JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Effects of *Ginkgo biloba* Constituents on Fruit-Infesting Behavior of Codling Moth (*Cydia pomonella*) in Apples

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ABSTRACT: Codling moth, *Cydia pomonella* (L.), is a cosmopolitan pest of apple, potentially causing severe damage to the fruit. Currently used methods of combating this insect do not warrant full success or are harmful to the environment. The use of plant-derived semiochemicals for manipulation with fruit-infesting behavior is one of the new avenues for controlling this pest. Here, we explore the potential of *Ginkgo biloba* and its synthetic metabolites for preventing apple feeding and infestation by neonate larvae of *C. pomonella*. Experiments with crude extracts indicated that deterrent constituents of ginkgo are present among alkylphenols, terpene trilactones, and flavonol glycosides. Further experiments with ginkgo synthetic metabolites of medical importance, ginkgolic acids, kaempferol, quercetin, isorhamnetin, ginkgolides, and bilobalide, indicated that three out of these chemicals have feeding deterrent properties. Ginkgolic acid 15:0 prevented fruit infestation at concentrations as low as 1 mg/mL, bilobalide had deterrent effects at 0.1 mg/mL and higher concentrations, and ginkgolide B at 10 mg/mL. On the other hand, kaempferol and quercetin promoted fruit infestation by codling moth neonates. Ginkgolic acids 13:0, 15:1, and 17:1, isorhamnetin, and ginkgolides A and C had no effects on fruit infestation-related behavior. Our research is the first report showing that ginkgo constituents influence fruit infestation behavior and have potential applications in fruit protection.

KEYWORDS: Codling moth, *Cydia pomonella*, *Ginkgo biloba*, insect feeding

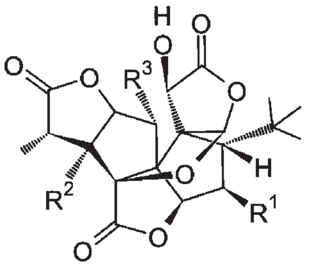
INTRODUCTION

Pharmacological products based on extracts from *Ginkgo biloba* (L.) are some of the most popular herbal products in the world.[1] Most of the extracts are made from the leaves, standardized for their content of terpene trilactones, and flavonol glycosides, and they are used for improvement of peripheral or central blood circulation. The most popular extract, EGb 761 (a standardized extract of *G. biloba* leaves originated by Willmar Schwabe Pharmaceuticals, Germany), contains 24% flavonoids, 6% terpene trilactones, and no more than 5 ppm of alkylphenols.[1]

Ginkgo terpene trilactones, ginkgolides (**1**−**3**), and bilobalide (**4**) (Figure 1) have attracted research attention mostly due to their unique chemical characteristics and action as potent and selective platelet-activating factor antagonists in humans.[2] Ginkgo flavonoids (**5**−**7**) (Figure 2) preferentially inhibit catalytic activity of human cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1),[3] the enzyme that activates many procarcinogens, inactivates some anticancer drugs and is thought to play an important role in carcinogenesis, particularly in ovarian, prostate, breast, and lung cancers.[4,5] The alkylphenols are often considered undesirable components of ginkgo extracts and usually are removed from pharmacological ginkgo products; however, antitumor activities have been reported for these compounds.[6] Although, technically, ginkgo 6-alkyl salicylic acids (**8**−**11**) (Figure 3) are not alkylphenols, many researchers use similar or the same methods of extraction of these compounds from ginkgo products or tissues.[1]

The codling moth, *Cydia pomonella*, is the major, cosmopolitan, and most difficult to combat insect pests of apples. If not controlled, it may cause annual losses reaching 80% of the fruit,[7] which translates to losses exceeding 1.7 billion dollars in the United States only.[8] The grower has very limited options for control of this insect because the invasive stage, the neonate larva, shortly after hatching from the egg, burrows into the fruit and stays there until its development is completed. Sprays with broad-spectrum organophosphate neurotoxin, azinphos-methyl, are still a popular control measure, even though this insecticide has to be applied in excessive amounts of 1.7 kg/hectare due to codling moth resistance against azinphos-methyl that has accumulated over years. This pesticide has been linked to health problems of agricultural workers and raises serious concerns from the U.S. Environmental Protection Agency. In fact, the outer layer of waxes covering the apple has to be washed off to minimize azinphos-methyl residues before the fruit reaches the consumer. This insecticide has already been banned in the European Union and will be phased out in the United States by 2012, and the New Zealand Environmental Risk Management Authority made a decision to phase out azinphos-methyl by 2014. Some newer chemical agents such as Lufenuron raise objections by Swedish Chemicals Agency due to toxicity to fresh water zooplankton. Banning this biocide was approved by the European Parliament in 2009. Insect ryanodine receptor activator, Rynaxypyr, chloride channel activator, emamectin benzoate, and disruptors of nicotinic/γ amino butyric acid (GABA)-gated chloride channels, such as Spinetoram, have been implemented against codling moth in the United States and in Europe, but these biocides have limited use in the areas where codling moth

