CHAPTER I

INTRODUCTION

1.1 Propolis

"Propolis" or bee-glue is a sticky brownish resinous material collected by foraging workers (Bankova *et al.*, 2000) from the leaf buds of numerous tree species. Propolis is a complex resinous bee product with a physical appearance that varies widely, depending on many factors. The color may be cream, yellow, green, light or dark brown. Some samples have a friable, hard texture, while other samples may be elastic and gummy as shown in Fig. 1.1.

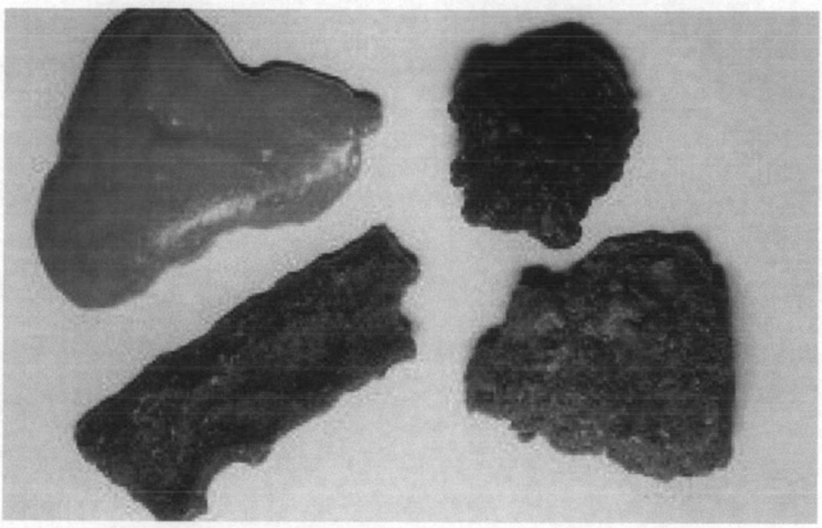

Fig. 1.1 The variety of bee propolis colour.

The term propolis is derived from the Greek *pro* (for, in front of, at the entrance to) and *polis* (community or city) and means a substance in defence of the hive. Bees use propolis for diverse purposes, among them to seal openings in the hive. In addition to avoiding the entrance of intruders, this contributes to maintain the hive inner temperature at to seal openings in the hive. In addition to avoiding the entrance of intruders, this contributes to maintain the hive inner temperature at around 35 °C. It is believed that propolis not only hardens the cell walls but also contributes to the attainment of an internal aseptic environment. The entrance to the hive is also lined internally with propolis. Evidence that leaves no doubt as to the antimicrobial properties of propolis comes from another use of the product in the hive: bees cover the carcasses of intruders that were killed and are too heavy to be thrown off the hive with propolis. The process comes close to an embalming effect, because the dead bodies dry out without undergoing putrefaction (Ghisalberti, 1979). This is obviously important to protect the hive from a widespread bacterial and fungal infection. Recognition of the antiseptic efficacy of propolis is ancient. Aristotle recommended the use of propolis to treat abscesses and wounds. Roman soldiers carried propolis as an emergency medicine for war wounds. A medicine containing vaseline and propolis (propolisin vasogen) was used for wound treatment during the Boer war (Matsuno, 1997). Propolis composition is extremely complex. The main constituents are beeswax, resin and volatiles. The bees secrete beeswax, while the latter two constituents are obtained from plants. But contrary to the well-known habit of visiting flowers for collection of nectar and pollen, bees usually take plant materials for propolis from plant secretions or by cutting fragments of vegetative tissues. The biological activity of propolis is assigned to these plant derived substances. Propolis is obviously an animal product, a considerable proportion of its components, chiefly those upon which rest its biological activity, are plant derived. The resin contains most of the compounds found in alcohol extracts which were consumed by people from many countries as food complements or alternative medicine. Furthermore, propolis contains other constituents, such as pollen and amino acids (Gabrys *et al.*, 1986, Marcucci, 1996). Over 300 known substances in propolis were registered. And in the last 10 years, were added a great deal of other substances (Marcucci *et al.*, 1995, Bankova *et al.*, 2000). Recently, propolis started gaining appreciation as a means for the treatment of health problems. Up to that time, propolis was regarded by beekeepers as an unwanted hive by-product, since it had no market value and its production meant a decline in the amounts of honey obtained. Starting with a gradual rise in interest from people from several countries in the mid 1980s, propolis ended up as an important product in complementary and alternative medicine. Japan is the leading importer of propolis, with a manifest preference for propolis from Brazil. Presently, many beekeepers in Brazil develop means to maximize propolis production and produce it as their major product. One of them is to leave longitudinal slits on both sides of the wooden box that shelters the bee colony. This method relies on the bee's behavior of depositing propolis seals on all openings which more detected in the hive (Fig. 1.2). A great enthusiasm characterizes present-day propolis research, driven by positive results in pharmacological tests, dealing not only with anti-microbial activity, the first and as yet the most investigated effect in propolis research, but also with a wide diversity of effects, including immune activation and cytotoxicity (Banskota *et al.*, 2001).

