Isomeric Flavonoids of Artemisia annua (Asterales: Asteraceae) as Insect Growth Inhibitors Against Helicoverpa armigera (Lepidoptera: Noctuidae)

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Source: Florida Entomologist, 96(3) : 897-903

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.096.0325

ISOMERIC FLAVONOIDS OF *ARTEMISIA ANNUA* (ASTERALES: ASTERACEAE) AS INSECT GROWTH INHIBITORS AGAINST *HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE)

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ABSTRACT

Artemisia annua (Asterales: Asteraceae) is one of the important natural sources of antimalarial compounds i.e., artemisinin and artemisinic acid. Also this plant is cultivated on a large area in India under industry-farmer partnerships. With a view to enhance the added value of the raw material of *A. annua* and its chemical constituents, we evaluated methanolic extract of powdered *A. annua* leaves and different compounds isolated from the extract for toxicity and inhibition and disruption of growth and development of the African pod borer, *Helicoverpa armigera*(Hübner) (Lepidoptera: Noctuidae). Methanol extract of *A. annua* and eight known constituent compounds [artemisinic acid, artemisinin, scopoletin, arteannuin-B, deoxy-artemisinin, artemetin and isomeric flavonoids (casticin and chrysosplenetin)] were bio-assayed for larval mortality, abnormal development, and growth inhibition. The methanol extract severely affected 100% of the larva treated, i.e., larvae gained very little weight, some larvae died, some formed larval-pupal intermediates, some pupae died and a few abnormal adults (adultoids) emerged. The mean weight of treated larvae reached only 0.026 g compared to the 0.270 g in the control and at par with larvae treated with 2% neem seed kernel extract (0.035 g) and 0.02% w/w azadirachtin (0.059 g).Among *A. annua* constituent compounds, the isomeric flavonoids exhibited a strong reduction in mean larval weight (58.5%), and growth inhibition (50.0%) as compared to the control. Extracts of *A. annua* and its isomeric flavonoids appear to have potential for developing novel biopesticides.

Key Words: sweet wormwood, chemical constituents, African podborer, biopesticides

RESUMEN

Artemisia annua (Asterales: Asteraceae) es una de las fuentes naturales importantes de compuestos contra la malaria como la artemisinina y ácido artemisínico. También esta planta se cultiva en una extensión grande de la India bajo asociaciones entre la industria y el agricultor. Con el fin de aumentar el valor añadido de la materia prima de *A. annua* y sus componentes químicos, se evaluó el extracto metanólico de *Artemisia* y diferentes compuestos de la planta, su toxicidad y la interrupción del crecimiento y desarrollo del barrenador africano de la vaina, *Helicoverpa armigera*. Se hizo un bioensayo del extracto de metanol de *A. annua* junto con ocho compuestos conocidos, como el ácido artemisínico, artemisinina, escopoletina, Arteannuin-B, desoxi-artemisinina, artemetina y flavonoides isoméricas (casticina y chrysosplenetin), para determinar la mortalidad de las larvas y la inhibición del crecimiento. El extracto de metanol mostró un nivel de inhibición de crecimiento del 100% y la reducción en el promedio de peso de las larvas (0.026 g) en comparación con el control (0.270 g) y en parte con extracto de la semilla de neem (0.035 g) y azadiractina (0.059 g). Entre los compuestos constituyentes de *A. annua*, los flavonoides isoméricos exhibieron una reducción en el promedio del peso de las larvas (58.51%) y la inhibición del crecimiento máximo (28.57%) en comparación con el control. Los extractos de *A. annua* y sus flavonoides isoméricos tienen aparentemente un potencial para el desarrollo de nuevos bioplaguicidas.

Palabras Clave: ajenjo dulce, componentes químicos, barrenador africano de la vaina, biopesticidas

The African podborer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous pest, which damages a wide range of 300 plant species including various ornamental, medicinal, aromatic, and agricultural crops. Initially larvae feed on tender leaves, and thereafter when on legumes, they bore into the pods and feed within them. A single larva may destroy 30-40 pods before pupation. The total annual losses of agricultural production caused by this pest alone are estimated for 29.2% in chickpea, *Cicer arietinum* L. (Fabales: Fabaceae) in India, where it has 7-8 generations per year (Fitt 1989).