Received: June 15, 2011
Accepted: September 12, 2011
Revised: September 11, 2011
Published: September 12, 2011



1 $R_1 = R_2 = H$
2 $R_1 = OH$, $R_2 = H$
3 $R_1 = R_2 = OH$

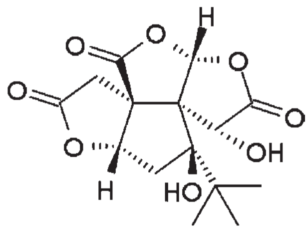


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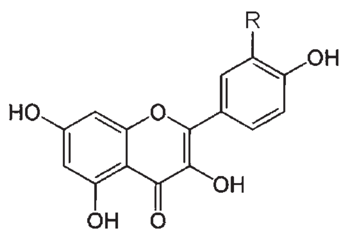
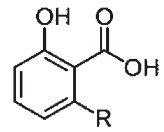
Figure 1. Chemical structures of *G. biloba* terpene trilactones studied for their potential in preventing apple infestation by neonate larvae of codling moth, *C. pomonella*; ginkgolide A (**1**), ginkgolide B (**2**), ginkgolide C (**3**), and bilobalide (**4**). Only ginkgolide B and bilobalide exhibited insect deterrent properties.



5 R = H
6 R = OH
7 R = OCH_3

Figure 2. Chemical structures of *G. biloba* flavonoids studied for their potential in preventing apple infestation by neonate larvae of codling moth, *C. pomonella*; kaempferol (**5**), quercetin (**6**), and isorhamnetin (**7**). None of these glycosides exhibited insect deterrent properties. On the contrary, kaempferol and quercetin stimulated fruit infestation.

have partial third generations: The neonates attempt to infest apples during or close to harvest when using insecticides is not allowed. Insecticides based on natural pathogens of codling moth such as bacteria or viruses are expensive and to become effective must be ingested in large quantities; thus, the fruit damage is often done before larvae die. Pheromone strategies are based on behavioral manipulation such as mating disruption or attract-and-kill are efficient on large and well-managed apple plantations.[9]



8 R= $C_{13}H_{27}$ **(C13:0)**
9 R= $C_{15}H_{31}$ **(C15:0)**
10 R= $C_{15}H_{29}$ **(C15:1)**
11 R= $C_{17}H_{33}$ **(C17:1)**

Figure 3. Chemical structures of *G. biloba* 6- alkyl salicylic acid studied for their potential in preventing apple infestation by neonate larvae of codling moth, *C. pomonella*; ginkgolic acid 15:0 (**8**), ginkgolic acid 15:1 (**9**), ginkgolic acid 13:0 (**10**), and ginkgolic acid 17:1 (**11**). Only ginkgolic acid 15:0 exhibited insect deterrent properties.

They do not, however, resolve problems caused by dense codling moth populations, migration of gravid moths from adjacent unmanaged areas in mosaic landscapes typical of small scale apple production,[9] or insecticide resistance.[10] Recently, studies on microencapsulated kairomones against codling moth showed promising results;[11] however, further research on alternative strategies of combating codling moth is needed. Discouraging the neonates from burrowing into the fruit with feeding deterrents of plant origin, originally proposed elsewhere,[12−14] may become a new strategy.

It is peculiar that ginkgo potential effects on behavior in insects (and particularly in pest insects) are understudied. On one hand, the fact that this plant and derived chemicals are used in medicine should facilitate the process of their registration for pest management due to minimum side effects on humans. On the other hand, the long-known, exceptional resistance of ginkgo to pests and pathogens[15] clearly indicates that this plant has pest-deterring properties. Among the few papers that explored insect-deterring potential of this plant, there are two studies that showed deterrent effects of extracts from ginkgo foliage on feeding by two insect pests of cabbage: *Pieris brassicae* and *Pieris rapae*.[16,17] Indirect methods used in those studies left an open question whether feeding was actually inhibited, or perhaps, ginkgo extracts merely acted as a constipation factor. Effects of anacardic acids on insect development and feeding were studied in only one insect species, the Colorado potato beetle, *Leptinotarsa decemlineata* (Say).[18] Despite the wealth of data about diversity of flavonoids in plants, few of these compounds have been tested against insects and even less is known about their effects on insect feeding.[19,20] There is evidence that some flavonoids have deterrent and antifeedant activity in the crucifer pest *Mamestra configurata*;[21] however, reactions of codling moth larvae to flavonoids were not studied. To the best of our knowledge, effects of ginkgo trilactone terpenes on insect feeding were not studied at all. In a single paper, researchers solely concentrated on insecticidal properties of these compounds in a hemipteran, *Nilaparvata lugens*.[22]