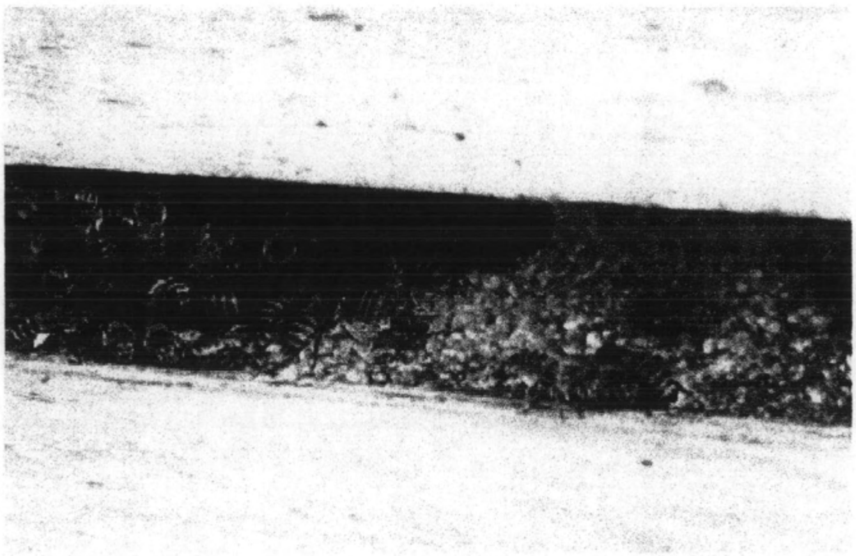

Fig. 1.2 Honeybee hive with a lateral slit, which enhances propolis production. Propolis was removed from the left side and bees are working to seal the resultant opening with new propolis deposits. (Salatino *et al.*, 2005)

Besides pharmacological activity, an important point in propolis research refers to its botanical origin and the consequent variation in chemical composition when samples from different locations, and even from the same locality, are compared. Propolis samples produced in Europe and South America share several characters; antimicrobial, antiviral, wound-healing, immune stimulating, antiinflammatory and anesthetic activities. However, similar as these samples might be in their biological activities, they are quite different chemically (Kujumgiev *et al.*, 1999), because different plants in Europe and South America provide resin for propolis production in the two continents. In typical European propolis the major pharmacologically active constituents have long been identified as flavonoids, the most common and abundant being galangin. It is now well established that such a chemical profile is a consequence of the fact that bees collect propolis resin mainly from vegetative poplar

(*Populus nigra*, Salicaceae) buds in Europe (Maciejewicz *et al*., 2001). In the tropics, poplars are seldom cultivated; plants are sources of propolis resin. For example, the flowers in Cuba (Cuesta-Rubio *et al*., 2002) produce resin, which bees collect for propolis production so alternative. In both cases, the major compounds are polyprenylated benzophenones and flavonoid are minor constituents.