Various control methods have been deployed to manage *H. armigera* in different crops under field conditions among which chemical control measures have been found easy to apply, quick to act and to have relatively low application costs, etc. However, synthetic pesticides pose serious threats to man and environment through residues, they decimate beneficial insects and thereby facilitate resurgence of pest populations, and pests develop resistance to pesticides, which results in widespread control failures, etc.

Among various plant extracts and phytomolecules evaluated in past, neem seed kernel extract(NSKE) containing azadirachtin obtained from Indian neem, *Azadirachta indica* A. Juss (Sapindales: Meliaceae), has been commercialized world over as a plant bio-pesticide for controlling various field pests of agriculture (Schmutterer & Ascher 1986). The plant is cultivated on a large area under industry-farmer partnerships in India. Nevertheless, commercial cultivation of Indian neem has usually been found insufficient to adequately supply the demand raw material. For this large dedicated plantations would be required. Also chemical synthesis of azadirachtin is still too expensive to be scaled up for industrial production. Therefore, there is an unrelenting search for potential bio-resources and novel insecticidal principles to meet the great global demand.

Owing to the discovery of the antimalarial drug, artemisinin,its precursor artemisinic acid, and arteannuin B from sweet wormwood, *Artemisia annua* L. (Asterales: Asteraceae) (Klayman 1985), this herb has been subjected to detailed chemical investigation (Bhakuniet al. 2002). With regard to our study, *A. annua* was cultivated on the CIMAP research farm, Lucknow, India for the extraction and isolation of artemisinin. This paper deals with isolation, identification and feeding deterrence activity of the herb *A. annua* extract and its major constituents, artemisinic acid, artemisinin, deoxyartemisinin, arteannuin B, artemetin, and a mixture of isomeric flavonoids, casticin and chrysosplenetein against *H. armigera.*

Clearly sweet wormwood, *A. annua* L. (Asterales: Asteraceae) has become an important industrially useful economic crop and major natural source of antimalarial compounds; containing artemisinin and artemisinic acid (Klayman 1985; Cyranoski 2004). In India, the technologies for production of this crop and the isolation of antimalarial compounds have been developed by our institute, which has led to bridging-in industrial-farmer partnerships to produce large amounts of raw materials to meet the growing demand of antimalarial compounds (Singh et al. 1988). The essential oil of *A. annua* has been found to influence the ovarian development, median neurosecretary cells activity, and haemolymph proteins with nymphal mortality, and insect growth regulator activity in *Dysdercus koenigii* Fabr. (Hemiptera: Pyrrhocoridae) (Rao et al. 1999), and to exhibit repellent and toxicity against stored insect pests, *Callosobruchus maculatus* and *Tribolium castaneum*(Shakil et al. 2000).The glandular trichomes of *A. annua* shoots are reported to contain possible plant biopesticides (Dayan & Duke 2003). *Artemisia annua* extract and artemisinin have also shown their bioactivity against *Epilachna paenulata* Germ. (Coleoptera: Coccinellidae) and *Spodoptera eriania* (Cramer) (Lepidoptera: Noctuidae) (Maggi et al. 2005). A constituent of *A. annua*, scopoletein, has shown insect feeding deterrence and growth inhibitory activities against *Spodoptera obliqua.* (Walker) (Lepidoptera: Arctiidae) (Tripathi et al. 2011). However, none of the earlier workers have yet investigated the effect of *A. annua* extracts and major constituents for biopesticidal activities against the African pod borer, *H. armigera*.

Materials and Methods

Insect Culture

In the initial culture, *H. armigera* larvae were reared in the laboratory at 26 ± 1 °C and 60-70% RH on a semi-synthetic artificial diet as method described by Singh & Rembold (1992). Vitamins were used as supplied by HiMedia Laboratories Pvt. Ltd., Mumbai, India. The required quantities of ingredients were weighed by electronic semi-microbalance; Sartorius- CPA225D (M/S Sartorius weighing technology GmbH bender Land star, 94-108/37075, Germany).Agar powder was added to water, heated to boiling, and then the yeast powder, sucrose, chickpea powder and vitamins were gradually added, mixed until homogenous and then cooked for an additional 2-3 min.

