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INHIBITORY ACTIVITY OF CERTAIN NATURAL PRODUCTS ON THE GROWTH OF *ASCOSPHAERA APIS*

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ABSTRACT:

This work was carried out to study the effect of some essential oils (amalaki; celery; chamomile; cinnamon; cloves; fennel; fenugreek; garlic; ginger; henna; jojoba; onion; pepper; peppermint; rose; thyme; violet; and worm-wood) and some honeybee products (honey and propolis) against *Ascosphaera apis* causing chalkbrood disease in honeybee larvae under laboratory condition. The highest reduction of mycylium growth was obtained by cinnamon; cloves; rose; thyme oils and propolis, 74.44, 71.11, 66.11, 71.44 and 68.11% respectively. Celery; chamomile; garlic; jojoba; pepper and peppermint oils, were exhibited the moderate inhibition against the causal pathogen since the growth reduction to 50.0, 46.78, 48.11, 56.33, 55.89 and 40.78%, respectively. While, fennel; ginger; henna; onion and worm-wood oils had a little inhibition against *A. apis*, where the growth reduction to 20.33, 25.89, 27.44, 29.67 and 18.11%, respectively. While, some products such as amalaki; fenugreek; violet oils and fennel honey don't show any inhibition effects against the growth of the fungi.

INTRODUCTION:

Ascosphaera apis **(Olive and Spiltoir) is a fungal pathogen causing chalkbrood disease in honeybee, *Apis mellifera* L., larvae. It is common in most beekeeping countries (Bailey and Ball, 1991). This disease rarely kills colonies but results in persistent loss of brood, which weakens colonies, leading to a reduction in honey surplus.**

Several histological studies have been made on larvae infected with chalkbrood (Carrera *et al*., 1987; Bamford and Heath, 1982 and Puerta *et al*., 1994). Nevertheless, there is still controversy about the route of invasion of the fungus into larvae.

Infection seems to be initiated by ascospores (Heath, 1982), although some authors suggest that infection is directly produced by invading hypha (Gilliam *et al*., 1978). Larvae can ingest the fungus at an early stage, but only stretched larvae, inside capped cells, present symptoms of the disease. Gilliam (1978) and Gilliam *et al*. (1978) demonstrated that eggs and pupae are not susceptible to laboratory infection.

For the control of bee pests and diseases chemicals used, which must be constrained and legally recommended otherwise bee products will be contaminated enough to be dangerous for humans (Delaplane, 1997). Currently, there are no products available for the management

of chalkbrood, despite reports that the disease has become more prevalent recently (Gilliam and Vandenburg, 1990). Possibly the increasing interest in the use of alternative therapies in the result of the development of antibiotic resistance in some microorganisms becoming a major problem. Some of these alternative therapies are essential oils; propolis and honey. Several studies have shown essential oils to be effective in controlling bee diseases such as chalkbrood (Higes *et al.*, 1998).

Essential oils are the result of a vapour hydrodistillation plant species, which are thus separated because of being immiscible in water. They are complex mixtures in whose composition there are mainly terpenic compounds, and phenols, which are being continuously studied, e.g., as natural biocide agents (Pedro *et al.*, 2006). Colin *et al*., 1989 using *in vitro* tests, demonstrated fungicidal activity of essential oils of *Thymus vulgaris*; *Satureja Montana* and *Origanum vulgare* against chalkbrood. In similar work, Davis and Ward, 2003 studied the antifungal efficacy of over 50 natural products and they found that, a number of essential oils were particularly efficacious at controlling, *in vitro*, the growth of *Ascosphaera apis*.

Honey is the natural sweet substance produced by honeybees from the nectar of plants. Honeybee products and some of its therapeutic values were mentioned in the Holy Quran. The ancient Egyptians used honey in combination with other herbs and on its own, to treat wounds and diseases of the gut (Zumla and Lulata, 1989).

Propolis or "bee glue" is a well-known substance that beekeepers find in their hives. It is one of the natural materials being used in human medicine and veterinary (Caillas, 1978) with a large spectrum of biological action.