Propolis chemical types, determined by its plant origin

The materials available to bees for "manufacturing" of propolis are substances actively secreted by plants as well as substances exuded from wound of plants: lipophilic materials on leaves and leaf buds, resins, mucilages, gums, lattices, etc. The composition of the plant source determines the chemical composition of bee glue. Combined with the knowledge of active principles, it gives clues to standardization and quality control, allowing the specification of propolis types that have distinct chemical composition. The present knowledge on the most important biologically active chemical constituents of propolis from different geographic locations and the corresponding plant sources is represented in Table 1. The table defines chemical types of propolis which have to be regarded as distinct entities in the process of standardization and quality control. It is important to remember that conclusions concerning the biological activity of one of these propolis types can by no means be automatically transferred to another one. In Table 1, the most studied propolis types are mentioned. Of course, there are many other propolis source plants and corresponding chemical types of propolis. For example, the one found in some Mediterranean regions (Sicily, the Adriatic coast) has the main components as diterpenic acids (Trusheva *et al*., 2003). In Brazil, 11 propolis types other than Alecrim (green) propolis have been described, but their occurrences are much more restricted (Park *et al*., 2002). However, it is important for researchers studying biological activity of propolis to be aware of the existence of the problem and to be able to distinguish between different propolis types. It is essential to have detailed and reliable comparative data on every type of biological activity, combined with chemical data, in order to decide if some specific areas of application of a particular propolis type can be formulated as preferable. The biological tests have to be performed with chemically well characterized, and if possible, chemically standardized propolis. A reliable criterion for chemical standardization of different propolis types is needed. However, such generally accepted criteria do not yet exist for any propolis type. The recent studies were directed towards the possibilities to standardize poplar type propolis. (Salatino *et al*., 2005)

Table 1.1 Propolis types according to their plant origin and their chemical compositions. (Salatino *et al*., 2005)

Propolis type	**Geographic origin**	**Plant source**	**Main biologically active substances**
Poplar propolis	Europe, North America, non-tropic regions of Asia	*Populus* spp. of section Flavones, *Aigeiros*,most often *P. nigra* L.	flavanones, cinnamic acids and their esters
Birch propolis	Russia	*Betula verrucosa* Ehrh.	Flavones and flavonols (not the same as in poplar propolis)
Green (alecrim) Propolis	Brazil	*Baccharis* spp., predominantly *B.dracunculifolia* DC.	Prenylated *p*-coumaric acids, diterpenic acids
Red (Clusia) propolis	Cuba, Venezuela	*Clusia* spp.	Polyprenylated benzophenones
Pacific propolis	Pacific region (Okinawa, Taiwan)	Unknown	C-prenylflavanones
Canarian propolis	Canary Islands	Unknown	Furofuran lignans

1.2 Stingless bee

Stingless bees, the highly eusocial hymenopteran insects belonging to subfamily Meliponinae (Wille, 1983), have no sting which honey bees use for defensive purposes. Workers of stingless bee species use other means of defense very aggressive. In some species, they ejected burning liquid while they were biting their predators (Radovic, 1981).

Stingless bees are easily distinguished from honeybees which belonging to subfamily Apinae by the reduced wing venation, presence of a penicillum (a brush of long setae on the outer, apical surface of the hind tibia), and a vestigial sting (Michener, 1990, 2000; Ruttner, 1988; Will, 1979, 1983). Honeybees and stingless bees are the only highly social bees, but stingless bees lead more diverse life styles, including obligate necrophagy (Camargo and Roubik, 1991; Roubik, 1982), and can recruit for resources such as dead animal, pollen, nectar, mud, resin, water, and nests (Roubik, 1989). Only some members of the stingless bee families store honey in quantities that can be harvested by man (Ruttner, 1988).