Freshly emerged adults were transferred into jars (25 × 20 cm) each containing a cotton swab dipped in 10% honey solution and covered with muslin cloth. The muslin cloth containing eggs was transferred to another jar with 60-70% R.H. to prevent desiccation of the eggs. Each freshly hatched larvae were transferred to Petri dishes containing artificial diet. After 3 days the larvae were transferred into multi-celled rearing trays to avoid cannibalism.

Plant Collection

The *A. annua* herb was collected from the Research Farm of the CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow. Shed-dried mature leaves were ground in a mill to make a fine powder. Neem seed kernel was collected from local markets in Lucknow and the seeds were ground into a powder.

Extraction

The powdered plant materials were soaked in methanol continuously for 24 h at room temperature and filtered through Whatman filter paper. This process repeated for 3 consecutive days, and then the solvent was evaporated using a rotary evaporator (Rotavapor- R-200 (M/S BUCHI, Labortechnik, Switzerland). The methanol leaf extract was refrigerated and held for bioassays.

Purified Compounds

To isolate artemisinin and other active compounds the shed-dried and powdered leaves of the *A. annua* herb were extracted with boiling *n*-hexane rather than methanol to reduce the bulkiness (weight) of the extract. After removal of the solvent, up to 75% the hexane extract was partitioned with acetonitrile-water (4:1) to remove fatty material. Salt (NaCl) was added to the acetonitrile-water fraction to remove the water. After concentration and drying the acetonitrile extract (45 g) was packed on silica-gel column (150 × 13 cm). The column was eluted with *n*-hexane-ethyl acetate (95:5, 90:10, 85:15, 80:20, and 70:30) and ethyl acetate (Singh & Bhakuni 2004). During the course of elution 6 pure compounds we isolated, i.e., artemisinic acid (**1**), mp 129-131 °C;artemisinin (**2**), mp 151-153 °C;scopoletin(**3**),mp 201-202 °C;arteannuin B (**4**), mp 151-152 °C;deoxyartemisinin (**5**), mp 110-111 °C;and artemetin (**6**),mp 159-161 °C. These compounds were identified by comparison of their physical (mp) and spectral (IR, ^{1}H, ^{13}C NMR and MS) data as reported in the literature (Linuma et al. 1980; El-Marakby et al. 1987, 1988; Blasko et al. 1998; Patel et al. 2010; Tripathi et al. 2011**)**. From the latter hexane-ethyl acetate (70:30) fractions, a TLC single compound was obtained as pale yellow granules,mp 170-174 °C; IR λ(KBr): 3400(OH), 1660(CO), 1600,1509,1460,1345,1260, 990 cm^{-1}; MS: m/z [M]$^{+}$ 374 for $C_{19}H_{18}O_{8}$. ^{1}H-NMR analysis of the flavonoid mixture showed it to be 2 isomeric flavonoids in the ratio of 55:45. ^{1}H-NMR(300 MHz, $CDCl_3$): δ 3.72s, 3.87s, 3.92s, 3.95s, 3.99s (8 × OCH_3 of isomers), 6.50s (H-8, common), 6.97d, 7.05d (H5' of isomers), 7.60d, 7.71d (H2' of isomers), 7.67dd, 7.74dd (H6' of isomers), 11.61s, 11.69s, 11,71brs (4 × OH of isomers); ^{13}C-NMR/DEPT (75MHz, CD_3COCD_3): δ 55.86, 55.98, 56.36, 59.78, 60.18 (8× OCH_3, 4× OCH_3 each of isomers), 91.24 (C8, common), 106.54 (C10, common), 111.61, 115.35 (C2'of isomers), 112.20, 115.64 (C5' of isomers), 121.41, 122.90 (C6' of isomers), 123.62(C1', common), 132.64 (C6, common), 138.75 (C3, common), 146.88 (C3', common), 147.83 (C4', common), 150.46, 150.47 (C5 of isomers), 152.67, 153.05 (C2 of isomers), 156.20 (C7, common), 159.59 (C8a, common), 179.26 (C4, common). From the detailed ^{1}H and ^{13}C NMR spectral analysis of the 2 isomeric mixture and comparison with the ^{13}C NMR data reported in the literature (Agrawal 1989; Bhakuni et al. 2001)it was possible to identify the mixture as 5,3'-dihydroxy, 3,6,7,4'-tetramethoxyflavone (**7,** casticin) and 5,4'-dihydroxy, 3,6,7, 3'-tetramethoxyflavone (**8,** chrysosplenetein).