Several authors have reported on the antimicrobial activity of propolis on fungi (Lindenfelser, 1967; Brumfit *et al.,* 1990 and Tosi *et al.,* 1996). Pepeljnjak *et al.,* (1982) found that, for pure propolis extracts, a concentration of 15-30 mg/ml was needed to inhibit the growth of *Candida albicans*; *Aspergillus flavus*; *A. ochraceus*; *Penicillium viridicatum* and *P. notatum*.

Obaseiki-Ebor *et al*., (1983) found that, at 0.5% v/v of distillated honey was fungicidal to *Candida albicans* and fungistatic to *Penicillium spp.* and *Aspergillus niger*. Radwan *et al*., (1984) stated that, growth of colonies from 30-60% of the fungi from sewage; soil; air; and trap water was found to be prevented by 25% honey.

The aim of the present work was to conduct a laboratory study on the efficacy of some natural products (e.g. propolis; honey and essential oils) against the chalkbrood fungus, *Ascosphaera apis*.

MATERIALS AND METHODS:

The present work was carried out in Plant Pathology Dept., Faculty of Agriculture, Assiut University.

1- Isolations and identification of the causal pathogen:

Larvae mummies were collected from different localities of Assiut Governorate in 2008 season. They were washed with tap water, surface sterilized for three minutes with 2% sodium hypocholorite solution, then rinsed several times in sterilized distilled water and dried between folds of sterilized filter papers. The surface sterilized mummies were plated on to Potato Dextrose Agar (PDA) medium and incubated at 27±1ºC. After 4-5 days incubation period, the developed fungal colonies were purified by hyphal tip and single spore isolation techniques. Identification of the fungal isolates was carried out by using the morphological

characteristics of mycelia and spores (Bailey & Ball, 1991), and confirmed by Assiut University Mycological Center. Assiut, Egypt.

2- Tested agents:

Ethanol propolis extract (EPE) prepared by ten grams of crude propolis, collected from Sahel Seleim, Assiut Governorate, were dissolved in 90 ml ethanol 70% (v/v). The mixture was shaken for 1/2 hour and left at room temperature for 24 h. This procedure was repeated daily for 5 successive days. The extraction was kept in a screw-capped tube and refrigerated until use. Fennel honey extracted from honeybee colonies from apiary at Assiut region. In addition to propolis and fennel honey, eighteen essential oils were obtained from El-Captain Company (Cap Farm) for extracting Natural oils; Herbs and Cosmetics El-Obour City, Cairo, were tested to controlling chalkbrood disease (Table 1).

Table (1): Identification of the tested essential oils

Oil	Common name	Scientific name
1	Amalaki	*Emblica officinalis*
2	Celery	*Apium graveolens*
3	Chamomile	*Matricaria chamomilla*
4	Cinnamon	*Cinnamomum cassia*
5	Cloves	*Eugenia caryophyllus*
6	Fennel	*Foeniculum vulgare*
7	Fenugreek	*Trigonella foenum-graecum*
8	Garlic	*Allium sativum*
9	Ginger	*Zingiber officinale*
10	Henna	*Lawsonia inermis*
11	Jojoba	*Simmondsia chinensis*
12	Onion	*Allium cepa*
13	Pepper	*Piper nigrum*
14	Peppermint	*Mentha piperita*
15	Rose	*Rosa hybrida*
16	Thyme	*Thymus vulgaris*
17	Violet	*Viola odorata*
18	Worm-wood	*Artemisia absinthium*

3-Effect of certain natural products on *Ascosphaera apis* under laboratory conditions:

Laboratory works were directed to study the effect of some natural products on linear growth of *Ascosphaera apis*. Natural products essential oils; fennel honey and EPE were added singly to PDA medium at 1000 ppm in order to test their direct effect on the tested fungi. Tested products were filtrate sterilized by Seitz filter before added to the medium. Petri dishes (9 cm in diameter) containing PDA medium were inoculated in the center with disks (5 mm) of the isolate fungi growing 7 days old culture. Four replicates were used for each treatment. Plates containing PDA medium without tested materials were used as control. Plates were incubated at 27±1°C. Linear growth of fungi was measured in mm. when fungal growth filled up control plates. Percentage of reduction in linear growth of the tested oils was determined using the following formula:

$$R = [C - (T/C)] \times 100$$

Where: R=Percentage of growth reduction, C=Diameter of the control hyphal growth, T= Diameter of the treated hyphal growth.