Stingless bees are of great importance for the pollination of many wild plants (Augspurger, 1980; Nogueira-Neto, 1970) in addition to tropical crops (Roubik, 1990). Some species swarm out from the nest to attack a large intruder more aggressively than the workers of *Apis*. However, instead of stinging, they bite, and can be very effective because they crawl into the eyes, nose, ears, or hair; their mere presence is a terrible nuisance and their ability to nip with the jaws makes almost any enemy retreat (Michener, 1974). Species of one subgenus, *Trigona* (*Oxytrigona*), drop the caustic secretion from the enlarged mandibular glands (Kerr and Costa, 1961) on the skin of the intruder and then bite it into the flesh with the mandibles. The result is very painful and causes lesions that last for many days and may cause almost permanent scar.

The tribe of Meliponini is generally divided into five different genera; (1) *Melipona* lives only in tropical America; (2) *Meliponula* of which only a single species is found in Africa; (3) *Trigona* is spread in all the tropics and occupies 16 subgenera having a large variation in morphology and biology; (4) *Dactylurina* has only a single species in Africa; (5) *Lestrimelitta* is found in Africa and tropical America (Crane, 1999; Michener, 1974; Rinderer, 1986).

1.2.1 *Trigona (Tetragonula) laeviceps* Smith, 1857

The taxonomic hierarchy of *Trigona laeviceps* has been recognized as follows (Sakagami *et al*., 1983; Schwarz, 1939):

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Family Apidae

Subfamily Meliponinae

Tribe Meliponini

Genus *Trigona*

Species *Trigona laeviceps* Smith, 1857

Trigona laeviceps is the most common species in Thailand. Distribution of *Trigona laeviceps* in Thailand is shown in Fig. 1.3.

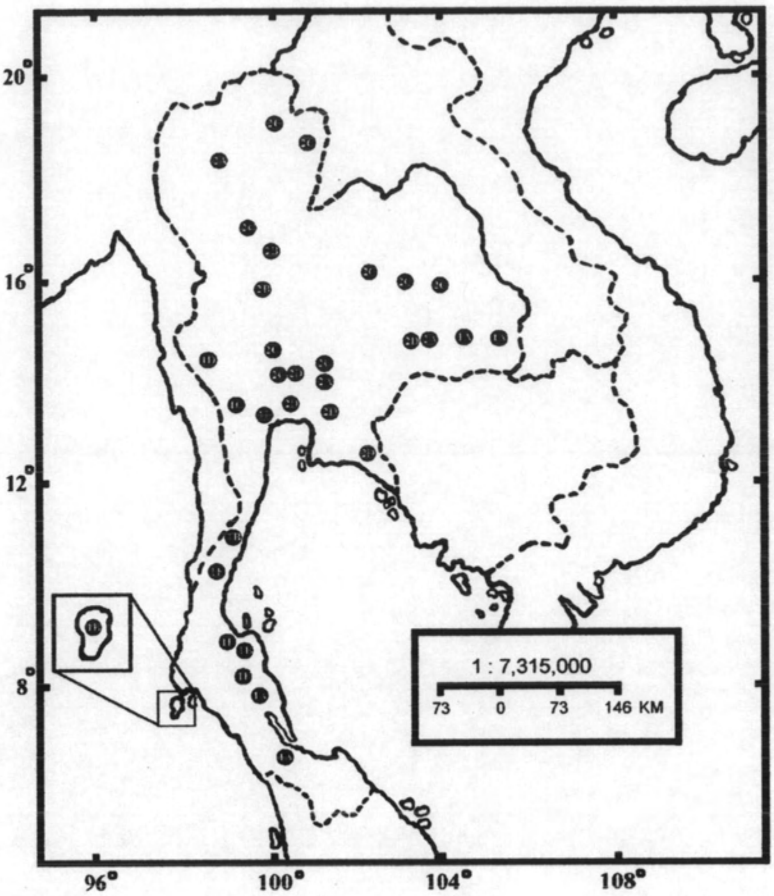

Fig. 1.3 Distribution of *Trigona laeviceps* in Thailand (Phuphisut, 2004)

Its nesting site is usually found in a cavity of human architecture. Its nest entrance diameter is about 1.0 cm. Morphological characters of this species are as followed; body and wing length are each 3.5-4.0 mm., body predominantly dark, fore wings rather uniformly transparent or slightly infuscate (Sakagami *et al*., 1985) (Fig. 1.4).