Bioassays

Bioassays were carried out with 10 insects by the method described by Neoliya et al. (2005). The methanol extract of *A. annua* herb and its each compound were incorporated in semi synthetic diet (%w/w),and the medicated diets were provided to 10 individual third instars of *H. armigera* for each bioassay. A 2% concentration of each *A. annua* herb extract and a 0.1% (w/w) of each its constituent compounds was incorporated in the diet and provided to individual larvae until they pupated. Control larvae were fed untreated diet. The neem seed kernel extract (NSKE) (2% concentration in methanol) was taken as a standard to compare the treatments. For each treatment 10 individual larvae were used and placed in separate cells of a rectangular multi rearing tray. The treated and untreated diets were replaced as necessary until each larva had completed the pupal molt. After 5 days of treatment the weight (g) of each individual larva was determined. The observation on percentage mortality were corrected using Abbott's formula (Abbott 1925), and growth parameters, sub-lethal effects; larval- pupal intermediates, pupal-adult intermediates and adultoids were observed and recorded until adult emergence from each treated and untreated(control) diet fed to individual larva for growth inhibition, which consists of lack of weight gain, developmental deformities and death in the treated and subsequent stages (Table 1, Fig. 1).We calculated percent growth inhibition, and we defined growth inhibition of treated larvae as the combination of prevention of weight gain, larval and pupal mortality, and developmental abnormalities including formation of larval-pupal intermediated and abnormal adults (adultoids).

TABLE 1. GROWTH INHIBITORY AND DEVELOPMENTAL EFFECTS ON *HELICOVERPA ARMIGERA* OF COMPOUNDS AND EXTRACTS DERIVED FROM *ARTEMISIA ANNUA*.

Treatments*	% Conc.	% Lm	% Lpi	% Pm	% Normal adults	% Adultoids	% Growth inhibition§
Artemisinic acid	0.10	—	—	—	100	—	00.0
Artemisinin	0.10	—	10	—	80	10	20.0
Scopoletin	0.10	—	—	10	70	20	30.0
Arteanuin B	0.10	—	—	—	90	10	10.0
Deoxy-artemisinin	0.10	—	—	10	70	20	30.0
Artemetin	0.10	—	10	10	80	—	20.0
Isomeric flavonoid	0.10	20	—	30	50	—	50.0
Azadirachtin	0.02	100	—	—	—	—	100.0
NSKE	2.00	100	—	—	—	—	100.0
Hexane extract	2.00	20	10	10	50	10	50.0
Methanol extract	2.00	100	—	—	—	—	100.0
Control	0	0	0	0	100	00	00.0

Abbreviations: Lm-larval mortality, Lpi-larva-pupa intermediate, Pm-pupal mortality.
*Each treatment consisted of 10 individual test insects.
§Growth inhibition is defined as the combination of prevention of weight gain, larval and pupal mortality, and developmental abnormalities including formation of larval-pupal intermediates and abnormal adults (adultoids).

Statistical Analysis

The trials were arranged using a randomized complete block design, and the data were subjected to one way analysis of variance (ANOVA). Data was statistically analyzed by statistical software 4.0 version available in our institute based on Panse & Sukhatme (1967) and Singh & Chaudhary (1979). The least significant variance (LSD) at P = 0.01 and P = 0.05 probability was used to test the significant differences among treated means.