There is an emerging body of evidence, coming from our lab, that chemicals from ginkgo affect feeding behavior in codling moth neonates. By now, we have reported that ginkgo foliage has an emetic effect toward codling moth neonates[23] and that the neonates avoid apples treated with 10 mg/mL of crude alcohol extracts from ginkgo leaves.[24] In a search for more uniform plant material than season- and weather-dependent foliage, we recently tested several ginkgo herbal products marketed as EGb 761 extracts for their codling moth deterrent activity. These extracts contain standardized amounts of terpene trilactones, flavonoids, and alkylphenols. One of them, containing exceptionally high amounts of 6-alkyl salicylic acids, was found deterrent in our screen tests and subjected to further investigation. In particular, we studied insect deterrent activities of terpene trilactone, flavonoid, and alkylphenol fractions of this extract and investigated insect deterrent activities of synthetic ginkgo terpene trilactones, flavonoids, and 6-alkyl salicylic acids known to be present in herbal medicines marketed as EGb 761.

MATERIALS AND METHODS

Insects. Codling moth pupae from Agricultural Research Service, U.S. Department of Agriculture Yakima Agricultural Research Laboratory in Wapato, WA, were stored at 25 °C, 70−80% relative humidity, and 16:8 (L:D). Moths were allowed to oviposit on polypropylene Ziploc bags (S.C. Johnson, Racine, WI). Neonates were collected 0.5−1.0 h posthatch and subjected to bioassays immediately.

Chemicals. Dehydration alcohol (v/v mixture of 80% ethanol, 10% isopropanol, and 10% of methanol) was purchased from EMD (Gibbstown, NJ). Ginkgolic acids 13:0 (**10**), 15:1 (**9**), and 17:1 (**11**) and ginkolide C (**3**) were purchased from Nacalai USA (San Diego, CA), Ginkgolic acid 15:0 (**8**) was purchased from Alexis Biochemicals (San Diego, CA). The remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Crude Ginkgo Extracts. In 2007 and 2008, we purchased five batches of tablets or capsules marketed by Nature's Bounty (Bohemia, NY) and AJG Brands (Boca Raton, FL) as standardized ginkgo extracts. One hundred fifty milligrams of the powder obtained by crushing a tablet or opening the capsule was placed in a 2 mL Eppendorf tube with 1 mL of dehydration alcohol, vortexed briefly, kept for 10 min at room temperature, and centrifuged at 2000*g* for 10 min. The liquid fraction was pipetted off, transferred to a preweighed Eppendorf tube, and concentrated in vacuo. After desiccation, Eppendorf tubes were reweighed to determine the final mass of the residue, which was subsequently resuspended with dehydration alcohol to the desired concentration. For further studies, the extracts were coded as A, B, C, D, and E, depending on the lot number, the manufacturer, and the retailer.

Bioassay. We used a laboratory bioassay described previously.[24] Briefly, apple plugs were formed by forcing a plastic soda straw through a 20 mm thick section of apple containing both epidermis and flesh of the apple. The straw with a plug in it was cut to a length of approximately 15 mm, the plug was positioned with the epidermis of the apple protruding 2 mm from the straw, and the entire assembly was dipped in liquid paraffin wax. Excess wax was removed from the epidermis of the plug using a warm spatula. Five microliters of either control (dehydration alcohol only) or experimental solution was applied to the epidermis of each apple plug and allowed to dry. Using a small amount of modeling clay, four plugs (two controls, two experimental) were arranged with one control and one experimental plug facing each other at either side of a 60 mm × 15 mm polyurethane Petri dish. A short piece of glass rod (1.3 mm diameter) was placed between the two pairs of plugs to form a bridge allowing tested codling moth neonate to choose between either control or experimental plug regardless of which direction the larva traveled along the glass rod. A single codling moth neonate was placed with a fine camel brush, on the glass rod, equidistant from the apple plug pairs, the Petri dish was covered, placed upon a light table, and covered with a semitranslucent dome to avoid a nondirectional light source, which could bias the results as codling moth neonates are known to exhibit mild phototropism.[25] Assays were evaluated after 20 h to determine which plug was fed upon. The feeding deterrence index was calculated according to Jones[26] by dividing the number of the neonates feeding on apple plugs treated with ginkgo extracts or synthetic ginkgo metabolites by the number of the neonates feeding on the plugs treated with dehydration alcohol only, subtracting this figure from 1, and multiplying the result by 100. The bioassays were performed in accordance with current policies of Missouri State University Office of Research Compliance and National Research Council Guide for the Care and Use of Laboratory Animals.

