Journal of Chemical Ecology, Vol. 20, No. 7, 1994

CHEMICAL COMPOSITION OF NORTH AMERICAN BEE PROPOLIS AND BIOLOGICAL ACTIVITY TOWARDS LARVAE OF GREATER WAX MOTH (*Lepidoptera: Pyralidae*)

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(Received February 19, 1993; accepted March 3, 1994)

Abstract—Bee propolis is a sticky amalgamation of plant resins collected by honeybees (*Apis mellifera* L.) and used in the hive for filling cracks and repairing combs. Propolis contains a diversity of compounds of plant origin, and is reported to have medicinal, antimicrobial, insecticidal, and phytotoxic properties. We examined the physical and chemical composition of North American samples of bee propolis from several sites in North America and tested for bioactivity against larvae of the greater wax moth (*Galleria mellonella* L.), a common apiary pest. The amount of methanol-extractable resin in samples from Ohio and Georgia ranged from 24% to 79% by weight. Propolis collected from hives in Ohio was more chemically diverse (over 30 compounds detected by paper chromatography) than material from south Georgia (fewer than 10 major compounds) and contained a lower proportion of methanol-insoluble beeswax. The paper chromatographic surveys revealed little variation in the chemical profile of specific hives over a six-month period and no differences between propolis from adjacent hives. Four flavonoids were identified from propolis collected in Ohio: kaempferol, galangin, 3,3′-dimethoxyquercetin and 3-methoxykaempferol. When mixed into artificial diet, fractionated propolis reduced larval growth of the greater wax moth, but not dramatically. An array of phenolics reported from propolis (caffeic acid, chrysin, ferulic acid, galangin, kaempferol, and quercetin) were bioassayed individually for effects on larvae, but none reduced larval growth at the

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0098-0331/94/0700-1783$07.00/0

concentrations tested, suggesting that wax moths are tolerant of some phenolics in their diet.

Key Words—*Galleria mellonella*, *Apis mellifera*, Pyralidae, Apidae, Lepidoptera, Hymenoptera, greater wax moth, honeybee, propolis, plant resins, phenolics.

INTRODUCTION

Propolis is a gluelike substance that honeybees (*Apis mellifera* Linnaeus) collect from plant exudates and use in the hive to fill cracks and repair comb. It is reported to have bacteriostatic properties that help control the microflora in the hive, and antibacterial, antifungal, antiviral, and phytoinhibitory properties of propolis and its constituents have been demonstrated by a number of workers (Ghisalberti, 1979; Pepeljnjak et al., 1985; Serkedjieva, 1992). Over 70 compounds, mostly flavonoids, have been reported from propolis in Europe (Bankova et al., 1982, 1983; Ghisalberti, 1979; Maciejewicz et al., 1985; Nenov et al., 1983; Popravdo et al., 1982), but the composition and biological activity of propolis in North America is largely unstudied (but see Lindenfelser, 1967). Recently, propolis mixed into artificial diet was found to reduce growth and survival of greater wax moth larvae (*Galleria mellonella* L. Pyralidae: Lepidoptera), (Eischen and Dietz, 1987), which feed on honeycomb and pollen and are pests of apiaries. Eischen and Dietz (1987) suggested that the biological activity of propolis might provide new opportunities for the biological control of the greater wax moth, since the use of insecticides in active hives is problematic. This project was undertaken to characterize the chemical composition of bee propolis from several apiaries in the eastern United States and to evaluate the effects of propolis fractions on survival and growth of wax moth larvae. Several phenolics commonly found in European propolis (caffeic acid, chrysin, ferulic acid, galangin, kaempferol, and quercetin) were also assayed for activity against greater wax moth larvae.

METHODS AND MATERIALS

Source of Propolis. Propolis of Italian honeybees (*A. mellifera ligustica* Spinola) was collected at three sites in the United States: western Ohio, north Georgia (Athens), and south Georgia (Claxton). In western Ohio, hives were surrounded by predominantly deciduous forest; in north Georgia by a variety of deciduous trees and conifers, and in south Georgia, hives were located in pine forest.

