturation of the potential inhibitory chemicals due to high temperatures during extraction (Dubey *et al*., 2009).

Other reason may be due to the extracting solvent used in the present study. Although it is well accepted that water is one of the most practical solvent universally used to extract active ingredients, the aqueous extracts of these plants didn't show any inhibition effects against *A. apis*. This might indicate the relative impracticability of water as extracting solvent for the active antifungal compounds of these plants.

In this regard, many reports declared that organic solvents either used in single or mixed forms especially polar ones are most preferable for extraction of biologically active plant ingredients (De Pasquale *et al*., 1995; Ferrero *et al*., 2007). Since nearly all of the identified components

from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanolic or methanolic extraction (Nostro *et al*., 2000). Methanol and ethanol are reported as efficient extracting solvents for saponins and sterols (De Pasquale *et al*., 1995; Hui *et al*., 2007; Ivanovska *et al*., 1996), alkaloids (Ivanovska *et al*., 1996), polyphenols (Ferrero *et al*., 2007) terpenoids (Taylor *et al*., 1996) while dichloromethane is efficient in terpenoids extraction (Cowan, 1999a; Mendoza *et al*., 1997).

Therefore, initial screenings of plants for possible antimicrobial activities typically begin by using crude alcohol extractions and can be followed by various organic extraction methods. In fact, many studies avoid the use of aqueous fractionation altogether. The exceptional water-soluble compounds, such as polysaccharides and polypeptides, including fabatin (Zhang and Lewis, 1997) and various lectins, are commonly more effective as antiviral. Occasionally few tannins and terpenoids are found in the aqueous phase of some plants, but they are more often obtained by treatment with less polar solvents (Cowan, 1999).

The stimulatory effect of some plant extracts, observed in the present study, on *A. apis* growth may be attributed to the increase of some growth factor, which may found in high concentrations in the crude plant extracts and stimulate fungal development (Fiori *et al*., 2000).

5.3. The nature of antifungal effect of *P. granatum* extract

The results of the present study showed alternations in the normal growth profile of *A. apis* after exposure to different concentrations of *P. granatum* flower extract (5%, 10% and 20%) compared to the control (0 concentrations), and this alternation still significant, even after 5 days of incubation on extract free media. From these findings, it appears that the *P. granatum* flower extract exhibits a favorable fungistatic activity against *A. apis* at the different concentrations tested.

Similar results were also reported by Frias and Kozusny- Andreanid, (2009) who found that, the pomegranate extracts (8%) acted as fungistatic against dermatophyte fungi. Another study

done by Dahham *et al*. (2010) also reported high fungistatic activity of pomegranate peel against *Penicillium citrinum* and *Aspergillus niger*.

The fungistatic activity from pomegranate was attributed to the phenolic compounds; lignans (a phytoestrogen) and punicalagin (ellagitannin) (Jayaprakasha *et al*., 2006; Russalind *et al*., 2006).

In other studies Cushnie and Lamb, (2005) and Zheng *et al*. (2012) found that, the antifungal activity of different plant extracts was attributed to the presence of flavonoid compounds which are grouped into anthocyanins and anthoxantins. As anthocyanins (glycosides of anthocyanidin) are the most important group of water-soluble plant pigments in flowers and fruits of *P. granatum* (Aviram and Rosenblat, 2006). It is therefore possible to suggest that the observed activity of the extract from *P. granatum* flower in the present study is due to the presence of that compounds

5.4. The toxicity of *P. granatum* flower extract on *Apis mellifera*

In apiaries, when formulating new treatment agent to be transported by worker bees to larvae through the food, one should take into consideration the possibility of altering the taste properties of food as compared to "pure" sugar feeds. Therefore, the prerequisite to be observed in such undertakings is to make sure that the attraction of a newly formulated product for bees is the same or higher than that of pure sugar syrup thereby guaranteeing the ingestion of such product by worker bees and delivering this food together with the extract to larvae in order to control the chalkbrood disease.