Screen Tests of Ginkgo Crude Extracts for Preventing Fruit Infestation. Five different crude ginkgo extracts were bioassayed for insect deterrent activity at 10 mg/mL. Twenty to 32 larvae were used for each extract testing. Four extracts (coded B−E) did not show statistically significant insect deterrent properties, but extract A effectively prevented fruit infestation: Out of 25 codling moth neonates tested, only one infested extract-treated plug ($P < 0.01$, Fisher exact test). Qualitative high-performance thin-layer chromatography (HPTLC) using a procedure for visualization of ginkgolic acids[27] showed significant presence of 6-alkyl salicylic acids and other polar constituents in extract A. Using HPTLC and serial dilution method,[28] we found that extract A contained at least 1% of total alkylphenols, which is in accordance with earlier reports that some ginkgo homeopathic products available on specialty markets may contain up to 2.2% of these compounds.[1] Extract A was produced in a form of 600 mg tablets by Nature's Bounty and withdrawn from retail sales in 2008. Crude alcohol extract from these tablets is referred to as "crude standardized ginkgo extract" throughout the remaining part of this paper and was either subjected to bioassays for insect deterrence in a dose-dependent manner or to crude extraction of terpene trilactones, flavonol glycosides, or alkylphenols.

Crude Extraction of Ginkgo Terpene Trilactones. The procedure described elsewhere[29] was used. Briefly, 25 mL of crude standardized ginkgo extract was boiled for 1 h in 0.5 L of 5% aqueous H_2O_2. After passing through a Buchner funnel, the remaining solution was extracted three times with ethyl acetate (250/125/75 mL). The organic layer was washed with a saturated solution of Na_2SO_3 followed by water and 80% aqueous NaCl (saturated aqueous solution diluted to 80%). After it was dried over Na_2SO_4, the solvent was removed to yield an amorphous yellow powder, which was subsequently dissolved to desired concentration in dehydration alcohol.

Crude Extraction of Ginkgo Flavonol Glycosides. Here, we used the procedure described elsewhere.[30] Briefly, 40 mg of evaporated crude standardized ginkgo extract was dissolved in 2 mL of 50% aqueous methanol. The mixture was concentrated in vacuo, and then, the residue was redissolved in 1 mL of water and extracted with ethyl acetate three times (3 × 600 μL). The ethyl acetate layers were combined, concentrated in vacuo, and dissolved to desired concentration in dehydration alcohol.

Crude Extraction of Ginkgo Alkylphenols. Alkylphenols were extracted from crude standardized ginkgo extract by partitioning with hexane[1] and subsequent HPTLC of the hexane fraction. Briefly, 400 μL of crude extract was placed in an Eppendorf tube, and 100 μL of double-distilled water was added. Next, 1 mL of hexane was added to the tube, the mixture was vortexed for 1 min, kept at room temperature for 10 min, and centrifuged at 2000*g* for 10 min, and the hexane fraction was collected to a separate tube, concentrated in vacuo, and dissolved to the desired concentration in dehydration alcohol. Because partitioning to hexane extracts also other fat-soluble constituents of ginkgo, the aforementioned procedure was followed by HPTLC using a recommended procedure.[27] Briefly, 5 μL of hexane fraction and 0.1 mg/mL of ginkgolic acid 15:0 as standard were applied onto Merck 10 cm × 10 cm silica gel 60 F_{254} glass plates with CAMAG Nanomat 4 HPTLC plate spotter. The plates were developed for 6 min in a twin trough 10 cm × 10 cm horizontal development chamber (CAMAG Scientific Inc., Wilminton, NC) with 5 mL toluene:ethyl acetate:acetic acid (8:2:0.2; v/v) and air-dried. The plates were inspected at UV 366 nm, the bands corresponding to the standard scraped from the plate, and collected into