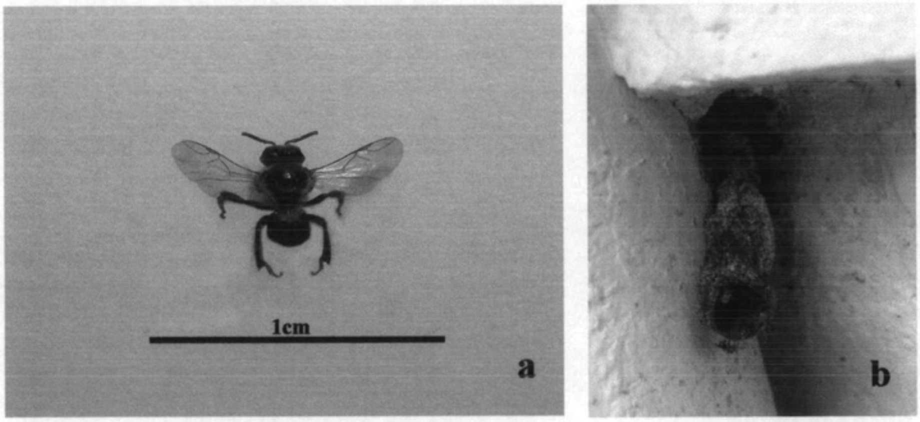

Fig. 1.4. *Trigona laeviceps* (a) (Phuphisut, 2004) and its nest entrance tube (b).

In Thailand, all species of stingless bees are commonly known as "Channarong". They are found throughout Thailand and are important pollinators of natural plants. Because of their visit a wide range of crops (polylecty), tolerant of high temperatures, active throughout the year (Amano *et al*., 2000) and absconding of entire colonies as a response to disturbance is extremely rare in stingless bees (Eltz *et al*., 2003). Individual colonies are generally perennial and reported maximum life spans range from 10 to 26 years (Roubik, 1989; Wille, 1983). Two species of stingless bees, *Trigona laeviceps* and *Trigona pagdeni*, they are kept by farmers in hive for agricultural pollination (Phuphisut, 2004).

1.2.2 Nest architecture and colony characteristics of *Trigona laeviceps*

Meliponini live in perennial colonies and have distinct female castes: queen (or gyne if not yet mated) and worker. The distribution of the Meliponini is confined to the tropical and southern subtropical areas throughout the world. In general they build their nests in empty tree trunks of different tree species with trunks of about 30-50 cm in diameter. The hollow space they occupied apparently had been formed through a process of decay and rotting, hollow branches or mainly dead trees, although some species build completely exposed nests. They spread 1- 2 mm thickness of resin around the entrance to their hive and, mixed with wax to form cerium, are used to make the structures inside the nest. Resin is also used to seal holes in the hives, exclude draught, protect against external invaders and mummify their carcasses.