The following characteristics of propolis from the three sites were compared: (1) relative amounts of methanol-soluble resin and insoluble residue

(beeswax), (2) the proportions of material in different solvent fractions, and (3) the chemical profile of the fraction richest in phenolics and other ultraviolet-absorbing compounds (ethyl acetate). Seasonal and between-hive variation in chemical composition at one site (north Georgia) was evaluated by comparing paper chromatograms of the ethyl acetate fraction of monthly samples from three adjacent colonies (ca. 2 m apart). At this site, propolis was sampled from June 1987 to May 1988 excluding winter months (September through February) when hive activity was low. Propolis was collected by scraping the lid and upper edges of the topmost super of multiple hives at each site and stored at −4°C until analysis.

Fractionation of Propolis and Chromatographic Survey. Frozen propolis (three Ohio samples and three Georgia samples) was ground into a coarse powder and extracted in four volumes of 80% methanol (24 hr each). The insoluble residue that remained (mostly beeswax) was removed by filtering and dried at room temperature. The methanol-soluble portion of propolis was fractionated as follows: extract was evaporated in vacuo to a near-aqueous solution (aqueous methanol) and partitioned with petroleum ether (petroleum ether I) followed by ethyl acetate. The ethyl acetate fraction was then partitioned with petroleum ether (petroleum ether II). The percent composition of resin and wax fractions of Ohio (three samples) and north Georgia (two samples) propolis were compared using Student's t test ($P < 0.05$). One sample from south Georgia was also fractionated, but was not included in the analysis because the extraction procedure was not replicated.

For bioassays, the ethyl acetate fraction of one sample of Ohio propolis was further fractionated on a Sephadex LH 20-100 flash column eluted with a methanol gradient (50%–100%). Fractions (100 ml) were collected as they eluted and combined into four major fractions (designated A, B, C, and D in order of elution) based on the similarity of ultraviolet-absorbing compounds on two-dimensional paper chromatograms.

After preliminary surveys of chromatograms of the four major fractions (aqueous methanol, petroleum ether I, petroleum ether II, and ethyl acetate) from each site, efforts to identify compounds were restricted to the ethyl acetate fraction, which contained the majority of ultraviolet-absorbing compounds. Compounds in these fractions were isolated using two-dimensional descending paper chromatography (2D-PC) developed in two solvent systems: TBA (*t*-butyl alcohol–acetic acid–H_2O, 3:1:1, v/v, long dimension) and 15% acetic acid (glacial acetic acid–H_2O, 15:85, v/v, short dimension). Chromatograms were observed under long-wave ultraviolet light (365 nm) with and without ammonia fuming.

Isolation and Identification of Compounds. Compounds were isolated using 2D-PC developed in a variety of solvent systems, including water, Forestal's solvent system (HOAc–H_2O–HCl, 30:10:3) or the BAW system (*n*-butanol–

HOAc–H_2O, 4:1:5) (Markham, 1982; Mabry et al., 1970). Compound purity was verified by thin-layer chromatography (polyamide developed in chloroform–methanol–methyl ethyl ketone–acetone, 15:10:5:1, v/v (Stahl, 1969). Flavonoid identifications were based on spectral characteristics (following Markham, 1982; Mabry et al., 1970; Jay et al., 1975), R_f values from two solvent systems (one-dimensional paper chromatograms in TBA and 15% acetic acid), and co-chromatography with standards (3,3′-dimethoxyquercetin and 3-methoxykaempferol supplied by T. Mabry).

