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Insecticidal activity and physiological property of *Annona squamosa* (L.) seed extracts against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera:Tephritidae)

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University of Hawaii, 1991

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INSECTICIDAL ACTIVITY AND PHYSIOLOGICAL PROPERTY OF
ANNONA SQUAMOSA (L.) SEED EXTRACTS AGAINST THE
MEDITERRANEAN FRUIT FLY, CERATITIS CAPITATA
(WIEDEMANN) (DIPTERA:TEPHRITIDAE)

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION
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By

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ABSTRACT

Insecticidal activity and biological properties of A. squamosa seed extracts against C. capitata were evaluated in the laboratory. Extracts were effective against the medfly from egg to adult stages. Extract bioassays with respect to medflies clearly demonstrated that A. squamosa contained a factor toxic to insect. Assays successfully guided extract fractionation to yield 1 or 2 active insecticidal compounds. Insect active fraction was 2.5×10^{-3} and 2.07×10^{-3} as toxic as malathion to male and female flies, respectively.

Topical application of sublethal doses of A. squamosa seed extracts caused no abnormal behavior among the flies. Insects were able to feed, undergo courtship behavior, mate and oviposit eggs. The extract did not affect adult longevity but exhibited a chemosterilant effect by reducing egg hatchability. Significantly, less eggs hatched when dipped in $> 0.313\%$ of extract. Measurement of ovarian length of treated flies revealed that extract affected oocyte development. Ovarian growth was significantly reduced which suggests interference with vitellogenesis. The extract was a repellent and an oviposition deterrent. Significantly, fewer flies were attracted to treated papaya tissues. Chronic exposure to A. squamosa seed extract treated diets affected insect growth and development. Larval period was significantly prolonged and pupation was

reduced. Delays in development occurred only during larval period with no treatment effect on length of pupal period.

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I. INTRODUCTION

The Mediterranean fruit fly or medfly, Ceratitis capitata (Wiedemann), is a pest of economic importance which limits the development and expansion of agriculture in many localities. It occurs in areas with a great diversity of agriculture, ecological habitats and host plants. It is highly plastic in its ability to adapt to different ecological conditions and man-made situations. In subtropical and temperate regions where it is not established, the possibility of accidental introduction is a great concern because this species has a high biotic potential and a wide host range which includes more than 200 fruits, nuts and vegetables (Harris 1977; Hagen et al. 1981). Adult females lay their eggs into the rinds of fruit, with preference for nearly mature fruit. During oviposition, the insect punctures fruit causing cosmetic damage and leaving sites for subsequent spoilage. When eggs hatch, the larvae feed and tunnel inside the fruit. In older fruit, damage is detected rather late when the fruit initially ripens and subsequently rots when microorganisms invade the tissues.

Various control measures are available to combat the pest. Eradication of a C. capitata population was first achieved with poison bait sprays (Steiner 1969). Use of

sterilized insects was subsequently developed for suppression and eradication purposes (Steiner 1972; Cunningham et al. 1980). The use of pesticides is often considered the most potent control measure for pests. However, chemical control alone has a number of limitations; yet most plant protection operations are still based on this unilateral approach. This is particularly true in developing countries where knowledge and structure to develop and implement new pest control technologies are generally lacking. Continuous or heavy usage of some insecticides has created serious problems such as direct insect toxicity to parasites, predators, pollinators, fish and man (Munakata 1977), pesticide resistance (Brown 1968; Georghiou and Taylor 1977; Schmutterer 1981; Wais et al. 1981), susceptibility of crop plants to insect pests (Pimentel 1977) and increased environmental and social costs (Pimentel et al. 1980). Pesticide prices continuously increase over the years, and in developing countries this has led to a situation where farmers can no longer afford to apply required amounts of chemicals on some crops. Even more important is the fact that current efforts to increase food production may be curtailed by these high prices.

Although synthetic pesticides will remain a primary measure for agricultural pest control during the foreseeable future, it is evident that society cannot tolerate the way

conventional chemicals are used (Doutt and Smith 1971). This impasse can be avoided by the development of pest management systems based on the judicious application of insecticides and the improved use of new and alternative control methods (Smith 1974). Implementation of less expensive and safer crop protection measures incorporating pesticides in a rational manner is necessary.

The existence of naturally occurring insecticidal plant components has been known for centuries. However, relatively few of these compounds are used in crop protection today. Increasing problems associated with the use of modern synthetic insecticides have caused renewed interest in naturally occurring pesticides. Because these compounds are often less toxic and persistent than their synthetic counterparts and are in some instances a component of mammalian diets, they are assumed to be environmentally more acceptable and less hazardous to humans. Of special interest are biologically active compounds which are natural components of food plants.

The plant kingdom is a rich source of various compounds with high potential for development as effective control agents. Interactions between phytophagous insects and plants over the ages have led to the evolution of numerous secondary plant chemicals which influence insect behavior, development, and physiology (Thorsteinson 1960; Beck 1965;

Fraenkel 1969; Hedin 1977). These chemicals can be used to control specific pests in appropriately designed strategies. As naturally evolved ingredients of the biosphere, such plant derived products have a potential advantage over synthetic compounds in terms of ecological suitability. Their development as successful pest control agents can also be economically feasible, especially if the source materials are plants available in abundance (e.g., common weeds, prolific herbs, shrubs and trees having a wide and rich distribution).

It is for the above reasons that the following study was conducted. The objectives of the study were to: investigate the insecticidal activity of Annona squamosa seed extract; isolate and characterize the active fraction of the extract; and evaluate the biological effects of the extract against the medfly.

II. REVIEW OF LITERATURE

Biologically Active Natural Products from Plants

Plants produce an extremely diverse array of biologically active natural products that adversely affect the growth and development of other organisms. Usually these products are considered to be defensive substances useful to the plants in discouraging or preventing attack from herbivores and microorganisms or in protecting the plants from stress exerted by the environment and competing species. These compounds are called secondary plant substances because explicit physiological functions are rarely known (Fraenkel 1959; Seigler and Price 1976; Jones 1979) even though many are actively metabolized within the plants (Robinson 1974). Numerous functions have been suggested for various members of this complex array. Among these are: regulators of plant growth biosynthetic activities; storage forms of plant growth regulators, energy reserves, transport facilitators, and waste products; detoxication products of environmental poisons; shields against excessive radiation; and effectors of allelochemical interactions between plants and their competitors and between plants and heterotrophic organisms (Fraenkel 1959; Whittaker 1970; Whittaker and Feeny 1971; Luckner 1972;

Nakanishi et al. 1974; Rice 1974; Robinson 1974; Harborne 1977).

Secondary plant substances have been implicated as defensive agents for plants in plant-plant (allelopathic) (Rice 1974), plant-pathogen (Bell 1974) and plant-herbivore interactions. The role of plant secondary chemicals in mediating the interactions of insects and their host plants is well established (Burnett et al. 1974; Rodman and Chew 1980; Berenbaum 1981; 1983; Bowers 1983). Such compounds may serve as feeding or oviposition stimulants and attractants, deterrents or toxins (Schoonhoven 1982; Blum 1983; Bordner et al. 1983; Bryers et al. 1986). There are several reviews of evidence for antiherbivore function of secondary substances (Schoonhoven 1972; Beck and Reese 1976; Feeny 1976; Rhoades and Cates 1976). Two main lines of evidence have been used to show antiherbivore activity of these substances. The first involves deterrence and the second antibiosis. In the first method, the preference of herbivores for a series of plant tissues is compared with the concentration of secondary metabolites in these tissues. If the herbivore is deterred, a negative correlation should result. In the second method, herbivores are fed various plant tissues and some fitness measures such as growth rate, fecundity or number of viable progeny are measured, and the

correlation between secondary metabolite content and herbivore fitness is examined.

Among secondary plant substances that have a negative effect on herbivore fitness or a deterrent effect on herbivore grazing are alkaloids, pyrethrins, rotenoids, long chain unsaturated isobutylamines, cyanogenic glycosides, phytoecdysones and juvenile hormone (JH) analogs, cardenolides and saponins, sesquiterpene lactones, non-protein amino acids, glucosinolates and isothiocyanates, oxalates, protoanemonin, hypericin, fluorofatty acids, selenoamino acids, 6-methoxybenzoxazoline, gossypol, condensed tannin, phenolic resin and phenol oxidase and proteinase inhibitors of the soybean trypsin inhibitor type (Rhoades and Cates 1976; Rodriguez et al. 1976; Ganjian et al. 1983; Romeo and Simmonds 1989), phytohemagglutinin (Janzen et al. 1976), chromenes (Bowers et al. 1976; Isman 1989; Klocke et al. 1985, 1989), and acetogenin (Alkofahi et al. 1989).

Modes of action of some secondary substances are known or suspected. Tannins act by forming relatively indigestible complexes with leaf proteins, thereby reducing the rate of assimilation of dietary nitrogen (Feeny 1969). Cyanogenic glycosides are hydrolyzed by enzymes to yield a sugar and a hydroxynitrile which dissociates to release hydrogen cyanide, a substance potentially lethal to

organisms with an electron transport system involving cytochromes (Rehr et al. 1973a). Insect hormone analogs (Slama 1969) and antihormones (Bowers et al. 1976) interfere with insect metamorphosis and development. Toxic non-protein amino acids, instead of dietary amino acids, are incorporated into proteins, leading to defective protein structure (Rehr et al. 1973b), and cardenolides disturb muscle action (Roeske et al. 1976).

Behavioral and Hormonal Effects of Secondary Substances

Scattered reports are available that show plant substances exhibit JH-mimicking activity in insects. Thujic acid (5,5-dimethyl-1,3,6-cycloheptatrien-1-carboxylic acid), isolated from the heartwood of western red cedar, Thuja plicata Donn ex. D. Don. showed significant JH activity after injection into pupae of the yellow mealworm, Tenebrio molitor L. (Barton et al. 1972). Sterculic acid (2-octyl-1-cyclopropene-1-octanoic acid) isolated from the oil of Sterculia foetida L. seeds, greatly affected the life cycle of the blow fly, Phaenicia sericata (Meigen), when fed to the larvae (Thompson and Barlow 1973). Nymphs of Dysdercus koenigii (F.) treated with dorsal applications of acetone extract of the roots of Iris ensata Thunb. either failed to reach adulthood or molted into adults with crippled wings. An extract of I. ensata leaves did not cause this effect (Saxena and Shrivastava 1972). Topical application of the

steam-distilled oil of Tagetes minuta L. to D. koenigii (F.) nymphs prevented adult molt, but the oil was inactive on larvae and pupae of mosquito, Aedes aegypti L. and pupae of the house fly, Musca domestica L. (Saxena and Shrivastava 1973).