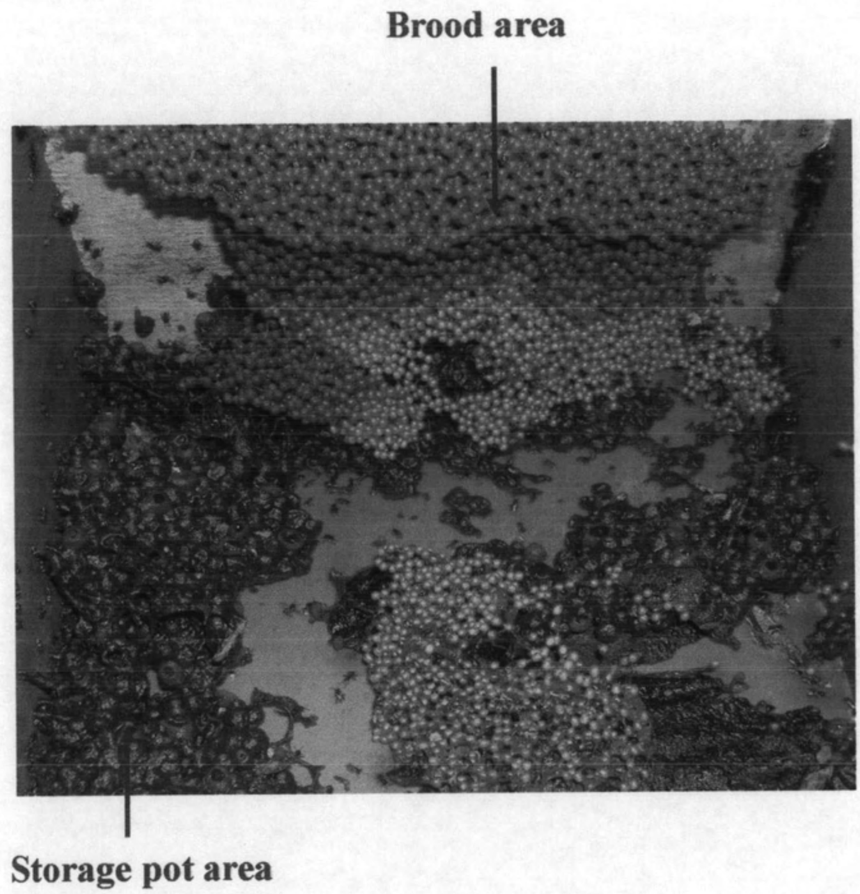

Fig. 1.5 Nest architecture of *Trigona laeviceps* in the hive

Nest architecture and colony characteristics of *Trigona laeviceps* were shown in Fig. 1.5. The oval brood cells (4.5-5 mm long) are arranged in amorphous clusters. The cells are connected to each other by pillars. New cells are brownish, but appeared yellow after the wax removal exposed the cocoons. Brood clusters are not covered with an involucrum. Several separate clusters of brood cells are found in the nests. There are also several clusters with storage pots. Honey and pollen are stored in separate pots of the same size (5-7 mm diameter, 12-15 mm high), but pollen pots are found closer to the brood masses. Several separate masses of plant resin and resinous material with a strong smell are found at the bottom of the nest. Males and workers are reared in identical cells. Queen cells, elliptical, are sporadically constructed at irregular positions in the brood nest. The number of queen cells is found from zero to seven. The volume of the nests is from 2.0 to 8.5 liters. The estimated number of brood cells is from 650 to 3,000 cells and the number of adult bees is estimated from 487 to 1,150 bees. The total volume of stored (pollen and honey) food is estimated from 1.0 to 5 liters. (Chinh *et al.*, 2004)

1.3 Chalkbrood disease

Chalkbrood disease in honeybees *Apis mellifera* L. is caused by an infection of *Ascosphaera apis* which affects the developing brood. Larvae ingest the fungal spores when feeding, permitting the disease to develop in the stretched larvae after sealing. The strentched larvae are killed and later dry, leaving a mummified cadaver reminiscent of a small piece of chalk, which becomes dark if fruiting bodies of the fungi are formed (sporulated mummies). At the peak of the disease, mummies are easily detected at the entrance to the hive as house bees remove them from their cells. Most fungi associated with *Apis mellifera* colonies are not a problem for beekeepers, only chalkbrood disease or ascosphaeriosis, caused by *Ascosphaera apis* is a problem. *Ascosphaera apis* was firstly recognized and classified by Spiltoir and Olive (1955). This fungus 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, all decline. Occasionally, severe cases of chalkbrood disease have been reported to kill colonies, but this is unusual.