Table 1. Effects of Crude Extracts from *G. biloba* on Apple Feeding by Codling Moth Neonates

type of crude extract and concn (mg/mL)	no. of neonates feeding: treated fruit	no. of neonates feeding: control fruit	deterrence index
crude standardized			
45	0[c]	20	100
15	0[c]	20	100
10	1[b]	12	91.7
3	3[a]	11	72.7
1.5	9[a]	28	67.9
0.3	12	15	20.0
0.03	13	15	13.0
alkylphenols			
5	1[c]	19	94.7
2	4[c]	29	86.2
1	11	23	52.2
0.2	10	18	44.4
0.1	11	14	21.4
flavonol glycosides			
10	10[b]	34	70.6
3	8	12	33.3
1	18	17	Not deterrent
0.1	6	7	14.3
0.3	10	10	0
terpene trilactones			
10	7[b]	35	80.0
3	12[a]	32	62.5
1	11[b]	34	67.6
0.1	14	29	51.7
0.01	25	33	24.2

[a] $P < 0.05$. [b] $P < 0.01$. [c] $P < 0.001$ in Fisher's exact test.

Table 2. Effects of Synthetic *G. biloba* Terpene Trilactones on Apple Feeding by Codling Moth Neonates

type of terpene trilactones and concn (mg/mL)	no. of neonates feeding: treated fruit	no. of neonates feeding: control fruit	deterrence index
ginkgolide A (**1**)			
10	16	26	38.5
1	28	47	40.4
0.1	12	13	7.69
ginkgolide B (**2**)			
10	6[c]	25	76.0
1	17	23	26.1
0.1	14	16	12.5
ginkgolide C (**3**)			
10	18	20	10
1	24	30	20
0.1	15	17	11.8
bilobalide (**4**)			
10	8[b]	32	75
1	31[a]	62	50
0.1	20[a]	46	56.5
0.01	13	17	23.5
0.001	15	15	0

[a] $P < 0.05$. [b] $P < 0.01$. [c] $P < 0.001$ in Fisher's exact test.

an Eppendorf tube. Alkylphenols were then eluted from these extracts with methanol.[1] Next, the methanol fraction was collected to a separate tube, concentrated in vacuo, and dissolved to the desired concentration in dehydration alcohol.

More Details on Testing Ginkgo Extracts and Synthetic Metabolites. Ginkgo extracts and synthetic ginkgo metabolites were tested for deterrent effects at concentrations ranging from 0.01 to 60 mg/mL. The number of tests per concentration (each test performed with a different neonate) varied between 13 and 93. Additionally, a choice assay with apple plugs was conducted at the highest effective dose of ginkgo crude standardized extract, 45 mg/mL. Here, behavior leading to choice of experimental or control plug was observed in 16−20 neonates under a dissecting microscope. In particular, it was observed whether the neonates attempted to feed on either of the plugs.

Statistical Analysis. The null hypothesis that 50% of neonates would choose control plugs and 50% would choose experimental plugs was tested using Fisher's exact test ($\alpha = 0.05$).

RESULTS

Effects of Crude Extracts. Crude standardized extract from *Ginkgo* prevented fruit feeding at concentrations equal or higher than 1.5 mg/mL. (n = 13−37, $P < 0.05$, Fisher's exact test) (Table 1). Concentrations of 0.3 and 0.03 had slight deterrent effect numerically, but these effects were not statistically significant. The crude alkylphenol extract had strong deterrent effects at 2 and 5 mg/mL with deterrence indexes of 86 and 95, respectively (n = 20−33, $P < 0.001$, Fisher's exact test) (Table 1). Lower concentrations were ineffective. Crude flavonol glycosides showed moderate deterrent properties only at 10 mg/mL with a deterrence index of about 70 ($n = 44$, $P < 0.01$, Fisher's exact test) (Table 1). Deterrent effects of terpene trilactones were found at 1, 3, and 10 mg/mL (n = 42−45, $P < 0.01$, Fisher's exact test) (Table 1) with a deterrence index ranging from 63 to 80.

Effects of Synthetic Ginkgo Terpene Trilactones. Ginkgolide B (**2**) showed significant feeding deterrent properties against codling moth neonates at 10 mg/mL but not at lower concentrations (n = 31, $P < 0.001$, Fisher's exact test) (Table 2). Bilobalide (**4**) had deterrent properties at a wider range of concentrations: 0.1, 1, and 10 mg/mL (n = 42−93, $P < 0.01$, Fisher's exact test) (Table 2). Ginkgolide A (**1**) and ginkgolide C (**3**) did not exhibit feeding deterrent properties against codling moth neonates (n = 22−75, $P > 0.05$, Fisher's exact test) (Table 2).