Secondary plant chemicals often have adverse effects on animal physiology, development and survival (Schoonhoven 1972; Feeny 1975; Beck and Reese 1976; Rhoades and Cates 1976). It is possible that all herbivores are vulnerable to some extent even though they may be adapted to tolerate or even utilize certain groups of these chemicals. The host selection behavior of many herbivorous species is such that plants containing potentially harmful chemicals are rejected (Jones 1972; Bernays and Chapman 1977). In contrast, some insects are so adapted that they can sequester certain secondary plant chemicals and so acquire toxic properties that provide a defense against predators (Brower 1969). When reared on other plants lacking the chemicals, such insects may be eaten readily by predators so that selection favors individuals that consume plants containing the appropriate secondary plant substances.

Oviposition deterrence to frass from insect larvae fed plant material is a form of chemically mediated behavior that regulates insect populations. Unlike pheromones and other insect-produced bioregulators, such deterrents appear

to be secondary plant substances that inhibit oviposition on contact with the insects at the ovipositional sites (Rothschild and Schoonhoven 1977; Renwick and Radke 1980, 1981; Sharma et al. 1981). Hedin et al. (1977) listed many secondary plant substances that influence insect behavior and development. Some of these compounds are important in plant defense acting as feeding deterrents, repellents or toxins to insects.

Insecticides Derived from Natural Products

Secondary products from higher plants represent an enormous diversity of biologically active compounds that have been exploited as pesticides and sources of new pesticide chemistries (Duke and Lydon 1987). A remarkable number of insecticidal plants were recognized first as fish poisons and received special scientific attention (Leonard 1960). Most of these plants have been principally used against body pests such as lice, roaches and ants (Fisher 1940; Seiferle 1942; Busvine 1946; Charles 1954; Bergmann et al. 1958). Those that were insecticidal received only the most rudimentary entomological study and few were subjected to detailed chemical examination although active principles were isolated. Recently, studies on the use of naturally occurring pesticides for pest control have been intensified. Many investigators isolated, identified and screened chemical compounds from leaves and

seeds of many botanical families for insect deterrence and growth inhibition (Jacobson et al. 1975; Bernays and Chapman 1977; Doskotch et al. 1977; Jacobson 1977; Carpenter et al. 1979; Warthen 1979; Jurd and Manners 1980; Menn 1980; Reed et al. 1982). Quassinoids, isolated from plants belonging to family Simaroubaceae, were toxic and had antifeedant and growth inhibitory effects against tobacco budworm, Heliothis virescens (F.), and fall armyworm, Spodoptera frugiperda (Smith) (Klocke et al. 1985) and decreased feeding by Mexican bean beetle, Epilachna varivestis Muls., and the Southern armyworm, S. eridania (Cram.) (Leskinen et al. 1984). When fed to aphids, quassinoids decreased Myzus persicae (Sulz.) feeding (Polonsky et al. 1989). Growth and development of corn earworm, Heliothis zea (Boddie) larvae fed on artificial diets containing L-azetidine-2-carboxylic acid (AZC) were markedly disrupted (Adeyeye and Blum 1989). AZC, a naturally occurring homolog of proline, caused severe retardation of larval development, larval mortality resulting from exuvial ligature, death at larval-pupal ecdysis, formation of grossly deformed pupae, and non-sclerotization of the anterior ventral half of the pupal integument. Azadirachtin is a tetranortriterpenoid isolated from neem seeds, Azadirachta indica A. Juss (Butterworth and Morgan 1968). It has growth regulating activity and inhibits feeding and disrupts the growth and development of

many insects (Schmutterer and Ascher 1984). Powell et al. (1981) isolated trewiasine from Trewia nudiflora seed. This maytansinoid was toxic to striped cucumber beetle, Acalymma vittatum (F.) and codling moth, Cydia pomonella (L.).

Economic Importance of A. squamosa

As described by Quisumbing (1951), sugar apple or sweetsop, A. squamosa, is a small tree 3 to 5 m in height when mature. Leaves are somewhat hairy when young, oblong and 8 to 15 cm in length, with a petiole 1 to 1.5 cm long. Flowers occur singly in leaf axils and are about 2.5 cm long. They are pendulous, hairy, 3-angled and greenish white or yellowish. Fruits are somewhat heart-shaped, 6 to 9 cm in length and marked by polygonal tubercles (Fig. 1). When the fruits ripen, they are light yellowish green. Flesh is white, sweet, soft and juicy and has a mild agreeable flavor.

A. squamosa belongs to family Annonaceae which includes about 120 genera and more than 2000 species. Economically, the family is of appreciable importance as a source of edible fruits. Oils from seeds of some plants may be used for the production of edible oils and soap (Lebouef et al. 1982). Woods of some Annonaceous plants have been employed for alcohol production (Lebouef et al. 1982). Fragrant flowers of ylang-ylang (Cananga odorata) are an important raw material for perfumery (Lebouef et al. 1982). Many

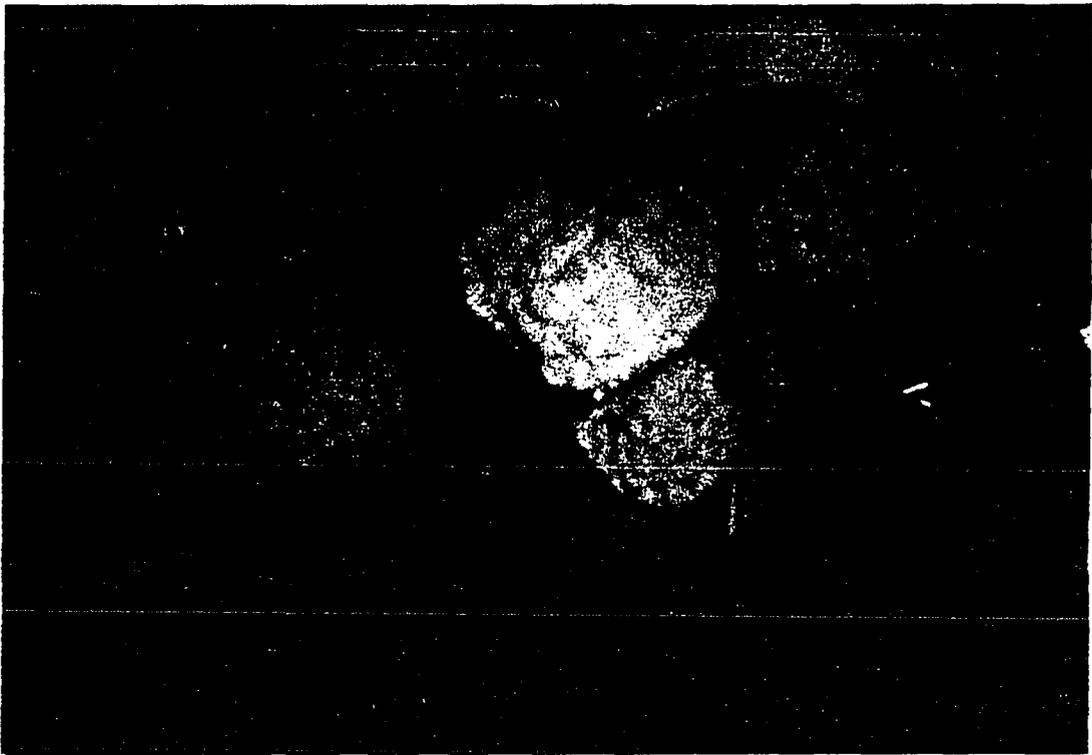


Fig. 1. The sweetsop, A. squamosa, fruit.

members of this family are used in folk medicine for various purposes.

The genus Annona consists of about 120 species; all are grown for their fruit (e. g., A. muricata (soursop), A. reticulata (custard-apple), and A. squamosa (sweetsop)). They are distributed mainly in tropical America but a few are natives of Asia and Africa. In the Philippines, A. squamosa fruit is a favorite item in the dining tables of Filipinos because its pulp possesses a pleasant aroma and a refreshing sweet taste. Various plant parts of some species are used in medicine, for stupefying fish and for insecticidal purposes. Annona dried seeds are used as an insecticide throughout many parts of the tropical world (Jacobson and Crosby 1971). Insecticidal potency of seed is high and is ascribed to the presence of a glyceride or glycerides of a hydroxylated unsaturated acid or acids of high molecular weight (Harper et al. 1947). Leaves, roots and bark also exhibit insecticidal properties.

Sanyal and Ghose (1934) claimed that A. squamosa leaves, unripe fruits and seeds contain vermifugal and insecticidal properties. Crushed seeds, in a paste with water are applied to the scalp to destroy lice. This mixture is also used as an abortifacient if applied to the os uteri in pregnant women. Bruised leaves, with salt, make a good cataplasm to induce suppuration. Fresh leaves

crushed between fingers and applied to the nostrils alleviate short fits and fainting. Ripe fruit, bruised and mixed with salt, is applied as a maturant to malignant tumors to hasten suppuration (Nadkarni 1927). Yamaguchi et al. (1964) found that A. squamosa possesses cytotoxic and antitumor activity in vitro or in vivo against experimental tumors. In a study conducted by Bhakuni et al. (1969), leaves of A. squamosa exhibited antitumor activity. Chemical investigation of the roots and bark of this plant yielded a compound which showed relaxant activity in frog's rectus abdominis and a laxative action in human (Krishna Rao et al. 1978). Green fruit is astringent and is used in the Philippines as a remedy for dysentery and diarrhea (Santos et al. 1953; Valenzuela et al. 1953). The bark is a powerful astringent and tonic while leaves are used as an anthelmintic (Nadkarni 1927). Seeds are a powerful irritant to conjunctiva and roots are considered a drastic purgative (Nadkarni 1927; Sanyal and Ghose 1934).