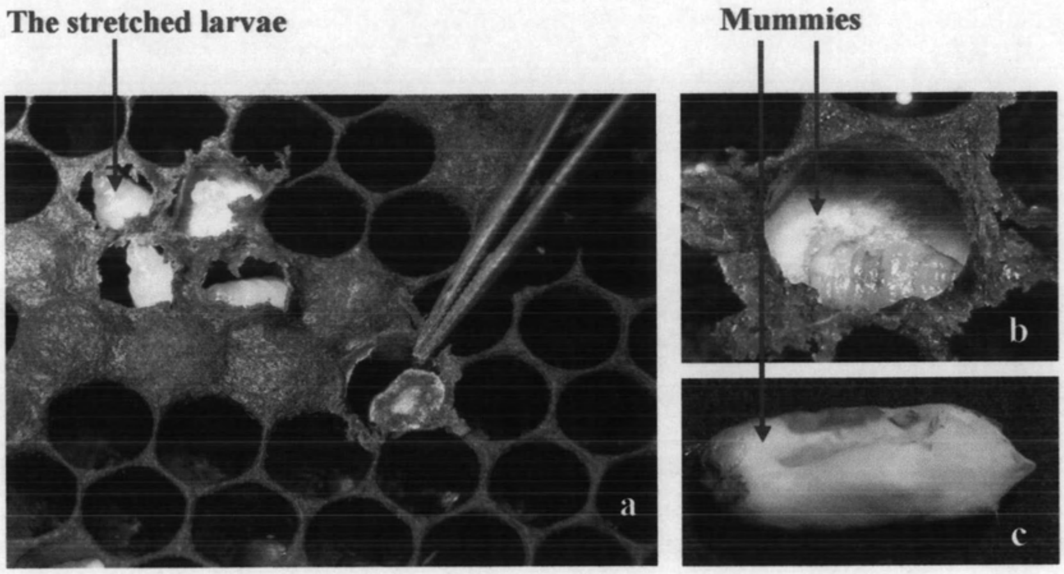

Fig. 1.6 Honeybee larvae which were infected by chalkbrood disease.(a: Stretched larvae are killed by the disease, b and c: The cadaver reminiscent of a small piece of chalk.)