The results of the present study showed that, the level of ingestion of syrups containing low concentrations of *P. granatum* flower extract was similar to that of pure sugar syrup (control) which may indicate that such addition of the extract did not affect the attraction of bees to this newly formulated product.

As foraging insect, honeybees are affected to different degrees by plant materials especially when offered as foodstuff. For example, many botanical compounds, isolated from several plant species, seem to act as insecticides (Mohamed *et al*., 2010). Extracts of *Cicer arietinum*,

Myristica fragrans and *Raphanus sativus* were found to be poisonous to worker honeybees, where, they killed 80, 70 and 55% of the experimental bees respectively (Dubey *et al*., 2009). Other compounds may act as a potent antifeedant or as an insect growth regulator ((Dubey *et al*., 2009). Hence, it was necessary to investigate about the potential effect of *P. granatum* extract on bees. Although the oral toxicity of different natural products (extracts and oils) has been already studied on the bees (Ebert *et al*., 2007), to our knowledge this is the first report regarding the use of *P. granatum* flower extract by oral administration *in vitro* assays.

When the *P. granatum* flower extract was administered in sugar syrup by systemic way, the result of the present study showed minimal mortality of *A. mellifera* when fed on low concentrations of *P. granatum* extract. At higher concentrations however, the mortality may reach to high levels.

In the groups which received high concentrations of *P. granatum* flower extract (8.8% and 22.4%), low consumption was accompanied by the highest bee mortality rate. The fact may have been due directly to starvation which may be interpreted by the high viscosity of these high concentrations of the extract. High viscosity of the solution would hinder absorption and feeding by honeybees and would result in starvation and finally death of bees.

Based on the results of the present study, it is worth to mention that, these results are most relevant to adult worker. It is possible that the adult workers could feed the extract to larvae, and that the larvae may be more sensitive. It is also possible that the extract fed to the queen or drones could affect their reproductive capacity. However, exposure of these individuals is buffered through the workers (Ebert *et al*., 2007). Unless the only source of food for the entire hive is the treated sugar water, there will be a dilution effect where the treated sugar water is mixed in the hive with nectar from outside sources.

Even in the presence of such dilution effect the highest safe concentration documented in the current research, 25%, is still 40 times higher than the recorded MIC (0.625) of *P. granatum* extract for *A. apis* spores.

As the ultimate goal of this study was to use *P. granatum* extract to treat hives for *A. apis* infection, testing the toxicity of the extract to the workers may considered as first step. The

next phase should to determine if safe doses of this extract can be fed to bees to effectively manage *A. apis* in field applications (Apiary trial).

Considering that it is safe to larvae and other colony members, the *P. granatum* extract is thought to be promising candidate for their future use for controlling the chalkbrood disease. The aqueous extract of *P. granatum* however, should further be studied for its phytochemical constituents in order to elucidate the active principle within the extract which can turn out to be a novel antifungal agent of the future.

Chapter 6

Conclusion and recommendations

6.1. Conclusion

- The results of the present study confirm the presence of chalkbrood disease in honeybees' apiaries in Gaza Strip.

- The results of the present study found that, the extract of pomegranate flowers were the most effective against *A. apis*, followed by guava, cinnamon and bottlebrush plant extracts.

- *Punica granatum* (pomegranate) flower extract has a fungistatic effect on *A. apis* mycillium growth.

- The pomegranate extract was not found to be lethal at low concentrations to adult workers responsible upon feeding the colony members.

6.2. Recommendations

- It is recommended to use *Punica granatum* (pomegranate) flower extracts or its purified compounds as a control agent for chalkbrood disease in honeybees.

- It is recommended not to use the extract of *Annona squamosa* leaves against chalkbrood disease.

- It is recommended to study the effect of new plant species extracts and materials on chalkbrood disease.

- It is very important to control *A. apis* and other honeybee diseases by means of biological control to achieve the best organic beekeeping.

- It is very important to intensify studying of the epidemiology of bee diseases in Palestine and searching for appropriate control methods and strategies.

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