Toxic Principles of A. squamosa

Insecticidal principles of Annona have received considerable chemical investigation (Harper et al. 1947). Activity was attributed to the benzyl-isoquinoline alkaloid, anonaine (C₁₇ H₁₅ O₂ N, Fig. 2) (Santos 1930, 1932; Reyes and Santos 1931). Anonaine was isolated from A. reticulata bark by initial extraction with alcohol, removal of resins,

reextraction into ether and precipitation as the insoluble hydrochloride (Santos 1930; Reyes and Santos 1931; Gopinath et al. 1959). It was identified in extracts of other Annona species including A. cherimolia (Lebouef et al. 1982), A. glabra (Yang and Chen 1974), A. montana (Yang and Chen 1979; Leboeuf et al. 1982), A. muricata (Tattersfield and Potter 1940), A. palustris (Tattersfield and Potters 1940) and A. squamosa (Reyes and Santos 1931; Tattersfield and Potters 1940; Bhakuni et al. 1972; Krishna Rao 1978). It is also a constituent of Cananga odorata (Lebouef et al. 1982), Enantia polycarpa (Jossang et al. 1977), Isolona campanulata (Lebouef et al. 1982), Michela compressa (Yang 1962), Mitrella kentii (Lebouef et al. 1982), Nelumbo nucifera (Bernauer 1964), Neolitsea sericea (Nakasato et al. 1966), Polyalthia oliveri (Hamonniere et al. 1977), Pseuduvaria sp. (Lebouef et al. 1982), Pseuduvaria grandiflora (Lebouef et al. 1982), Schefferomitra subaequalis (Lebouef et al. 1982), Xylophia brasiliensis (Lebouef et al. 1982), Xylophia pancheri (Nieto et al. 1976), Xylophia papuana (Lebouef et al. 1982) and Doryphora sassafras (Chen et al. 1974). Other alkaloids are also present as are other types of compounds which may be active (Bhakuni et al. 1972; Krishna Rao et al. 1978; Bhaumik et al. 1979). A resinous mixture extracted from the seeds with ether, then precipitated with light petroleum was toxic to aphids (Harper et al. 1947) and a

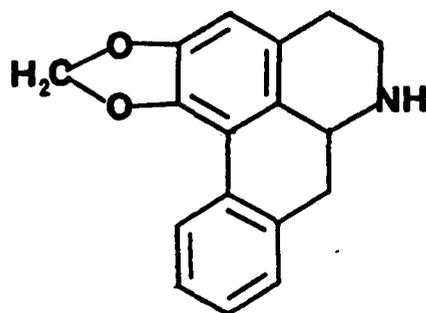


Fig. 2. Structure of anonaine (1,2-methylenedioxy-noraphorphine).

non-polar sesquiterpene fraction from the leaf wax was also insecticidal (Mackie and Misra 1956).

Insecticidal Properties of A. squamosa

Annona extractives were claimed to act as both contact and stomach poisons. Contact toxicity was equivalent to that of rotenone or nicotine; stomach poisoning was variable (Harper et al. 1947), and there was little or no ovicidal activity. In laboratory trials conducted by Tattersfield and Potter (1940), results showed contact insecticidal properties of A. muricata, A. palustris and A. reticulata to Aphis fabae Scop. and Macrosiphoniella sanborni (Gill.). A. reticulata was the most toxic with seeds, roots and stems possessing contact insecticidal properties. The most effective applications were against various aphids (Harper et al. 1947) and human body lice (Reyes and Santos 1931). Krishna Rao et al. (1978) obtained a volatile oil fraction from A. squamosa which showed repellent action against white ants and cockroaches.

No detailed toxicological or pharmacological data were reported for anonaine, although related alkaloids extracted from A. muricata (Meyer 1941) had a depressant action on rabbit heart.

III. INSECTICIDAL ACTIVITY OF A. SQUAMOSA SEED EXTRACTS

INTRODUCTION

Plants provide an abundant source of medicinal drugs and pesticidal compounds. These natural plant products may constitute new sources of pest control materials or their prototypes. The existence of naturally occurring insecticidal plant components has been known for centuries. However, relatively few of these compounds are used in crop protection today. Increasing problems associated with the use of synthetic insecticides caused renewed interest in these naturally occurring pesticides. There is a current worldwide effort aimed at screening plant species for bioactivity towards insect pests (Balandrin et al. 1985; Plimmer 1985). The presence of novel pesticidal components from some plants has been examined (Villani and Gould 1985; Lane et al. 1987). In particular, tropical plants are rich sources of bioactive chemicals. Plants from arid and semi-arid regions also possess insecticidal substances (Hedin 1982).

A. squamosa is one of those plant species reportedly screened for insecticidal properties. However, little information is available regarding its toxic effects on insects. Earlier studies showed that A. squamosa leaves,

unripe fruits, and seeds contained vermucidal and insecticidal properties (Sanyal and Ghose 1934). Although dried seeds are used as an insecticide throughout many parts of the tropical world (Jacobson and Crosby 1971), convincing data on toxicity to insects are not fully documented. Ethereal extracts of a closely related species A. senegalensis twigs were highly toxic to the large milkweed bug, Oncopeltus fasciatus (Dallas) (Jacobson et al. 1975). The toxic effect of A. squamosa seed extracts has never been evaluated against C. capitata, hence, this study. This paper aimed to determine insecticidal activity of the extract against C. capitata, and isolate and characterize the insect active fraction of the extract.

MATERIALS AND METHODS

Test Insects. Medfly eggs, larvae and pupae were obtained from the USDA Tropical Fruit and Vegetable Research Laboratory, Honolulu, Hawaii. Larvae were fed with a standard wheat diet (Tanaka et al. 1969) while adults were provided a sugar-protein hydrolysate mixture (3:1, w/w) and water.

Extraction of A. squamosa Seeds. Extraction and isolation of alkaloids were done following the method of Villar del Fresno and Rios Canavate (1983) with slight

modification. A. squamosa fruits were purchased from a local market in the Philippines. Pulp was separated from the seeds, after which, the latter were washed with water, air dried and brought to Hawaii. Seeds were ground in a Wiley mill to pass a 20 mesh sieve. Finely ground seeds were defatted with hexane (50-60⁰, Soxhlet), prior to extraction. Extraction was done by overnight soaking of 1 kg seeds in 3 liters of 95% ethanol. Extracts were separated from solids by filtration using suction. Plant material was extracted with ethanol twice and filtrate from each extraction was combined. Filtrates were then concentrated in vacuo using a rotary evaporator.

Isolation of Active Fraction from Seed Extract.

Fractionation of crude extracts and purification of active components were done with solvent partitioning, column and thin layer chromatography. Bioassays on C. capitata were used to follow insecticidal activity throughout extraction and isolation.

The dark-brown, crude seed extract concentrate was further extracted with chloroform. Chloroform extract was then dried with anhydrous sodium sulfate, filtered and evaporated in vacuo to a viscous brown syrup. Extract was chromatographed on silica gel. Column was gradually eluted with chloroform, followed by a mixture of chloroform and methanol (95:5, 90:10). The alkaloid containing fractions

were concentrated (semi-purified extract) and extracted with 5% HCl. The acidic solution was basified with 5% NH₄OH and extracted with ether-chloroform (1:1). The ether extract was washed with water, dried, concentrated (purified extract) and used in preparative thin layer chromatography. Plates were spotted with the extract and were developed in 9:1 chloroform-methanol. Spots were visualized under UV light and those with similar R_f values were scraped off from the plates. Isolates were recovered from the adsorbent with acetone which was completely removed by evaporating in vacuo.

Characterization of the Active Fraction. The active fraction was partially characterized based on its physical and chemical properties. Solubility of the fraction in different solvents, (i. e., acetone, benzene, chloroform, diethylether, dilute acid, dilute alkali, ethanol, hexane, methanol, methylene chloride, petroleum ether, water and xylene), and its R_f value in 9:1 chloroform-methanol were obtained.

A high pressure liquid chromatography of the fraction was further done. Chromatogram was run in a 1081 A liquid chromatograph (Hewlett-Packard model). A stainless steel Partisil PXS 10/25 ODS-2 (no. 1H) column was used. Column outlet was connected to the inlet of a variable wavelength detector (Gilson HM Holochrome UV/VIS) set at 254 nm.

Signal from the detector was fed into a recorder (3380S Hewlett-Packard integrator) which was set at 0.30 mV/min and 128 attenuation. Chromatographic conditions used were: eluent, methylene chloride-methanol (9:1) both HPLC grade; flow rate, 0.5 ml/min; pump setting, 150-151 and detector range, 0.01.

Bioassay of the Extracts and LD50 Determination.

Toxicity of the seed extract was determined in newly-emerged C. capitata adults. One ul acetone solution of the extract was topically applied on the abdominal venter of anesthetized flies using a Burkard microapplicator. Final concentrations used were determined from preliminary tests conducted on seed extracts. Each treatment was replicated six times with 10 insects per replicate. Treated flies were confined in mesh-cloth covered paper cups (9 cm diam x 6 cm height) provided with water and adult diet.

Equal numbers of insects were similarly treated with acetone and malathion (o,o-dimethyl-dithiophosphate of diethyl mercaptosuccinate, 95.0% purity) as control and standard insecticide, respectively. Mortalities were recorded 48 hr after treatment and LD50 values were computed by probit analysis (Finney 1971).

Toxic effect of the extracts was also determined on third instar larvae. Larvae were dipped in various concentrations of the extracts for five seconds and were

kept in petri dish (6 cm diam x 1 cm height) provided with larval food. The dish was put onto a bigger one (9 cm diam x 2 cm height) containing sand for popping larvae to pupate on. Each treatment consisted of five replications with 10 larvae per replicate. Control larvae were treated with acetone. Number of larvae that reached pupation and those that emerged into adults were recorded.

Data Analysis. Data were statistically analyzed using analysis of variance (ANOVA) procedures available in the Statistical Analysis System computer package (Ray 1982).

RESULTS

Extraction and Isolation of the Active Fraction.

Extraction of the ground A. squamosa seeds with ethanol produced a dark-brown crude extract concentrate and re-extraction with chloroform yielded a light brown solution. A viscous brown syrup was obtained upon the removal of chloroform in vacuo. From 1 kg of ground seeds for extraction, about 21.4 g of chloroform extract concentrate was obtained.