Effects of Synthetic Ginkgo Flavonoids. Numerically, isorhamnetin (**7**) showed a slight tendency to discourage some percentage of the larvae from infesting the fruit, but when analyzed with Fisher's exact test, none of synthetic ginkgo flavonoids exhibited statistically significant deterrent properties (n = 20−71, $P > 0.05$, Fisher's exact test) (Table 3). On the contrary, kaempferol (**5**) and quercetin (**6**) had statistically significant feeding stimulatory effects and facilitated fruit infestation at 10 mg/mL and higher concentrations.

Table 3. Effects of Synthetic *G. biloba* Flavonoids on Apple Feeding by Codling Moth Neonates[a]

type of flavonoid and concn (mg/mL)	no. of neonates feeding: treated fruit	no. of neonates feeding: control fruit	deterrence index
kaempferol (**5**)			
60	17	3	no deterrence*
30	35	9	no deterrence**
10	10	10	no deterrence
1	24	32	25
0.1	12	8	no deterrence
quercetin (**6**)			
30	21	4	no deterrence**
10	20	16	no deterrence
1	24	24	no deterrence
0.1	14	10	no deterrence
isorhamnetin (**7**)			
ca. 3[b]	19	28	32.1
1	43	28	no deterrence
0.1	26	29	10.3
0.01	18	23	21.7

[a] None of these substances exhibited statistically significant deterrent properties, but kaempferol and quercetin exhibited statistically significant feeding stimulatory properties instead. Statistically significant feeding stimulation was found at $^*P < 0.05$ or $^{**}P < 0.01$ in Fisher's exact test. [b] Saturated solution.

Table 4. Effects of Synthetic *G. biloba* 6-Alkyl Salicylic Acids on Apple Feeding by Codling Moth Neonates

type of 6-alkyl salicylic acid and concn (mg/mL)	no. of neonates feeding: treated fruit	no. of neonates feeding: control fruit	deterrence index
ginkgolic acid 15:0 (**8**)			
5	4[b]	22	81.8
2	5[a]	19	73.7
1	1[a]	12	91.7
0.5	11	15	26.7
0.2	12	16	25.0
ginkgolic acid 15:1 (**9**)			
10	7	19	63.2
5	15	30	50.0
1	14	24	41.7
0.5	13	16	18.8
ginkgolic acid 13:0 (**10**)			
10	11	19	42.1
5	14	14	0
1	17	23	26.1
0.5	13	17	23.5
ginkgolic acid 17:1 (**11**)			
10	8	20	60.0
5	10	22	54.5
1	18	19	5.3
0.5	10	16	37.5

[a] $P < 0.05$. [b] $P < 0.01$.

Effects of Synthetic Ginkgo 6-Alkyl Salicylic Acids. Ginkgolic acid 15:0 (**8**) had feeding deterrent effects against codling moth neonates at 1, 2, and 5 mg/mL (n = 13−24, $P < 0.05$, Fisher's exact test) (Table 4). Lower concentrations of ginkgolic acid 15:0 had no effect (Table 4). Deterrence indexes of 1−5 mg/mL ginkgolic acid 15:0 ranged from 74 to 92. Ginkgolic acid 15:1 (**9**), ginkgolic acid 13:0 (**10**), and ginkgolic acid 17:1 (**11**) did not exhibit feeding deterrent properties against codling moth neonates (n = 26−40, $P > 0.05$, Fisher's exact test) (Table 4).

Choice Behavior of the Neonates. In the experiments recording neonate behavior upon arrival to the apple plugs, all neonates exhibited similar behavior. The plugs were probed with mouthparts by the neonate. If the control plug was probed first, the neonates either borrowed to that plug or probed the experimental plug treated with 45 mg/mL ginkgo crude standardized extract, then abandoned that plug, and bored to the control plug. If the ginkgo-treated plug was probed first, it was abandoned immediately. Also, in some cases, when lower concentrations of ginkgo crude standardized extract or synthetic ginkgo constituents were tested, we saw evidence of feeding on either plug before making a choice.

DISCUSSION

There is an increasing body of evidence that some constituents extractable from the plants of medical importance influence fruit searching behavior and mechanisms of fruit infestation by codling moth neonates. Our earlier works[12,14,24,31] showed that extracts from *Artemisia absinthium* L., *Artemisia arborescens* × *absinthium*, *Artemisia ludoviciana* Nutt., and *Artemisia annua* L. prevent apple infestation by codling moth neonates. Two metabolites of *A. annua*, artemisinin or 1,8-cineole, also prevent fruit infestation by codling moth neonates.[14] Chloroform extracts from rabbitbrush, *Chrysothamnus nauseosus* Pallas, garlic, *Alium sativum* L., and tansy, *Tanacetum vulgare* L., also showed some potential for preventing apple infestation by young codling moth larvae in pilot studies.[12] Essential oils from tansy, garlic, rue, *Ruta graveolens* L., and patchouli *Pogostemon cablin* Blanco modify codling moth behavior related with fruit location, but whether these oils actually prevented fruit infestation was not studied.[13] Our current study expands this knowledge by finding that *G. biloba* extracts and synthetic ginkgo metabolites prevent apple infestation by codling moth neonates, the major, cosmopolitan pest of this fruit.