4- Statistical analysis:

Inhibition percentages of *A. pis* were transformed using arcsin method, then, analysis of variance (ANOVA) was carried out to determine if the treatments differed from control according to the method of Waller and Duncan (Waller and Duncan, 1969).

RESULTS AND DISCUSSION:

Pathogenicity capability of this isolate for Larvae mummies were carried out by spry 200 larvae with 30 ml of spore suspension and the larvae diseases were recorded after 7 days from spry (data not shown).

Data in Table (2), showed that cinnamon; cloves; ginger; thyme, celery; chamomile; garlic; jojoba; pepper, peppermint, fennel, henna, onion, rose, worm-wood oils and propolis caused antifungal activities against *Ascosphaera apis*. Cinnamon; cloves; rose; thyme oils and propolis, showed the strongest activity against *A. apis*. They produced inhibition rate 74.44, 71.11, 66.11, 71.44 and 68.11%, respectively.

Essential oils are complex mixture in whose composition, there are mainly terpenic compounds, and phenols, which are being continuously studied, *e.g.*, as natural biocide agents. Oils that contained oxygenated terpenes as major constituents showed the highest inhibition percentage (Pedro *et al*., 2006). *In vitro* (Stranks, 1977) reported that, citral and geraniol inhibited the fungus *Ascosphaera apis* which causes chalkbrood disease in the honeybee, *Apis mellifera*. Cinnamon oil exhibits activity against mycotoxigenic moulds, *Penicillium* spp. and *Clostridium botulinum*. Thymol, a major component of thyme oil, is highly active against *Aspergillus parasiticus* (Buchanan and Shepherd,1981) and *Clostridium botulinum* (Karapinar and Aktug, 1987).

Pepeljnjak *et al*., (1982) found that concentrations of 1500–3000 mg/ml. from pure propolis extract were needed to inhibit the growth of *Candida albicans* and *Aspergillus flavus*. The flavonoids in propolis (mainly piocembrin) have been considered to responsible for its inhibitory effect on *Candida* (Metzner *et al*., 1979).

Celery; chamomile; garlic; jojoba; pepper and peppermint oils, were exhibited the moderate inhibition against *Ascosphaera apis* where as the growth reduction to 50.0, 46.78, 48.11, 56.33, 55.89 and 40.78%, respectively. While, the lowest inhibitory reaction against *A. Apis* 20.33, 25.89, 27.44, 29.67 and 18.11% were recorded in case of the tested materials, fennel, ginger, henna, onion and worm-wood oils. The remaining essential oils and fennel honey were shown to be ineffectual against *Ascosphaera apis* in vitro test system. Our results are in agreed with several report, Efem *et al*., (1992) found that, growth inhibition of fungi causing surgical infections or wound contaminations, was complete in the media containing 100% unprocessed honey, partial in media containing 50% and no inhibition was produced by 20% honey.

Molan, 1992a stated that Fungi are generally much more tolerant than bacteria to the high osmotic effect. The same author (1992b) also stated that, no fungi can grow in fully ripened honey, but the more diluted honey becomes, the more species can grow in it.

Our results showed that, cinnamon; cloves; rose; thyme oils and propolis were efficacious in inhibition growth *Ascosphaera apis* in vitro.

From our results we can conclude that, some of natural products such as, cinnamon; cloves; rose; thyme oils and propolis may be particularly useful against *Ascosphaera apis*, pathogenic fungus documented to cause chalkbrood disease in honeybee. These materials, however, need to be researched more fully in the mode of actions of tested materials as well as the interaction between such materials and the pathogens before they may be commercially acceptable. We are continuing with this investigation to assess the practical value of the therapeutic application of these products.