At least 7 isolates were obtained through preparative thin layer chromatography (Fig. 3). The figure shows that the extract consisted of many components with a wide range of polarity, including a very polar fraction which remained

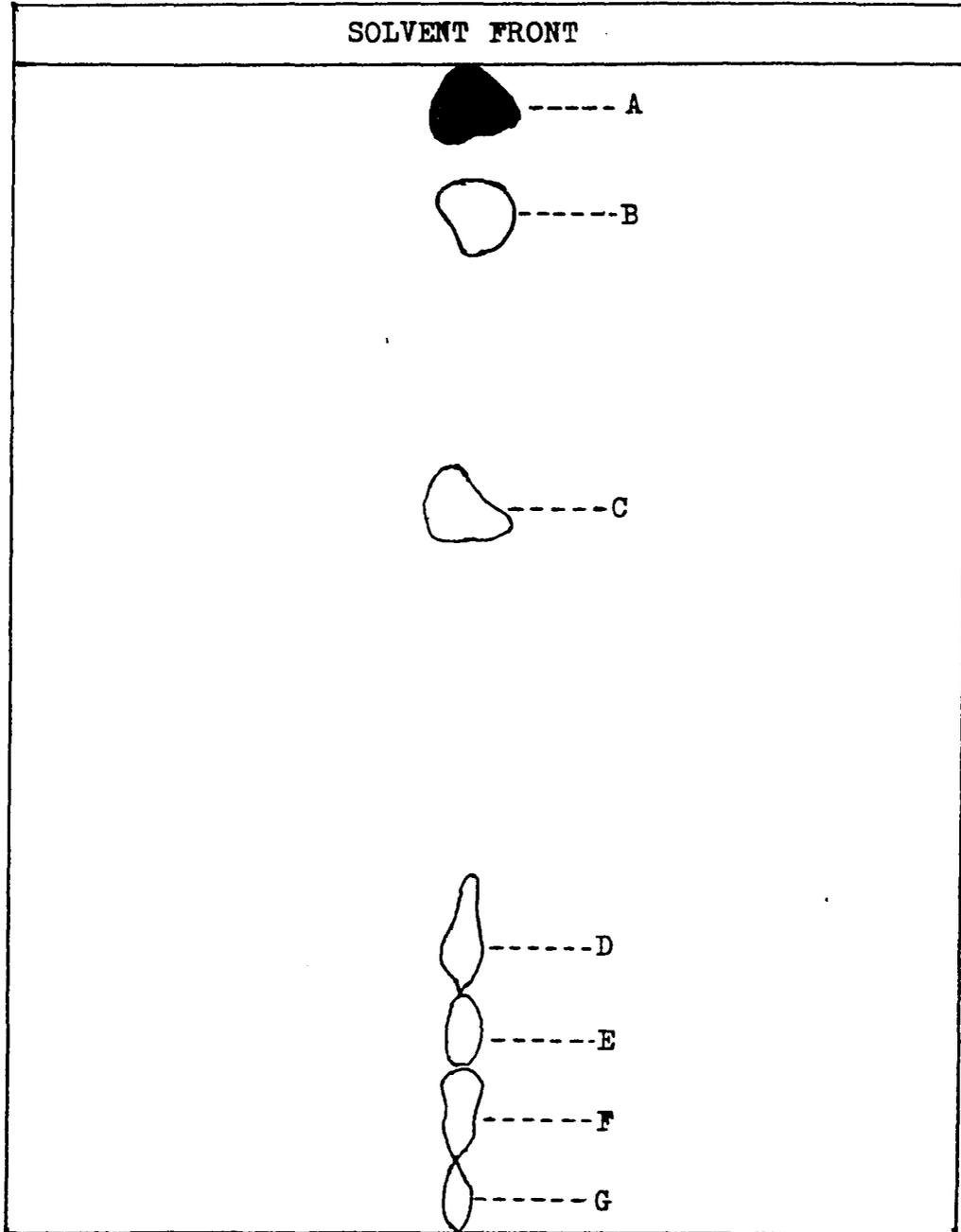


Fig. 3. Thin layer chromatogram of A. squamosa seed extract in 9:1 chloroform-methanol.

at the starting point of the plate. The first three fractions (A, B, C) appeared to be relatively pure, while the remaining fractions are still mixtures of two or more components. Such is suggested by the sizes and shapes of the spots obtained in the chromatogram. Fractions A (FA) and G (FG) had the highest (0.92) and lowest (0.01) Rf values, respectively, on silica gel-coated plates developed in chloroform:methanol (9:1) (Table 1). FA had the largest amount recovered (302.6 mg) while FG had the smallest (4.0 mg) based on one kg of A. squamosa seeds used for extraction.

To determine the insect active fraction from among the 7 isolates, each was bioassayed against C. capitata. When topically applied to the flies, FA and FG gave the highest (86%) and lowest (8%) percentage kill, respectively (Table 2). As a consequence, FA was included in the LD50 determination for comparison. Based on the percentage mortality of the treated flies, insecticidal activity of the fraction decreased as polarity increased.

Characterization of the Active Fraction. Upon recovery from the adsorbent, FA appeared as a light yellowish brown oil. The oil was completely miscible with acetone, chloroform, diethyl ether, methylene chloride, benzene and xylene. Solubility of FA in hexane and petroleum ether was relatively lower than that observed in the aforementioned

Table 1. Fractions isolated from A. squamosa seed extracts by preparative thin layer chromatography.

Fraction	Rf Value ¹	Weight Recovered (mg) ²	Recovery (%) ²
A	0.92	302.6	3.03×10^{-2}
B	0.86	239.9	2.40×10^{-2}
C	0.75	78.7	7.87×10^{-3}
D	0.13	30.5	3.05×10^{-3}
E	0.09	13.5	1.35×10^{-3}
F	0.04	7.7	7.7×10^{-4}
G	0.01	4.0	4.0×10^{-4}

¹

Solvent system used for development was 9:1 chloroform-methanol.

²

Based on 1 kg of plant material used for extraction.

Table 2. Insecticidal effect of the isolated fractions from A. squamosa seed extract on C. capitata.

Fraction ¹	Percentage Mortality ²
A	86.0
B	66.0
C	30.0
D	8.0
E	14.0
F	12.0
G	8.0
Control ³	0.0

¹ Evaluated at a set dosage of 100 ug/insect.

² Average of 5 replications with 10 insects per replicate. Mortality observations were obtained 48 hr after treatment.

³ Control was treated with acetone.

organic solvents. FA was insoluble in dilute acid, dilute alkali, ethanol, methanol and water.

High pressure liquid chromatographic analysis of FA showed four peaks with the first peak as the major component when the chromatogram was run in 9:1 methylene chloride-methanol (Fig. 4). Same number of peaks were obtained when the fraction was eluted with 9:1 chloroform-methanol, however, two major peaks were observed (Fig. 5). The number of peaks could be indicative of the number of components present.

Bioassays of the Extracts and LD50 Determination. Male flies were generally more susceptible to the toxic effect of the extracts than females (Table 3). The relative order of toxicity of the extracts to the males was: FA > purified extract > semi-purified extract > ethanol extract > chloroform extract. The same was true for the females except that chloroform extract was more toxic than ethanol extract. Malathion was: 1625X and 1649X more toxic than EtOH extract to males and females; 2362X and 2331X more toxic than CHCl₃ extract to males and females; 1312X and 1636X more toxic than SPE to males and females; and 1175X and 1593X more toxic than PE to males and females. FA was 2.55×10^{-3} and 2.07×10^{-3} as toxic as malathion to male and female flies, respectively.

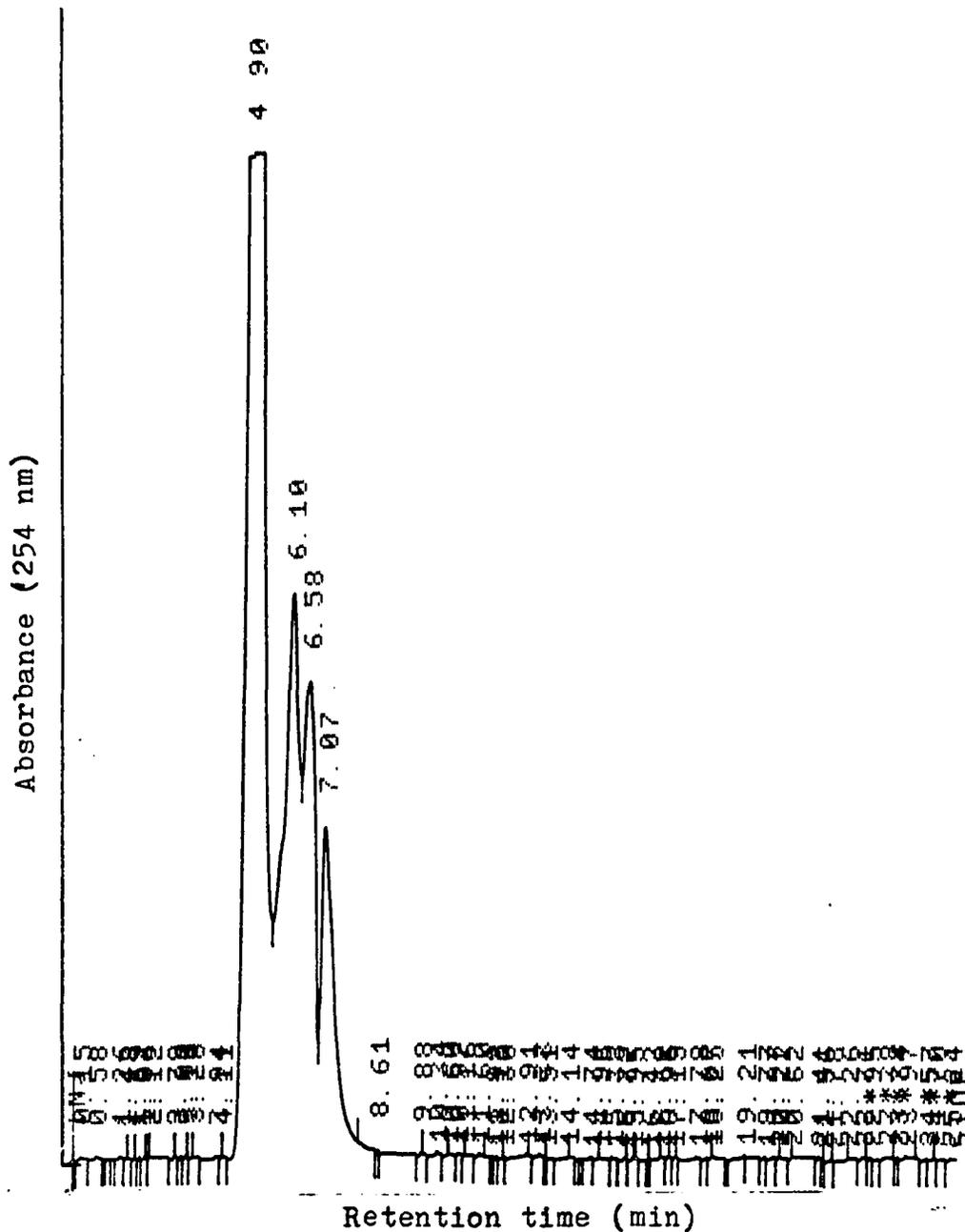


Fig. 4. High pressure liquid chromatographic analysis of FA. Conditions: eluent, methylene chloride-methanol (9:1); flow rate, 0.5 ml/min; pump setting, 150; detector range, 0.01 at 254 nm; attenuation, 128.