Ginkgo Constituents That Affect Apple Infestation Behavior. In our experiments, crude extract from standardized *Ginkgo* prevented apple infestation by codling moth neonates in a dose-dependent manner (Table 1). The behavior of the larvae choosing the apple plug to bore into indicates that *Ginkgo* is a gustatory-based deterrent rather than a substance perceived from a distance. The crude extract exhibited deterrent properties at concentrations as low as 1.5 and 3 mg/mL. At 10 mg/mL, over 90% of neonates avoided the fruit treated with the extract and higher concentrations completely prevented fruit infestation. Deterrent properties of crude standardized ginkgo extracts against codling moth neonates showed in this study are comparable with those of crude alcohol *A. annua* extracts against the same insect reported in our earlier study[14] and higher than those of *A. absinthium*, reported elsewhere.[31] The relatively high activity of crude ginkgo extracts may come from a higher effective dose of the most active components prior to fractionation, or perhaps, there is some synergy between active components in the original extract.

Unfortunately, further discussion of our results encounters difficulties due to scarcity of information on effects of ginkgo-derived chemicals on insect feeding. Crude terpene trilactones were significantly deterrent to codling moth at relatively low concentration of 1 mg/mL (Table 1), and this action could be explained by feeding deterrent action of bilobalide (**4**) and, perhaps, that of ginkgolide B (**2**) (Table 2). Again, we found only two papers reporting that these compounds were feeding deterrents in insects: fifth-instar caterpillars of *P. rapae* reacted to bilobalide and ginkgolide B by lower foliage consumption rates.[16] Ginkgolide B stimulated deterrent receptors in larvae of the same species.[17]

Crude ginkgo alkylphenol extract was strongly deterrent at 2 and 5 mg/mL in our experiments (Table 3), indicating that some ginkgo alkylphenols or 6-alkyl salicylic acids could be a deterrent against codling moth neonates. This assumption was further supported by our finding that ginkgolic acid 15:0 had strong insect deterrent properties at concentrations ranging from 1 to 5 mg/mL (Table 3). We found only one report that corresponds to our results: It was found that a similar concentration (2.5 mg/mL) of ginkgolic acid 24:1 reduced eggplant leaf feeding by the Colorado potato beetle by about 50%.[18] Our current data suggest that the character and the length of the alkyl chain in ginkgolic acid molecules influence insect deterrent properties of these compounds. However, the question why in our study only a saturated ginkgolic acid 15:0 (**8**) was feeding deterrent, whereas unsaturated ginkgolic acids are more abundant in pest-resistant plants,[18] requires further research.

Paradoxically, the most common research literature involves ginkgo flavonoids, which in our study had none or very weak deterrent effects against codling moth neonates and only at relatively high concentration of 10 mg/mL (Table 1). Quercetin, at very low concentrations of 30 μg/L, slightly stimulated feeding in foliophagous *M. configurata* larvae, whereas isorhamnetin was slightly deterrent in the same study.[21] Quercetin 3-*O*-glucoside promotes biting and consumption of leaves in *Bombyx mori* larvae.[32] Extracts containing quercetin and its derivatives also stimulate feeding in larvae of *Helicoverpa armigera*.[33] Codling moth neonates seem to react in similar manner to ginkgo flavonoids that prevail in EGb 761 extract: We observed slight stimulatory effects of kaempferol (**5**) and quercetin (**6**) and negligibly deterrent effects of isorhamnetin (**7**) (Table 4). Feeding-stimulatory effects of quercetin may be partially explained by the fact that this flavonoid is present in apple peel.[34] It is known that codling moth neonates use gustatory stimuli in the process of fruit selection,[23] and the stimuli from quercetin may help in location or assessment of quality of the fruit prior to its infestation by this insect. At the present stage of our study, we do not know what components of crude flavonol glycoside extract contributed to its slight deterrent properties. Thirteen flavonoids that have feeding deterrent properties against foliophagous caterpillars were reported,[32] but none of these compounds was found in *Ginkgo*.[1]