RESULTS AND DISCUSSION

According to the results of ANOVA, among the treatments with *A. annua* methanol extract, hexane extract and the *A. annua* constituent compounds (artemisinic acid, artemisisnin, scopoletin, arteanuin B, deoxyartemisinin, artemetin and isomeric flavonoids), the methanol extract and isomeric flavonoids significantly (P = 0.01 and P = 0.05) prevented gains in the weights of larvae in comparison to the control (F = 70.78, df = 11, P < 0.05) (Fig. 1).

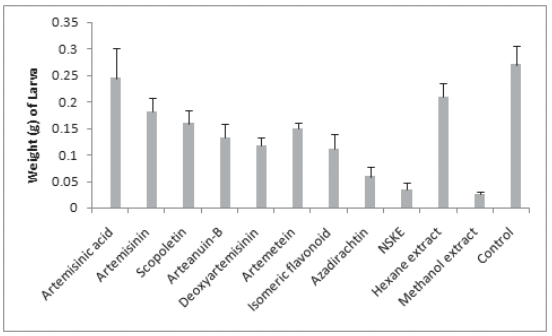

Fig 1. Growth inhibitory effects on *Helicoverpa armigera* of compounds and extracts derived from *Artemisia annua*, and from neem, i.e., neem seed kernel extract (NSKE) and azadirachtin.

Helicoverpa armigera larvae reared on the control diet required 12.0 days to reach the pupal stage (Table 2). In contrast those reared on diet laced with 2% methanol extract of *A. annua* required 23.1 days to reach pupation, and this prolongation of larval development was similar that of larvae reared on diet laced with 2% (w/w) methanol neem seed kernel extract, i.e., NSKE (25.2 days). Also the 0.1 % isomeric flavonoid treatment caused an increase in larval duration to 14.3 days.

These purified compounds were isolated from the *n*- hexane extract of the herb *A. annua*. However methanol, a polar solvent, was used to extract the herb for better mixing into the insect diet in the bioassays experiments. The methanol extract of the herb showed the presence of the isomeric flavonoids and the 6 other compounds by TLC. A significant and drastic reduction in mean larval weight (Fig. 1) was caused by the methanol *A. annua* leaf extract (0.026 g/larva) at 2 percent concentration. Also the isomeric flavonoid at 0.1 percent concentration (0.112 g/larva) substantially reduced larval weight compared to the control (0.27 g/larva). However, 2% methanol neem seed kernel extract and 0.02% azadirachtin reduced mean larval weight to 0.035 g/larva and 0.059 g/larva at 2% and 0.02% concentrations mixed in semi-synthetic diet, respectively (Fig. 1).

The pupal period (17.7 days) was also increased by the isomeric flavonoid in comparison to control (Table 2), but no other compound caused a

TABLE 2. EFFECTS ON THE DURATION OF THE LARVAL AND PUPAL STAGES OF *HELICOVERPA ARMIGERA* OF COMPOUNDS AND EXTRACTS DERIVED FROM *ARTEMISIA ANNUA*.

Treatments	Mean larval period (days)	Mean pupal period (days)
Scopoletin	12.0	15.3
Isomeric flavonoid	14.3	17.7
Arteanuin B	12.5	15.8
Deoxy- artemisinin	12.2	15.0
Artemetin	12.1	14.7
Artemisinin	11.4	11.5
Artemisinic acid	11.8	11.5
NSKE	25.2	16.1
Azadirachtin	21.8	—
Methanol extract	23.1	—
Hexane extract	12.3	—
Control	12.0	14.0
SEM	0.311	0.367
LSD($P = 0.01$)	1.15	1.370
LSD($P = 0.05$)	0.874	1.033
Df	11.0	8.0
F value	104.3	28.81

Purified compounds were administered at 0.1% of the diet and extracts were administered at 2% of the diet.