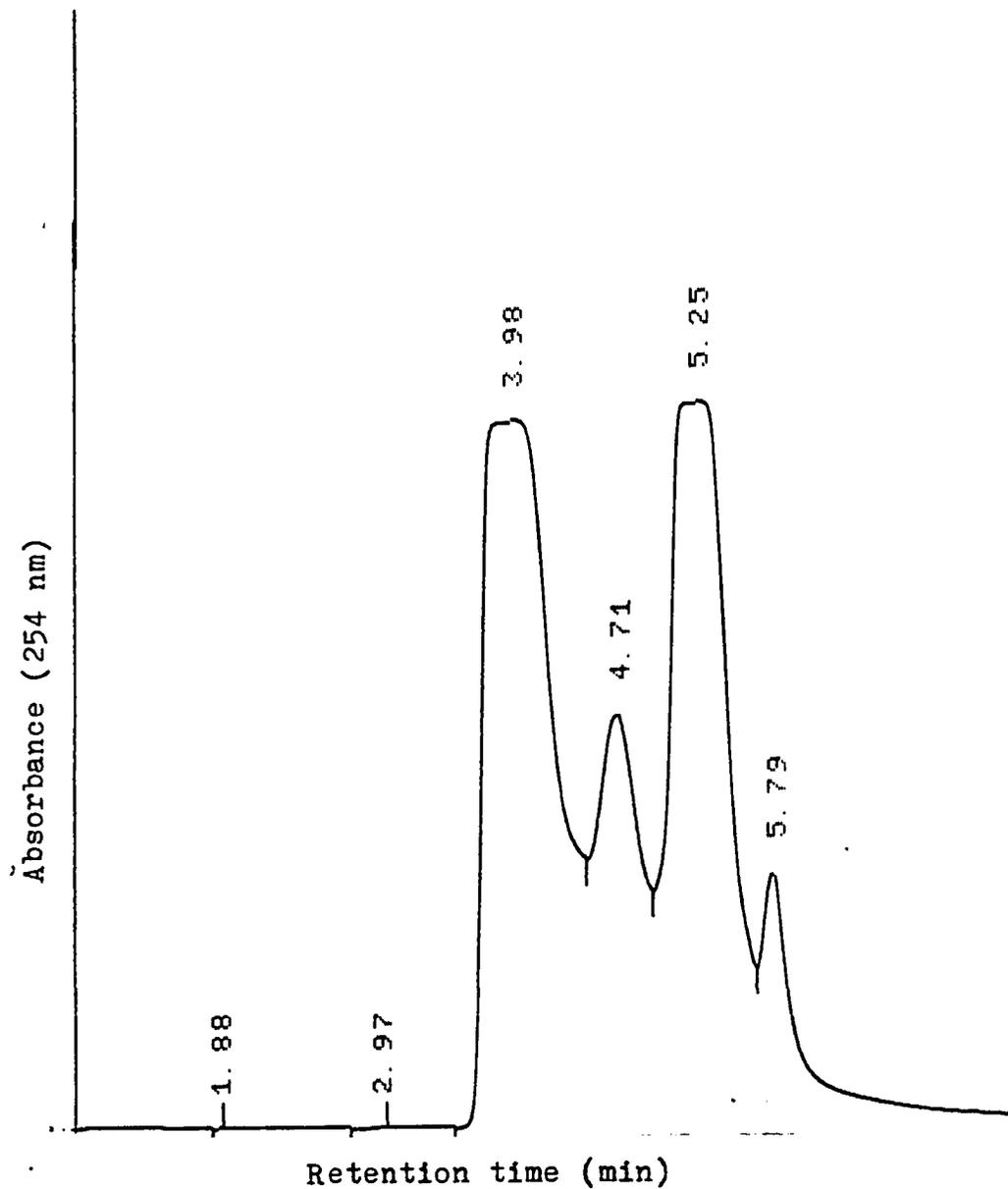


Fig. 5. High pressure liquid chromatographic analysis of FA. Conditions: eluent, chloroform-methanol (9:1); flow rate, 0.5 ml/min; pump setting, 150; detector range, 0.05 at 254 nm; attenuation, 256.

Table 3. Median lethal dose (LD50) of A. squamosa seed¹ extracts and malathion on C. capitata (L.) .

Chemical	Sex	LD50 (ug/mg insect)	Confidence Limits(95%)	
			Lower	Upper
EtOH extract	M	3.247	2.447	4.017
	F	4.948	3.657	6.550
CHCl3 extract	M	4.724	3.662	5.914
	F	6.995	5.440	9.415
² SPE	M	2.624	1.974	3.117
	F	4.909	3.556	6.574
³ PE	M	2.349	1.622	2.992
	F	4.780	3.882	5.794
⁴ FA	M	0.785	0.335	1.841
	F	1.448	0.033	1.836
Malathion	M	0.002	0.001	0.002
	F	0.003	0.002	0.003

¹

Chemicals were topically applied in 1 ul acetone solution to newly-emerged adults. Mortality was determined 48 hr after treatment and based on 60 insects per dosage applied. LD50 values were computed by probit analysis.

²

Semi-purified extract. Obtained after CHCl3 extract was column chromatographed.

³

Purified extract. Obtained after SPE was extracted with 5% HCl, 5% NH4OH and ether-CHCl3 (1:1).

⁴

Insect active fraction of A. squamosa seed extract.

To determine the toxic effect of the extracts on third instar larvae, they were dipped in various concentrations of the test compounds. Dipping the larvae in chloroform extract did not affect pupation and adult emergence (Table 4). Similarly, semi-purified extract did not influence emergence but a significant reduction in the number of larvae reaching pupal stage was observed when larvae were dipped in 25% extract. Survivors were allowed to continue development to the adult stage. However, there was no morphogenetic effect observed in the ensuing pupae and emerging adults.

Table 4. Effect of dipping C. capitata¹ larvae in acetone solutions of A. squamosa seed extracts .

Chemical	Treatment (%)	Pupation (%)	Adult Emergence (%)
CHCl ₃ ³ extract	0.00 ²	98a	94.0a
	1.56	90a	95.6a
	3.13	90a	95.8a
	6.25	86a	93.2a
	12.50	90a	87.8a
	25.00	88a	89.8a
SPE ³	0.00 ²	98a	94.0a
	1.56	88ab	95.4a
	3.13	96ab	94.0a
	6.25	90ab	88.8a
	12.50	88ab	93.2a
	25.00	86 b	93.6a

¹

Based on the mean of 5 replications with 10, third instar larvae per replicate. Larvae were dipped in acetone solutions of A. squamosa seed extracts for 5 seconds. Means followed by the same letter are not significantly different at 5% probability (DMRT). CHCl₃ extract, pupation, df=5, 29, F=0.88; adult emergence, df=5, 29, F=0.79; SPE, pupation, df=5, 29; F=2.21; adult emergence, df= 5, 29, F=0.45.

²

Control was treated with acetone.

³

Semi-purified extract.

DISCUSSION

Recent efforts have been devoted in search for alternatives to current arsenal of pest control agents. Plant-derived extracts and phytochemicals are again scrutinized for potentially useful products or as models for new classes of synthetic insecticides (Hedin 1982; Balandrin et al. 1985).

Extract bioassays with respect to C. capitata clearly showed that A. squamosa contained compounds toxic to the insect. Bioassays successfully guided the phytochemical fractionation of the extracts to yield 1 or 2 active insecticidal compounds. Thin layer chromatography of the extract, developed in 9:1 chloroform-methanol, yielded 7 fractions with varying degrees of polarity. Fractions tested positive for alkaloids when the plate was sprayed with Mayer's and Marquis' reagents. FA was scraped off the plate and re-chromatographed on TLC plate. A single spot was obtained when the chromatogram was developed in the same solvent system. However, FA resolved into 4 peaks with 1 major peak when high pressure liquid chromatography was done. This shows that HPLC has a higher resolution and is more powerful than TLC. FA also showed same number of peaks but with 2 major ones when 2-3 month old sample was analyzed

chromatographically which suggests a possible degradation of the compound when stored over a period of time.

Topical application of each fraction on C. capitata revealed that FA and FG were the most and least toxic fractions, respectively. Polarity of the fraction could have contributed to toxicity. Rate of penetration of any material through insect cuticle depends on cuticle nature as a membrane and nature of material, particularly its polarity. Insect cuticle is hydrophobic (i.e., lipophilic) therefore apolar compounds can easily penetrate through the membrane (Matsumura 1985). Because FA is apolar, it probably penetrated through the cuticle and carried by the hemolymph to the site of action. FG, a very polar fraction, was possibly not able to enter through the membrane.

Size is an important factor influencing susceptibility of insects to pesticides. Reinert (1970) proposed that effects of age and size on insect susceptibility can be ascribed to increase in fat content. In general, bigger insects contain more fats than their smaller counterparts. This explains why the males were generally more susceptible to A. squamosa seed extracts than females. The latter, being bigger, had more fats which trapped the toxic compound, thus, making it unavailable to the target site. Within an hour after treatment, flies were knocked down and

exhibited paralysis characteristic of poisoning by naturally occurring botanical insecticides as rotenone.

Laboratory screening of crude plant extracts is an initial step in their development as pest control agents. Convenience and reliability of bioassay systems are important considerations in search for new bioactive substances. The assay should employ a minimum amount of test materials to permit phytochemical screening and guide fractionation. Results of our study demonstrated the effectiveness of extraction and isolation procedures used in detecting A. squamosa seed extract's insecticidal property. However, gas chromatography-mass spectroscopy needs to be done to elucidate chemical nature of the active fraction. Further studies with other insect species are warranted to determine if the extract has a broad application to numerous target organisms.