Bioassay of Propolis Fractions and Selected Flavonoids. Greater wax moths used in the bioassays were less than three generations from the wild (collected from Wilbanks Apiary, Claxton, Georgia). Larvae were reared on a pollen–honeycomb diet (63:37) at 27°C in partial darkness as described by Eischen and Dietz (1987). Feeding assays were initiated by placing unhatched eggs in glass jars (approximately 120 ml) ventilated by 1-cm screened holes in the lid. Larvae were allowed to feed until the prepupal stage, then all jars were frozen and larvae were retrieved from the diet.

Diet for the assays was prepared by hand mixing propolis fractions (10% propolis g/g dissolved in 70 ml ethanol) into the pollen–honeycomb diet, then allowing the solvent to evaporate by spreading the diet in shallow pans over low heat (approximately 40°C) for several days. To ensure complete evaporation of solvent, diet was then ground into pea-sized chunks and lyophilized for 48 hr. Two control diets, one treated with ethanol and another with nothing added, were prepared simultaneously and handled the same as the treatment diets. After lyophilization, dried diet was weighed directly into glass jars and rehydrated with distilled water.

In the first bioassay, only the methanol-insoluble residue (beeswax) and aqueous methanol fraction were tested. In the second bioassay, the methanol-soluble fractions (petroleum ether I, petroleum ether II, and the four component fractions of the ethyl acetate fraction A, B, C, D) were tested. The following commercially available phenolics were also included in the second bioassay: kaempferol (0.05%), quercetin (0.05, 0.25, 0.5%), chrysin (0.05, 0.25, 0.5%), galangin (0.05%), caffeic acid (0.05%), and ferulic acid (0.05%). All were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, except for kaempferol, which was purchased from Sigma Chemical Co., St. Louis, Missouri. Each diet treatment was replicated in 15–20 jars containing 10–15 larvae per jar. Significant diet differences were detected by ANOVA on larval dry weights (log transformed to correct for unequal variances) and Dunnett's test (Steel and Torrie, 1960) used to compare treatment means to the solvent control. Larval survival was not analyzed because only the pooled survival across all replicates per diet treatment had been recorded.

RESULTS

Composition of Propolis—Geographic and Seasonal Variation The amount of methanol-soluble resin in propolis from different sites ranged from a low of 23.3% in south Georgia to 78.3% in Ohio. The remainder of the propolis consisted of methanol-insoluble residue (mostly beeswax). The proportion of insoluble residue was significantly higher in propolis from north Georgia than in samples from Ohio, in which the ethyl acetate fraction constituted a higher percentage of the gross weight (Table 1). In contrast, in samples from north Georgia, the petroleum ether II fraction was the largest fraction.

Judging from the paper chromatographic profiles of extracts, propolis collected in south Georgia appeared to have low chemical diversity, with fewer than 10 major compounds visible. Chromatograms of Ohio propolis typically contained more than 30 ultraviolet absorbant spots. We detected little qualitative differences within sites, nor was there evidence of seasonal or between-hive variation in the monthly samples taken from adjacent hives at the north Georgia site.

Isolation and Identification of Compounds. Eleven compounds with flavonoid like ultraviolet spectra were isolated from the ethyl acetate fraction of Ohio propolis. Four flavonoid aglycones were identified as kaempferol, galangin, 3,3′-dimethoxyquercetin, and 3-methoxykaempferol from their spectral characteristics and cochromatography with authentic standards. All but the latter have been reported from European propolis. The R_f values and changes in absorbance maxima after addition of shift reagents for four of the remaining compounds suggested that they were also methoxylated flavonoids, but their

TABLE 1. PERCENT OF EXTRACTABLE RESIN AND INSOLUBLE RESIDUE (WAX) IN FRACTIONATED BEE PROPOLIS COLLECTED FROM HIVES AT THREE GEOGRAPHIC LOCATIONS[a]