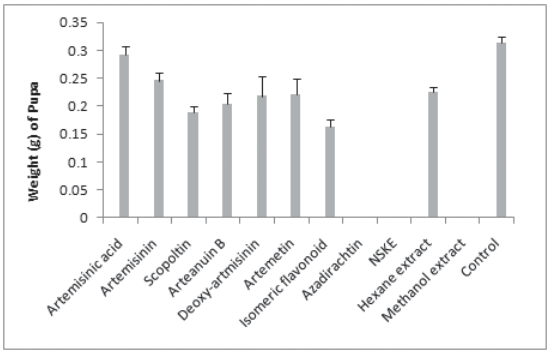

Fig 2. Effects on pupal weights of *Helicoverpa armigera* of compounds and extracts derived from *Artemisia annua*, and from neem, i.e., neem seed kernel extract (NSKE) and azadirachtin.

significant increase in pupal duration. However fifty percent adult emergence was observed in isomeric flavonoid treatment and other compounds were observed as 70 to 90% adult emergence. Out of the 7 major compounds tested, isomeric flavonoids (a mixture of casticin and chrysosplenetin) caused the greatest reduction in larval weight (58.5%) (Fig. 1) and 50.0% growth inhibition of larvae (Table 1) at 0.1% concentration in the semi synthetic diet compared to the control.

The dietary utilization experiment showed that *A. annua* extract and the isomeric flavonoid drastically reduced mean larval weights, and hence growth inhibition. Results showed that 100% of the larvae treated with the methanol extract of *A. annua* were severely affected, i.e., some died in the larval stage, some formed larval-pupal intermediates, some pupae died and a few abnormal adults (adultoids) emerged. Neither the methanol extracts of *A. annua* nor any of its constituent compounds caused direct contact toxicity to African pod borer larvae. NSKE is already known to be an effective insect feeding deterrent and insect growth regulator (Koul et al. 2003; Jaipal et al. 1983), and this was substantiated in our experiments (Figs. 1 and 2, Table 1). Simmonds & Stevenson (2001) have identified the isoflavonoid mixture of maackiain and judaicin extracted from chickpea (*Cicer arietinum* L.; Fabales: Fabaceae), and found it to deter feeding even at only 10 ppm, and, hence, to reduce the weight gain of early stadia of *H. armigera.* None of the earlier workers have reported the growth inhibition activity of isoflavonoids (mixture of casticin and chrysosplenetin) obtained from *A. annua* against *H. armigera*.

Botanicals have been found to alter the oviposition behavior and morphology in *H. armigera* (Bajpai & Sehgal 2003). Plant derived chemicals like azadirachtin interfere with food utilization and they may distort the midgut enzymatic profile of this pest (Babu et al. 1996). Bioefficacies of azadirachtin and other neem pesticides against the African pod borer have already been reported (Chakraborti & Chatterjee 1999). Besides Indian neem seed kernel, the effects of various other plant extracts, fractions and phytocompounds have been investigated on the African pod borer in the laboratory by various researchers. The growth and development of other herbivorous pests have also been found to be influenced by certain terpenes of the Asteraceae family (Salinas-Sanchez et al. 2012). *Artemisia annua*, which is a member of the Asteraceae, is an annual; easy to grow, do not block the land for long periods, the agro-technologies for its production are available to farmers and the raw material is abundantly available.

In present investigation, we found drastic loss in mean larval weight gain (90.5%) and greatly increased prolongation of the larval stage -like that caused by neem seed kernel extract - by diet containing a methanol extract of A. *annua* as compared to control, which also caused maximum growth inhibition activity of *H. armigera* (100%). This extract also causes a great prolongation of the larval and pupal periods. Based on the result of the present study, further investigations are warranted on the influence of *A. annua* extracts including its constituent compounds (Fig. 3) on biochemical changes in target pests in the quest of developing possible novel and potentially useful biopesticides. The isoflavonoid mixture (5,3'-dihy-