IV. BIOLOGICAL EFFECTS OF A. SQUAMOSA SEED EXTRACTS

INTRODUCTION

Natural plant compounds play an increasingly significant role in pest control, both as sources of insect control agents or models for the chemical synthesis of structurally related mimics. The need for new and safer insecticides necessitates exploitation of natural chemical defense mechanisms of plants (Menn 1980). Research works have been focused on plant chemicals which specifically affect insect feeding behavior, oviposition, and growth. (Hedin 1977). Deterrency, retarded growth, and development are used to document the antiherbivore role of plant allelochemicals (Rhoades 1979; Gunderson et al. 1985). Insect growth inhibitors may provide effective tools for integrated crop management systems by protecting crops from herbivory while preventing destruction of beneficial insect complex (Bernays 1983). Larval growth and development of corn earworm, Heliothis zea (Boddie) were markedly disrupted when insects were fed diets containing L-azetidine-2-carboxylic acid (Adeyeye and Blum 1989). Avoidance of Erysimum cheiranthoides for oviposition by Pieris rapae L. was attributed to the presence of water-soluble deterrents (Renwick et al. 1989). Salloum and Isman (1989) reported that petrol and ethanolic extracts of six asteraceous weeds

severely inhibited larval growth of variegated cutworm, Peridroma saucia (Hbn.). Potent insect growth inhibitors may have a use where resistance to insecticides is a problem (Bernays 1983) and where pest managers are integrating biological and cultural control methods in crop protection strategies.

Our earlier studies showed that A. squamosa seed extracts were toxic to C. capitata. This prompted us to further investigate biological effects of the extracts against the medfly. The study was conducted to determine chemosterilant and ovicidal effects of the extract and evaluate its influence on settling response, and growth and development of C. capitata.

MATERIALS AND METHODS

Test Insects. Medfly eggs, larvae and pupae were obtained from the USDA Tropical Fruit and Vegetable Research Laboratory, Honolulu, Hawaii. Larvae were fed with a standard wheat diet (Tanaka et al. 1969) while adults were provided a sugar-protein hydrolysate mixture (3:1, w/v) and water.

Chemosterilizing Activity. Sublethal doses (LD5, LD10 and LD20) of chloroform and semi-purified extracts were topically applied to newly-emerged flies, previously

anesthetized with nitrogen. Each concentration was determined from the dose-response curves obtained from the earlier toxicity study. After treatment, flies were kept in separate cages according to sex. Surviving flies were paired 48 hr after and single pairs in copula were kept in plastic cages (12 cm diam x 7 cm height) provided with water and adult diet. Treated males were paired with treated and untreated females and vice versa. Untreated pairs were included as controls. Each variant was replicated 10-15 times. Perforated plastic vials were inserted through a hole in the cage for egg deposition. A piece of dental wick soaked in guava juice was put inside the vial to induce oviposition. Insect pairs were observed daily and adult longevity, total egg production (fecundity) and egg hatchability were recorded. Spermathecae from mated females were also dissected out for confirmation of successful insemination.

Ovicidal Activity. Toxic effect of the extracts on C. capitata eggs was also evaluated. Twenty, newly-laid eggs were dipped for 10 seconds in various concentrations of the extracts dissolved in 10% Tween-20 (polyoxyethylene sorbitan monolaureate, Bio-Rad). Control eggs were dipped in solvent alone. Each treatment was replicated six times. Treated eggs were kept in 6 cm diam x 1 cm petri dish and incubated

at room temperature. Numbers of hatched larvae were counted and percent hatchability was computed.

Ovarian Growth in C. capitata. To determine the effect of A. squamosa extract on the medfly ovarian growth, the chemical should be applied to females prior to vitellogenesis. Based on light microscopic and SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analyses of developing oocytes and hemolymph protein patterns from C. capitata, females were considered previtellogenic within 24 hr after eclosion (Hsu et al. 1989). Therefore, newly-emerged, virgin females were topically applied with sublethal doses of the extract. After treatment, flies were caged and provided with food as in the toxicity test. At various time intervals (3, 5, 7, 10, 15 and 20 days after treatment), 40 females per treatment were sacrificed. Ovaries of treated and control medflies were dissected out and their average growth was determined by measuring the length with a stage micrometer.

Settling Response of C. capitata. To determine the effect of seed extracts on the settling response of adult medfly, a modified method of McDonald and McGinnis (1985) was followed. A 4 x 4 cm papaya section was perforated with 9 holes using a dissecting needle and painted with the required concentration of the extract, dissolved in acetone, with the aid of a camel's hair brush. Solvent was allowed

to evaporate, afterwhich, the sections were arranged randomly and equidistantly from each other but forming a circle inside a flexiglass cage (30 x 30 x 38 cm) (Fig. 6). One hundred anesthetized, 7 to 8 day old gravid females were released at the center and kept in the cage for 24 hr. Adult diet and water were provided to the flies. Number of insects that settled on each section was noted at 15 min interval for two hr and every hour, thereafter, for six hr to establish an index of host finding. Sum of fly observations for the entire period constituted an index of flies on papaya. Fruit section was dissected 24 hr after exposure and the number of eggs was counted.

Growth and Development. While short term experiments permit distinction between any toxic or repellent effects of test compounds, long term growth studies are a more realistic tests of cumulative effects of plant chemicals on the growth and development of insects. If a female lays eggs on a new potential host, newly-hatched larvae may be forced to feed on this plant. To approximate such a situation, effect of extract on the growth and development of medfly was studied by incorporating the chemicals with larval diet. Extracts were dissolved in acetone to provide treatment levels of 0.039, 0.078, 0.156, 0.313 and 0.625%. Five ml per treatment were added to 100 g diet, afterwhich, treated diet was mixed thoroughly with a stirring rod to

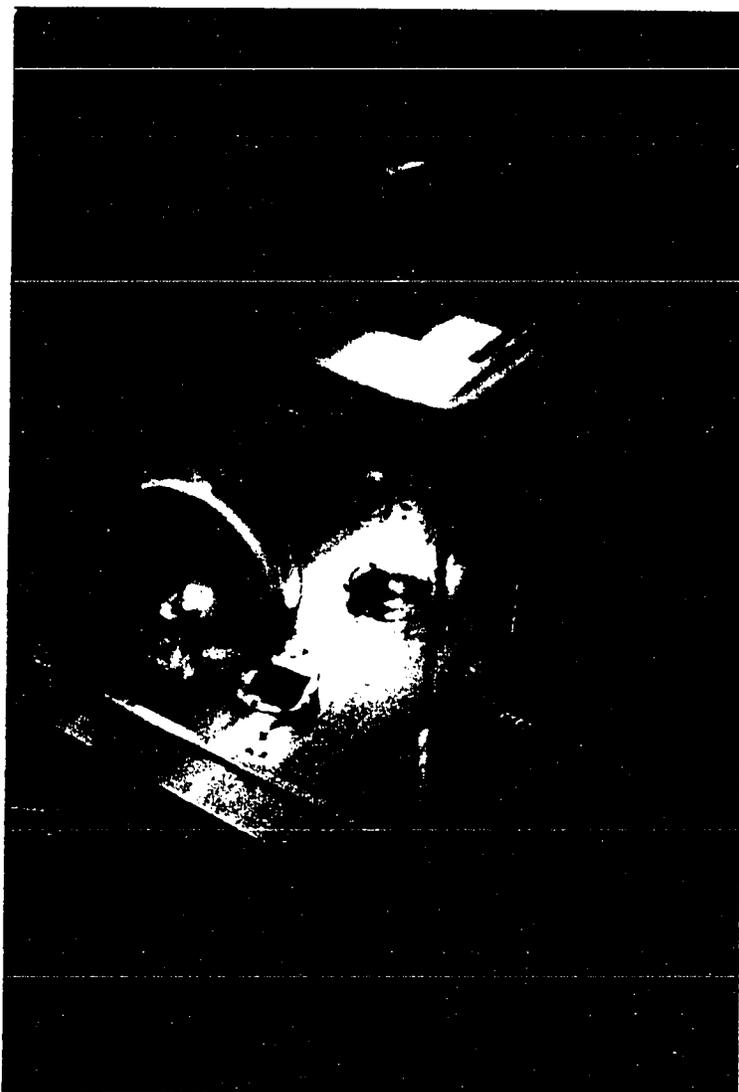


Fig. 6. A 30 x 30 x 38 cm flexiglass cage used in settling response study. Treated papaya sections were arranged randomly and equidistantly from each other but forming a circle.

ensure complete mixing. Control diet was similarly treated with acetone. Solvent was allowed to evaporate overnight in the hood and 20 g diet was poured onto a petri dish (6 cm diam x 1 cm height). Diet was infested with 10 newly-hatched, first instar larvae and the dish was put onto a bigger dish (9 cm diam x 2 cm height) containing sand for larvae to pupate on. Each treatment was replicated five times. Larval survival as percent pupating; growth rates expressed as average number of days to reach pupal stage; size expressed as pupal weight; and pupal survival as percent of adults emerging were determined. Average number of days to pupation was calculated as sum of the products of each daily count of larvae pupating, multiplied by number of days to reach pupation divided by total number of pupae.

Data Analysis. All data were statistically analyzed using analysis of variance (ANOVA) procedures available in the Statistical Analysis System (SAS) computer package (Ray 1982).

RESULTS

Chemosterilizing Activity. Topical application of sublethal doses of A. squamosa seed extracts did not cause abnormal behavior among treated flies. The insects were able to feed, undergo courtship behavior, mate and oviposit

eggs similar to controls (Table 5). The data indicate no significant interaction between the two main factors, dose and mating combinations. Chloroform extract did not cause any pronounced effect on adult longevity and the number of eggs laid. Although egg production in most matings was higher than the control, the differences were not statistically significant. However, a sterilant effect was noted when the F1 generation egg hatch was calculated. This was exhibited by a decreased hatch. Significant reduction in hatchability was observed when females were treated with > 8 ug chloroform extract per insect and mated with untreated males. Semi-purified extract treatment reduced mean female survival time, but it did not significantly affect longevity of both sexes (Table 6). At 9 ug per insect, however, mating semi-purified extract treated females with treated males significantly reduced egg hatching although egg production did not differ from controls. Although treating females alone with chloroform extract was sufficient enough to cause significant reduction in egg hatching, both males and females have to be treated with semi-purified extract to observe the same effect. However, regardless of the semi-purified extract dosage used, fecundity in various mating combinations was lower than in controls although no significant differences were observed. Treated males seemed capable of transmitting the

Table 5. Egg production, hatchability and longevity of C. capitata adults treated with CHCl₃ extracts ¹.