Table (2): Antifungal activity of the tested natural products against the growth of *Ascosphaera apis*

Antifungal activity / Tested products	Mean diameter of growth zone (mm) ± SE	Mean diameter of inhibition zone (mm) ± SE	Mean inhibition (%) ± SE
Amalaki	90±0.000	0.0±0.000	0.0±0.000 k
Celery	45.0±0.816	45.0±0.816	50.0±0.906 e
Chamomile	47.9±0.367	42.1±0.367	46.78±0.408 f
Cinnamon	23.0±0.204	67.0±0.204	74.44±0.227 a
Cloves	26.0±0.890	64.0±0.890	71.11±0.990 b
Fennel	71.7±1.179	18.3±1.179	20.33±1.308 j
Fenugreek	90±0.000	0.0±0.000	0.0±0.000 k
Garlic	46.7±2.357	43.3±2.357	48.11±2.621 ef
Ginger	66.7±0.286	23.3±0.286	25.89±0.318 i
Henna	65.3±0.531	24.7±0.531	27.44±0.590 hi
Jojoba	39.3±0.286	50.7±0.286	56.33±0.316 d
Onion	63.3±1.179	26.7±1.179	29.67±1.308 h
Pepper	39.7±0.408	50.3±0.408	55.89±0.453 d
Peppermint	53.3±2.357	36.7±2.357	40.78±2.619 g
Rose	30.5±0.000	59.5±0.000	66.11±0.000 c
Thyme	25.7±0.408	64.3±0.408	71.44±0.455 b
Violet	90±0.000	0.0±0.000	0.0±0.000 k
Worm-wood	73.7±0.408	16.3±0.408	18.11±0.453 j
Fennel honey	90.0±0.000	0.0±0.000	0.0±0.000 k
Propolis	28.7±0.286	61.3±0.286	68.11±0.318 c
Control	90±0.000	0.0±0.000	0.0±0.000 k

Means followed by different letters within the same column are significantly different (P < 0.05, ANOVA, LSD).

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النشاط التثبيطى لبعض المنتجات الطبيعية على نمو فطر اسكوسفيرا آبيس

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** قسم أمراض النبات– كلية الزراعة– جامعة أسيوط

تمت دراسة تأثير بعض الزيوت الطبيعية (زيوت أملج، الكرفس، البابونج، القرفة، القرنفل، الشمر، الحلبة، الثوم، الزنجبيل، الحنة، الجوجوبا، البصل، الفلفل الأسود، النعناع الفلفلي، الورد، الزعتر، البنفسج، الشيح)، وبعض منتجات نحل العسل (البروبوليس وعسل الشمر) على فطر اسكوسفيرا آبيس المسبب لمرض الحضنة الطباشيري في يرقات نحل العسل وذلك فى المعمل. وكانت أعلى نسب لتثبيط نمو الفطر هي 74.44، 71.11، 66.11، 71.44، 68.11٪ في البيئات المحتوية على زيوت كل من القرفة، القرنفل، الورد، الزعتر والبروبوليس على التوالي .بينما أظهرت المعاملة بزيوت كل من الكرفس، البابونج، الثوم، الجوجوبا، الفلفل الأسود والنعناع الفلفلى تأثيراً متوسطاً فى تثبيط نمو المسبب المرضى حيث كان التثبيط بنسبة 50.0، 46.78، 48.11، 56.33، 55.89، 40.78٪ على التوالى. في حين كان لزيوت الشمر، الزنجبيل، الحنة، البصل والشيحتأثيراً قليلاً على تثبيط نمو فطر اسكوسفيرا آبيس حيث كانت نسب التثبيط 20.33، 25.89، 27.44، 29.67، 18.11٪على الترتيب. بينما لم تظهر بعض المركبات أي تأثيرات مثبطة على نمو الفطر وهذه المركبات هي زيوت كل من الأملج، الحلبة، البنفسج وكذلك عسل الشمر .