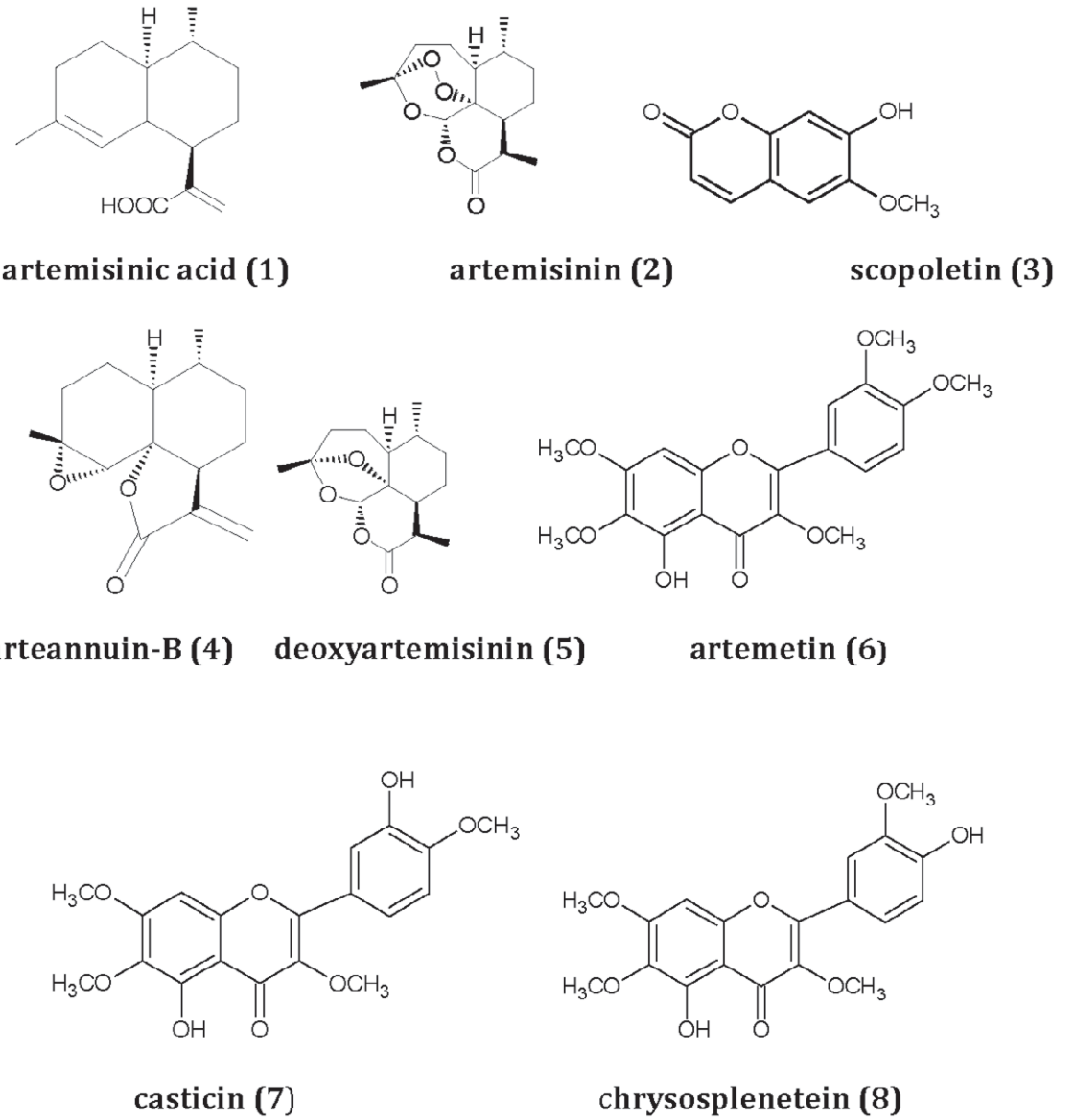

Fig 3. Structures of major compounds (1-8) isolated from *Artemisia annua*.

droxy, 3,6,7,4'-tetramethoxyflavone and 5,4'-dihydroxy, 3,6,7, 3'-tetramethoxyflavone) may be explored for formulating novel biopesticides for the management of major agricultural insect pests.

Acknowledgments

We thank the Director, CSIR-CIMAP, Lucknow, India, for valuable suggestions during the experimentation, Dr. R. K. Lal for help in statistical analysis and Dr. A. K. Gupta for providing the plant materials of *A. annua* from the Gene Bank of CSIR-CIMAP, Lucknow. First author (N.A.) is thankful to The University Grants Commission, India, for providing the Rajiv Gandhi National Fellowship during the investigation period.

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