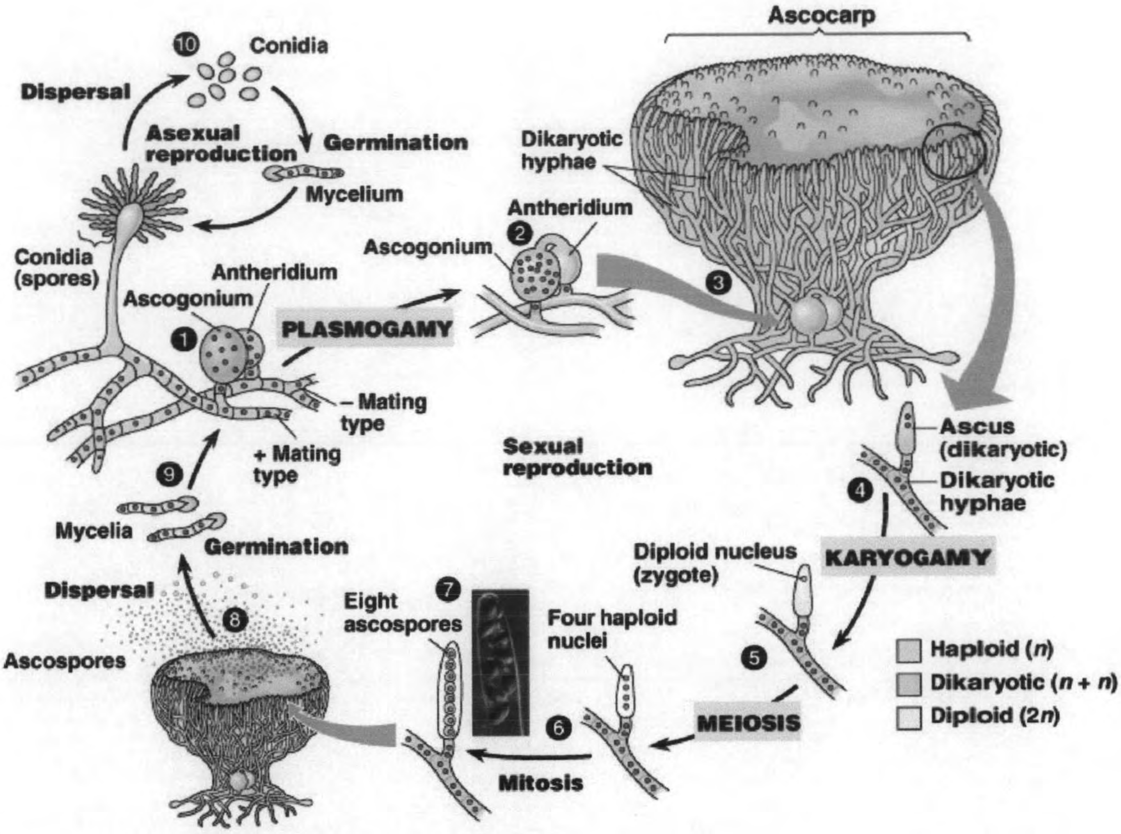

Fig. 1.7 The ascomycota life cycle

(http://www.biology.lsu.edu/heydrjay/1002/Chapter24/lifecycles/lifecycle.html)

The taxonomic position of *Ascosphaera apis*

Subdivision: Ascomycotina

Class: Plectomycetes

Order: Ascosphaerales

Family: Ascosphaeraceae

Genus: *Ascosphaera*

Species: *Ascosphaera apis*

Ascosphaera apis has white and septate hyphae. No conidial state seems to be present and the fungus is heterothallic, the strains being morphologically distinct only during reproduction. When + and - strains are grown in close proximity, sexual reproduction with the formation of spore cysts occurs. The colour of the spore cysts is olive green or brown and the form is globular. They occur in discrete areas or spread all over the agar when the fungus is cultured, and cover the surface of the mummy (except the head region). The spore cyst contains many spore balls and the ascospores are hyaline and smooth. Potato dextrose agar(PDA) is the most frequently used for culture. The culture plates must be maintained at 30°C under aerobic conditions.

The non-sporulate cultures which obtained from tinged hyphae, have produced white, cotton-like, compact and fast growing colonies that reached from 5 to 7 cm in diameter during 7 days. The sporulate cultures could be obtained from shallow rasping of black mummies, presented themselves as gray to black colonies, as a consequence of the abundance of ascocysts mixed with the mycelium, of centrifugal growth, and with radial sectors.

Management strategies, chemicals and the use of bees that show resistances to chalkbrood disease have all been shown some benefit although no individual control method will ensure a cure for the disease. The effective control of chalkbrood disease will probably require a combination of control methods. The fact that *Ascosphaera apis* is so widespread makes the possibility of its eradication unlikely. If the disease cannot be eradicated, then any chemical that is considered for using against chalkbrood disease must be demonstrated to produce minimal residues in honey or other bee products. Longterm use of any chemical for disease control is likely to result in the development of resistance to that chemical. However, the use of a chemical that controls chalkbrood disease and does not leave residues in honeybee products would be useful to beekeepers, even if *Ascosphaera apis* did develop resistance. Development of antibiotic resistance by a honeybee pathogen has already occurred in the beekeeping industry of several countries where American

Foulbrood disease has developed resistance to oxytetracycline in the long term use.

This research has attempted to test propolis from nest of the stingless bee *Trigona laeviceps* for the control of chalkbrood disease. The experiment followed up on this interest in biological properties of propolis. Propolis sample was collected from nest of the stingless bee *Trigona laeviceps* in Thailand. Fractions of the methanolic extract were tested for antifungal activity against chalkbrood disease in honeybees *Apis mellifera* L.

Therefore, the main objectives of this research are as follows:

1. To search for bioactive compounds isolated from nests of the stingless bee *Trigona laeviceps*.
2. To study antifungal activity of bioactive compounds isolated from propolis and their inhibitory effect against chalkbrood disease in honeybees *Apis mellifera* L.