Applied Aspects of Our Study. We have found three ginkgo metabolites that show promising potential in preventing fruit infestation by codling moth neonates: ginkgolic acid 15:0 (**8**), ginkgolide B (**2**), and bilobalide (**4**). Interestingly, both ginkgolic acid 15:0 (**8**) and bilobalide (**4**) exert insect deterrent properties at relatively low concentrations of 0.01 and 0.1%; thus, there is a potential of their use in codling moth control even as direct sprays, particularly by environmentally conscious growers. Most "green" insect controlling products are applied at 0.5% (e.g., Natural Guard Spinosad Landscape and Garden Insecticide) and higher concentrations, reaching sometimes 95% (e.g., Natural Guard Crawling Insect Control). (These products are registered with EPA at numbers 62719-314-7401 and 7401-449, respectively.) This strategy is particularly appealing in the case of bilobalide, since this compound is nontoxic to mammals (LD_{50} >1000 mg/kg), is not mutagenic, and is not phytotoxic at concentration of 0.2%.[22] Also, the gingkolic acids should be relatively easy to synthesize from salicylic acid, a commodity chemical, in a few steps. Moreover, numerous recent works on cloning and functional analysis of the ginkgo terpene trilactones biosynthetic genes[35−39] have been paving the way to improving production of these compounds through ginkgo genetic engineering. Some progress has also been made toward expressing ginkgolic acid in yeasts.[40] On the other hand, the apple genome has been recently sequenced[41] and reconstruction of the complete biosynthetic pathways for ginkgolic acids or bilobalide in transgenic apple is only a matter of time. It should be noted that gingkolic acids and bilobalide come from different metabolic pathways, so that a significant number of genes would have to be introduced into apple, making such an undertaking costly. Thus, probably, at the first steps of such a genetic modification only one of these pathways could be reconstructed in the apple. There may be some public resistance to having transgenic apple orchards, but we think that such a resistance may be mitigated by dissemination of knowledge about genetically modified organisms among the public and employing economical incentive systems for the farmer.

We think that ginkgolic acids or bilobalide could be expressed in apple, in amounts making the fruit unpalatable to codling moth larvae, but still acceptable for consumers. The fact that *Ginkgo* constituents have therapeutic values should contribute to minimizing undesirable side effects of such genetic modification on consumer health. Bilobalide can be safely consumed at relatively high rates[42] and has been shown to have strong neuroprotective effects[43] and strong antioxidant activity both alone and in combination with apple flavonoids.[44] There are some concerns about safety of ginkgolic acids: These compounds are suspected to have cytotoxic, allergenic, and mutagenic effects.[45] On the other hand, some homeopathic formulations of ginkgo extracts contain as much as 2.2% of ginkgolic acids (40000 times higher concentration than that considered safe!), and no reports have been filed on adverse effects of such tinctures.[1] Moreover, as nonpolar compounds, ginkgolic acids could be perhaps expressed in apple waxes that could be removed before the fruit reaches the consumer as it is done presently.

Despite a wealth of information about the methodology of analysis and extraction, the chemistry of *Ginkgo* has received little attention by insect behavioralists, toxicologists, and pharmacologists. This is amazing if we consider the exceptional resistance of this tree to insect pests, even generalist insect herbivores, like the Japanese beetle or the gypsy moth refuse to feed on ginkgo foliage.[46,47] Most works on the effects of *Ginkgo* on behavior pertain to some aspects of cognitive behavior or, less often, mating behavior in mammals. Our present report expands scarce knowledge about effects of *Ginkgo* on insect feeding behavior and suggests the application of two feeding deterrents from *Ginkgo* be added to the pest control strategies of a significant segment in horticultural industry. Assuming that these deterrents could be used as sprayable formulations, at the present stage of our study, it is too early to hypothesize about the possible composition of these formulations. Certainly, they should be protected from light, UV, rain, and microbes. Perhaps they could be used along with other biocides.

We are currently investigating the effects of ginkgo extracts on oviposition of codling moth, to test whether our feeding deterrents do not attract gravid females or stimulate oviposition. Additionally, we hypothesize that there are some unidentified ginkgo flavonoids that have insect deterrent properties. We also hope that our work will help attract more attention to this plant from general and applied entomologists.

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Funding Sources

This work was supported in part by Missouri State Life Science Research Board, Award #13249-2007, Missouri State University Graduate College, and Missouri State University Faculty Research statutory funds.

ACKNOWLEDGMENT

We thank Pauline Anderson and Jim Harris (YARL, Wapato, WA) for their assistance with insect breeding and three anonymous reviewers for their helpful comments.

ABBREVIATIONS USED

CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; EGb 761, a standardized extract of *G. biloba* leaves originated by Willmar Schwabe Pharmaceuticals, Germany

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