	Ohio (N = 3)	North Georgia (N = 2)	South Georgia (N = 1)
Insoluble residue	25.1 ± 7.1a	55.3 ± 6.8b	76.0
Extractable resin			
Aqueous methanol	0.80 ± 0.7a	1.7 ± 0.4a	1.1
Petroleum ether I	0.93 ± 0.4a	4.4 ± 0.6a	0.1
Petroleum ether II	8.0 ± 12.9a	37.6 ± 4.8a	0.1
Ethyl acetate	62.7 ± 8.2a	14.4 ± 3.9b	22.0

[a] Means with different letters are significantly different between Ohio and north Georgia sites, Student's t test ($P > 0.05$). N = number of propolis samples extracted.

structures were not elucidated. We did not detect several compounds commonly reported in European propolis, including ferulic acid, caffeic acid and cinnamic acid, based on cochromatography with authentic standards.

Effects of Propolis Constituents on Wax Moth Larvae. Neither the aqueous methanol fraction nor the methanol-insoluble residue significantly reduced growth of greater wax moth larvae compared to their respective control treatments (Table 2); in fact, the aqueous methanol fraction significantly increased larval weight. In the second bioassay, the propolis fractions (ethyl acetate A, B, C, D, petroleum ether I and II) reduced larval weight compared to the solvent control, but differences were not significant, perhaps due to the high variance in the solvent control weights (Table 3). Several of the selected phenolics, ferulic acid (0.05%), quercetin (0.05%), and chrysin (0.25% and 0.5%), positively affected larval weight, but not significantly.

DISCUSSION

The gross composition of North American propolis (percent beeswax and methanol-soluble resin) is variable, but within the range reported for European propolis (Ghisalberti, 1979). The proportion of beeswax to plant resin in propolis likely depends on the availability of plant resins and the specific use to which it is applied within the hive. Propolis used to repair honeycomb is often supplemented with larger quantities of wax to give it a firmer composition, while propolis applied in a thin coat to the surface of comb usually contains little or no wax (Meyer, 1956). Bees may also incorporate more wax into propolis during periods when resins are scarce or difficult to collect (Meyer, 1956). The low proportion of resin in propolis collected from south Georgia in this study may reflect a low availability of collectable resins in pine forests (Popravdo, 1977).

The chromatographic surveys of Ohio and north Georgia propolis revealed

TABLE 2. SURVIVAL AND DRY WEIGHT OF GREATER WAX MOTH LARVAE FED DIETS CONTAINING INSOLUBLE RESIDUE (WAX) AND AQUEOUS FRACTION FROM PROPOLIS

Treatment	Survival (%)[a]	Dry weight (mg)	N^b
Diet (control)	86	84.1 ± 4.2	19
Diet + insoluble residue	91	79.0 ± 4.9	20
Diet + solvent (control)	69	43.1 ± 4.5	15
Diet + aqueous fraction	81	62.1 ± 6.3	17

[a] Percent survival from first instar to end of experiment (all replicates pooled).
[b] N = number of replicate jars, each replicate consisting of 10–15 larvae.

TABLE 3. SURVIVAL AND DRY WEIGHT OF GREATER WAX MOTH LARVAE FED DIETS CONTAINING FRACTIONATED PROPOLIS AND SOME SELECTED PHENOLIC COMPOUNDS

Treatment	Dry weight (mg)[a]	Survival (%)[b]	N
Untreated diet	46.3 ± 8.7[c]	100	14
Solvent control	23.2 ± 7.8	81	14
Ethyl acetate A	12.0 ± 1.8	57	19
Ethyl acetate B	8.3 ± 1.5	36	17
Ethyl acetate C	11.1 ± 2.0	90	18
Ethyl acetate D	12.7 ± 2.2	98	18
Petroleum ether I	9.3 ± 1.4	98	18
Petroleum ether II	9.2 ± 1.4	77	17
Ferulic acid 0.05%	37.5 ± 7.0	100	13
Quercetin 0.05%	32.8 ± 6.2	99	15
Quercetin 0.25%	18.1 ± 4.3	72	15
Quercetin 0.5%	17.8 ± 4.0	72	14
Chrysin 0.05%	14.5 ± 2.9	72	14
Chrysin 0.25%	31.5 ± 20.5	53	14
Chrysin 0.5%	31.2 ± 8.7	73	12
Caffeic acid 0.05%	30.2 ± 4.1	96	13
Kaempferol 0.05%	21.0 ± 6.5	79	13
Galangin 0.05%	16.4 ± 2.9	84	13