Dose ug/insect	Pairing ² M x F	Longevity(days) ³		Total No. Eggs Laid	Eggs laid Per Day	Hatchability ⁴ (%)
		Male	Female			
0	U x U	42.9a	30.6abc	825.9a	29.6a	87.6a
5.0	T x T	58.7a	37.8a	974.3a	26.4a	77.8abc
	T x U	55.7a	29.5abc	963.3a	31.5a	90.1a
8.0	U x T	62.5a	29.5 bc	739.3a	33.5a	70.5abc
	T x T	57.6a	31.7abc	963.1a	30.8a	85.3ab
	T x U	55.7a	36.8a	1035.1a	31.5a	77.7abc
14.0	U x T	47.8a	27.9 c	787.3a	26.7a	63.8 bc
	T x T	33.8a	34.3abc	915.3a	30.1a	71.5abc
	T x U	40.6a	35.5ab	925.6a	27.9a	67.3abc
	U x T	40.1a	26.6 c	741.8a	29.6a	56.6 c

¹
Newly-emerged adults were topically applied with 1 ul acetone solution (LD5, LD10, LD20) of CHCl₃ and semi-purified extracts. Survivors were mated with treated or untreated partners 48 hr after the treatment.

²
T = treated; U = untreated.

³
Values are based on the mean of 10-15 observations.

⁴
Based on hatchability of eggs laid for 3 days.

Means followed by the same letter are not significantly different at 5% probability (DMRT). Male df=9,149 F=1.52; female df=9,149 F=2.38; total eggs laid df=9,99 F=1.12; eggs laid/day df=9,99 F=0.36; hatchability df=9,57 F=2.37.

Table 6. Egg production, hatchability and longevity of C. capitata adults¹ treated with semi-purified extracts .

Dose ug/insect	Pairing ² M x F	Longevity(days) ³		Total No. Eggs Laid	Eggs laid Per Day	Hatchability ⁴ (%)
		Male	Female			
0	U x U	42.9ab	30.6a	825.9a	29.6a	87.6ab
2.5	T x T	31.5 b	25.7a	766.3a	26.2a	93.6a
	T x U	32.7 b	22.2a	580.5a	27.2a	93.2a
4.0	U x T	44.0ab	22.5a	606.8a	33.5a	97.6a
	T x T	44.7ab	26.3a	714.6a	28.1a	79.1 b
	T x U	29.7 b	21.0a	652.3a	26.9a	89.9a
9.0	U x T	55.2a	22.0a	645.5a	32.9a	82.8ab
	T x T	38.5ab	22.7a	822.8a	32.3a	65.2 b
	T x U	34.4 b	23.7a	648.9a	24.1a	79.9ab
	U x T	27.1 b	25.9a	715.3a	24.4a	80.4ab

¹

Newly-emerged adults were topically applied with 1 ul acetone solution (LD5, LD10, LD20) of semi-purified extracts. Survivors were mated with treated or untreated partners 48 hr after the treatment.

²

T = treated; U = untreated.

³

Values are based on the mean of 10-15 observations.

⁴

Based on hatchability of eggs laid for 3 days.

Means followed by the same letter are not significantly different at 5% probability (DMRT). Male df=9,149 F=2.04; female df=9,149 F=0.93; total eggs laid df=9,98 F=0.66; eggs laid/day df=9,88 F=0.7; hatchability df=9,59 F=1.86.

toxic effect of the extract when mated with untreated females as indicated by a lower number of eggs laid. The F1 larvae were not monitored past eclosion in all the treatments.

Ovicidal Activity. Ovicidal property of the seed extracts was assessed by exposing newly-laid eggs to different concentrations of the test compounds. Egg hatchability was determined in each treatment group (Table 7). Hatchability decreased as concentration of the extract increased. Significantly, less eggs hatched when dipped in > 0.313% ethanol, chloroform, and semi-purified extracts.

When the unhatched eggs in the various treatments were examined microscopically, dead embryos were seen under the egg chorion. This suggests that the ovicidal action occurred late in embryogenesis because most treated eggs contained fully developed embryos.

Ovarian Growth in *C. capitata*. Effect of the seed extracts on oocyte development was investigated by measuring ovarian length of treated medflies. Chloroform and semi-purified extract treatments significantly reduced ovarian growth at > 5 ug and > 2.5 ug per insect, respectively (Table 8). This suggests possible interference with vitellogenesis. Inhibitory effect of chloroform extract was more pronounced at 3 and 5 days post treatment. At 7 days

Table 7. Ovicidal effect of A. squamosa seed extracts on C. capitata eggs ¹.

Concentration (%)	Hatchability (%)		
	EtOH extract	CHCl ₃ extract	² SPE
³ 0.000	98.3a	95.0a	95.0a
0.039	95.8a	92.5a	90.8a
0.078	94.2a	96.7a	92.5a
0.156	90.8a	84.2a	81.7a
0.313	74.2 b	57.5 b	66.5 b
0.625	31.7 c	34.2 c	45.0 c

¹

Based on the mean of 6 replications with 20 eggs per replicate. Means followed by the same letter are not significantly different at 5% probability (DMRT). EtOH extract df=5,35 F=69.26; CHCl₃ extract df=5,35 F=17.68; SPE df=5,35 F=8.25.

²

Semi-purified extract.

³

Control eggs were dipped in 10% Tween for 10 sec.

Table 8. Effect of A. squamosa seed extracts on the ovarian growth in C. capitata¹.

Chemical	Treatment (ug/insect)	Length (mm) Days after treatment					
		3	5	7	10	15	20
CHCl ₃ extract	0	1.18a	1.56a	1.58a	1.63a	1.52a	1.50a
	5.0	1.03 b	1.41 b	1.46 b	1.57a	1.46ab	1.40 b
	8.0	0.97 b	1.32 c	1.44 b	1.39 b	1.42 b	1.34 b
	14.0	0.75 c	1.25 c	1.42 b	1.36 b	1.40 b	1.37 b
Semi- purified extract	0	1.18a	1.56a	1.58a	1.64a	1.55a	1.50a
	2.5	1.11 b	1.46 b	1.47 b	1.45 b	1.42 b	1.37 b
	4.0	1.04 c	1.39 bc	1.43 bc	1.43 b	1.46 b	1.38 b
	9.0	0.94 d	1.34 c	1.40 c	1.40 b	1.46 b	1.38 b

¹

Based on the mean of 40 females. Newly-emerged insects were topically applied with 1 ul acetone solution (LD5, LD10, LD20) of CHCl₃ and semi-purified extracts. Means followed by the same letter are not significantly different at 5% probability (DMRT). CHCl₃ 3 DAT df=42,129 F=3.76; 5 DAT df=42,159 F=2.6; 7 DAT df=42,159 F=1.6; 10 DAT df=42,159 F=2.06; 15 DAT df=42,159 F=1.14; 20 DAT df=42,159 F=1.26; SPE 3 DAT df=42,159 F=3.45; 5 DAT df=42,159 F=1.98; 7DAT df=42,159 F=2.67; 10 DAT df=42,159 F=2.25; 15 DAT df=42,159 F=1.06 20 DAT df=42,159 F=1.39.

post treatment, flies started to recover from the effect although ovarian growth was still significantly reduced. Semi-purified extract inhibited growth markedly until 7 days post treatment. After this period, flies seemed to overcome the effect. Furthermore, at 20 days post treatment ovarian growth was still inhibited for both CHCL3 and semi-purified extract treated medflies.

Settling Response and Oviposition. When adult medflies were released at the center of the cage, they remained at the release site for varying periods of time before dispersing. Thereafter, the flies flew, hovered, walked or remained stationary before they settled on the papaya. At > 0.78%, significantly fewer flies were attracted to the CHCl3 treated sections compared to the controls (Table 9). A similar response was observed when the sections were exposed to the flies for 6 hr more. This suggests a possible repellent effect of the extract. While chloroform extract repelled the flies from settling on papaya, it did not influence the number of eggs laid by the females 24 hr after release.

Flies were significantly repelled from settling on papaya tissues treated with > 0.39% semi-purified extract at 2 hr after release (Table 10). When flies were kept in the cage for 6 hr more, the extract caused a significant reduction in the number of flies attracted to the sections

Table 9. Settling response of and oviposition by C.¹
capitata on CHCl₃ extract treated papaya sections .

Concentration (%)	No. of flies attracted		No. of eggs laid ²
	2 hr after	8 hr after	
0.00 ³	17.13a	34.38a	57.50a
0.39	9.63a	19.50a	38.63a
0.78	2.38 b	8.25 b	46.13a
1.56	2.50 b	6.75 b	32.63a
3.13	2.38 b	7.00 b	52.13a

¹ Based on the mean of 8 replications per treatment. Means followed by the same letter are not significantly different at 5% probability (DMRT). 2 hr after df=4,39 F=15.64; 8 hr after df=4,39 F=10.62; eggs laid df=4,39 F=0.91.

² Eggs were counted 24 hr after release.

³ Control was treated with acetone.

Table 10. Settling response of and oviposition by C.¹
capitata on semi-purified extract treated papaya sections .

Concentration (%)	No. of flies attracted		No. of eggs laid ²
	2 hr after	8 hr after	
0.00 ³	65.88a	93.00a	1485.67a
0.39	27.25 b	42.88ab	648.17ab
0.78	15.00 bc	23.50 bc	294.17 b
1.56	11.38 bc	29.75 bc	388.17 b
3.13	6.13 c	16.75 c	352.67 b

¹ Based on the mean of 8 replications per treatment. Means followed by the same letter are not significantly different at 5% probability (DMRT). 2 hr after df=4,39 F=8.82; 8 hr after df=4,39 F=5.43; eggs laid df=4,39 F=2.92.

² Eggs were counted 24 hr after release.

³ Control was treated with acetone.

at > 0.78%. Unlike chloroform extract ,however, semi-purified extract caused significant reduction in the number of eggs laid by females at > 0.78%. Results suggest that the semi-purified extract is an oviposition deterrent. Oviposition was deterred as the medfly laid one-fifth the number of eggs on papaya sections treated with > 0.78% extract as on controls treated with acetone only.