[a] Mean dry weight ± SE.
[b] Percent survival of larvae to prepupal stage (pooled replicates).
[c] Mean is significantly different from solvent control, Dunnett's test ($P > 0.05$)

a chemical diversity similar to that of European samples (Ghisalberti, 1979). Only one of the four compounds identified in our study (3-methylkaempferol) is newly reported for propolis. This compound occurs naturally in *Populus* and *Aesculus* (horsechestnut) (Harborne et al., 1975), two sources of resin utilized by bees. Kaempferol is a common flavonoid that has been isolated from many plants, including *Betula*, *Alnus*, *Populus*, and *Salix*, which are also propolis resin sources (Ghisalberti, 1979). Galangin occurs in *Populus* and *Pinus* (Harborne et al., 1975) and is common in European propolis (Ghisalberti, 1979), and 3,3-dimethoxyquercetin has also been reported from European propolis (Schneidewind et al., 1975). We did not detect quercetin in Ohio propolis, although it is quite widespread in the plant kingdom and has been found in numerous studies of propolis from other regions (Bankova et al., 1983; Ghisalberti, 1979; Nenov et al., 1983).

Several of the methanol-soluble resin fractions from propolis retarded growth of greater wax moth larvae, but not as dramatically as crude propolis extract (Eischen and Dietz, 1987). It is possible that fractionation of constituents may

have disrupted synergistic or cumulative toxic effects in crude extract, or the propolis used in this study had a different chemical composition. We cannot discount the possibility of loss of activity from autooxidation of compounds during the diet-drying process, although the level of heat used was low. The more oxidation-sensitive constituents in propolis are likely to be oxidized under natural hive conditions before extraction. The larval bioassays on the honey–beeswax–pollen diet exhibited high within-treatment variation, perhaps indicating incomplete mixing of diet components. The heterogeneity of the diet may have contributed to the retention of solvent, but the persistence of these effects after lyophilization suggests that additional factors were involved.

To summarize, we found little evidence of seasonal or hive-to-hive variation in the specific constituents of propolis, but significant differences between geographic locations. The assays of propolis fractions and individual compounds indicate that greater wax moth larvae are able to tolerate phenolics in their diet and may respond positively to some, although many plant phenolics are toxic or antifeedant towards insects (Levin, 1976; Shaver and Lukefahr, 1969; Isman and Duffey, 1983). This may be due to the low concentrations used in our assays (0.05–0.50%), since equivalent concentrations of quercetin and rutin do not inhibit tobacco bollworm, tobacco budworm, or pink bollworm growth (Shaver and Lukefahr, 1969). Dietary phenolics have also been reported to improve the performance of some insects (Bernays and Woodhead, 1982; Kato, 1978; McFarland and Distler, 1982). Although individual phenolics and fractionated propolis had little effect on greater wax moth larvae in our study, the extent of geographic variation in propolis composition and the possibility that toxicity decreases during fractionation makes it difficult to generalize about the biological activity of material from other sites. The degree of tolerance of greater wax moth larvae to dietary phenolics and the chemical variation in propolis from different geographic locations must be considered concurrently when evaluating the effect of propolis on natural greater wax moth infestations.

Acknowledgments—J.L. Bossart, D.A. Herms, J.K. Nitao, and two reviewers provided helpful comments in this manuscript. We thank Dr. J. Porter for generously allowing us to use his hives for sampling, V. Butz for advice on beekeeping, and T. Mabry for providing authentic flavonoid samples. This work was supported by a graduate student research award provided by the Botany Department at the University of Georgia.

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