Growth and Development. Chronic exposure to alkaloid diets demonstrated that survival and developmental characteristics of the insect were variously affected. Chloroform extract significantly reduced the number of larvae reaching the pupal stage at 0.625% (Table 11). The extract retarded larval growth at > 0.312% and caused 28% mortality at 0.625% compared with the controls. Feeding the medflies with chloroform extract treated diet did not affect the pupal size, adult emergence and the length of time between pupation and eclosion. Semi-purified extract had no effect on pupation, pupal size, eclosion and the length of time to eclosion (Table 12). However, the extract significantly prolonged the larval stage at > 0.078%. It appears that C. capitata can tolerate diets containing levels of semi-purified extract used here because the larval number reaching the pupal stage did not differ significantly from the control. Delays in development occurred only

Table 11. Development of C. capitata on A. squamosa seed CHCl₃ extract¹ treated diet .

Concentration (%)	Avg. Days to Pupation	Pupation (%)	Pupal Wt. ² (mg)	Eclosion (%)	Avg. Days to Eclosion
0.000 ³	6.70 cd	92.0a	10.2a	98.00a	9.79ab
0.039	6.50 d	92.0a	10.4a	95.20a	9.97a
0.078	6.80 cd	76.0ab	10.1a	95.00a	9.93ab
0.156	7.20 c	78.0ab	10.4a	94.92a	9.72ab
0.312	7.84 b	80.0ab	10.3a	97.14a	9.71ab
0.625	8.70a	72.0 b	10.2a	84.84a	9.61 b

¹ Based on the mean of 5 replications with 10 insects per replicate. Treated diet was infested with newly-hatched larvae. Means followed by the same letter are not significantly different at 5% probability (DMRT). Avg. days to pupation df=5,29 F=17.51; pupation df=5,29 F= 2.01; pupal wt. df=5,209 F=0.48; eclosion df=5,29 F=1.33; avg. days to eclosion df=5,29 F=1.57.

² Based on the mean weight of 35 pupae per treatment.

³ Control diet was treated with acetone.

Table 12. Development of C. capitata on A. squamosa seed semi-purified
¹
 extract treated diet .

Concentration (%)	Avg. Days to Pupation	Pupation (%)	Pupal Wt. ² (mg)	Eclosion (%)	Avg. Days to Eclosion
³ 0.000	6.7 b	92.0a	10.2a	98.00a	9.79a
0.039	6.9 b	82.0a	10.8a	97.14a	9.74a
0.078	7.1a	76.0a	10.6a	94.92a	9.65a
0.156	7.2a	88.0a	10.4a	94.92a	9.67a
0.312	8.2a	76.0a	10.3a	93.50a	9.77a
0.625	8.5a	72.0a	10.1a	87.78a	9.79a

¹
 Based on the mean of 5 replications with 10 insects per replicate.
 Treated diet was infested with newly-hatched larvae. Means followed
 by the same letter are not significantly different at 5% probability
 (DMRT). Avg. days to pupation df=5,29 F=17.14; pupation df=5,29 F=1.52;
 pupal wt. df=5,209 F=1.12; eclosion df=5,29 F=0.98; avg. days to eclosion
 df=5,29 F=1.68.

²
 Based on the mean weight of 35 pupae per treatment.

³
 Control diet was treated with acetone.

during the larval growth, with no treatment effect on pupal period length.

DISCUSSION

Secondary plant substances can affect adult longevity and egg number and viability. This has been demonstrated many times for various plants (Janzen 1973) and was recognized years ago as possibly related to plant chemistry (Evans 1938). A chemosterilant effect of A. squamosa extract was noted when the F1 generation egg hatch was calculated. A significant reduction in hatchability was observed when chloroform extract-treated females were mated with untreated males. Many oviposited eggs failed to hatch. This was particularly true at the levels of > 8 ug per insect. The common feature of inhibition induced by the extract is that sterility was not caused by suppression of egg production, but rather by deposition of defective eggs as a result of impaired embryogenesis. Ramos-Ocampo and Hsia (1986) found out that mating untreated female kelp flies with 2,4-DMBP-treated males significantly reduced survival time which suggests that the toxic effect of the chemical can be transferred through mating. This phenomenon referred to as "booby trapping" (Morgan 1967; Whitten and Norris 1967. Harwalkar et al. 1971; Moore et al. 1978;

Srivastava and Mishra 1984), however, was not observed in the A. squamosa extract-treated medflies. Results were not significantly altered by cross matings between control or treated males with untreated females. This could have been due to the chemicals used. The efficacy of A. squamosa seed extracts to inhibit the hatching of C. capitata eggs, when dipped in the various concentrations of the compound, is another useful attribute which renders the pest vulnerable at nearly all stages of its life cycle. Dead embryos were also observed in unhatched eggs which suggests that the ovicidal action seems to occur late in embryogenesis since most of the treated eggs contained fully developed embryos. It appears that A. squamosa extract probably interferes with some determinative information necessary for embryogenesis. Similar findings were reported by Ramos-Ocampo and Hsia (1986) when they exposed newly-laid Oncopeltus fasciatus eggs to different concentrations of European calamus oil and various propenylbenzenes. Likewise, when canavanine treated Dysdercus koenigii females were mated with untreated males, many of the oviposited eggs failed to hatch and those that developed were characterized by possessing incomplete embryogenesis (Koul 1983). Embryogenesis was generally initiated but terminated between blastoderm formation and blastokinesis (Saxena et al. 1977b). Furthermore, the ovicidal action of precocene 11 and diflubenzuron seems to

occur late in embryogenesis (Bowers et al. 1976; Grosscurt 1976).

Treated males were not able to transfer the toxic effect of the extracts to untreated females. A plausible explanation for this type of action is based on the well known facts that spermatogenesis, a process which includes all the successive divisions and transformations undergone by germ cells in the formation of spermatozoa in Diptera, begins 28 hr after egg hatching and viable sperms are already present 24-30 hr after the onset of pupation (Huettner 1930c; Kerkis 1933; Gleichauf 1936; Gloor 1943; Geigy and Aboim 1944). Therefore, even though the treatment was given to newly-ecdysed males many viable sperms had already formed and were available to females for fertilization. This could have resulted in a higher percent fertility of the eggs.

An insecticide effect on reproductive potential can be caused by a lowered incidence of mating, a shortened life span, the suppression of the reproductive organs and a direct toxic effect on the eggs (Kumar and Chapman 1984). Lack of oocyte and follicular growth is reflected externally by undersized ovaries which could be measured conveniently by a stage micrometer (Chang et al. 1987). Therefore, measurement of the ovarian length by a micrometer affords a reliable measure of oocyte development. Ovarian measurement

of A. squamosa extract treated medflies revealed that the chemical had an inhibitory effect on the growth of the ovary. A week after the treatment, treated flies started to recover from the effect although ovarian growth was still significantly reduced. Recovery of the growth is possibly due to degradation and subsequent loss of A. squamosa extract activity over a period of time. Chang et al. (1987) reported the same findings when they treated C. capitata females with benzyl-1,3-benzodioxole analogs. These compounds caused a temporary delay in ovarian growth and continued exposure of the analogs at the target site appears to be necessary for more permanent effects. They speculated that the temporary absence of ovarian growth in benzodioxole treated medflies can be attributed to JH antagonism since early studies showed that benzodioxole analogs and benzylphenols possessed anti-JH activity (De Loof et al. 1982; Van Mellaert et al. 1983b). The possibility that the same situation was happening in A. squamosa extract treated medflies can not be ruled out.

Plants have evolved natural chemical defense that protects them against herbivores. These chemicals afford plant protection by affecting insect development and survival on the host and influencing insect behavior. Host selection by insects involves one or more behavioral responses that may be mediated by secondary plant

substances. These include orientation, oviposition and feeding (Renwick 1983). However, these responses are not always clearly separated. For example, oviposition generally can not occur without orientation to the host plant, so analysis of factors affecting egg laying often requires consideration of stimuli that elicit landing. Among the chemical factors that affect settling response and acceptance of potential host plants for oviposition are attractants, repellents, stimulants and deterrents.

Settling response of C. capitata to A. squamosa seed extract treated papaya sections were significantly affected. The flies were apparently repelled by the extract as they did not settle on treated sections in as large numbers as on control papaya. Semi-purified extract seemed to be more repellent than chloroform extract. While a significant reduction in the number of flies attracted to papaya treated with > 0.78% chloroform extract was observed, it required only half of this concentration for the semi-purified extract to cause the same effect especially at 2 hr after release. Although chloroform extract repelled the flies from settling on papaya, it had no effect on oviposition. Semi-purified extract, on the other hand, caused a significant reduction in the number of eggs laid by the flies on the treated tissues. Unlike chloroform extract, semi-purified extract is both a repellent and oviposition

deterrent. The extract probably inhibited egg deposition when the flies contacted the chemical at the ovipositional site. Alternatively, the repellent effect of the extract might have minimized the number of females that could have laid eggs on treated papaya sections. Several authors have reported that plant secondary substances deter oviposition in phytophagous insects (Gupta and Thorsteinson 1960; Hsiao and Fraenkel 1968; Mitchell and Heath 1985; Renwick et al. 1989).

The strategy used by insects in foraging for ovipositional sites and the manner in which these areas are exploited influence the survival of the progeny. Phytophagous insects use a variety of chemical cues to effect population density regulation. The medflies and other tephritid fruit flies use olfactory cues, in addition to visual cues (Prokopy and Economopoulos 1976; Nakagawa et al. 1978; Cytrynowicz et al. 1984; Prokopy and Roitberg 1984) to seek and assess habitat, food and ovipositional resources (Prokopy and Roitberg 1984). Interfering with oviposition blocks insect infestation before any damage can be done. Oviposition deterrency exhibited by A. squamosa extract suggests that the chemical can be used to regulate fruit fly population. The identity of the compound needs to be known. Once it is identified, the deterrent compound can be tapped for pest control. It can be introduced into crop

plants through plant breeding or DNA recombinant techniques or the chemical can be sprayed directly on the plants to be protected.

Incorporation of A. squamosa seed extracts into the larval diet caused deleterious effects on the growth and development of C. capitata. Chronic exposure to alkaloid diet caused a longer larval period and consequently reduced the number of larvae reaching the pupal stage. Similar results were reported by Chan and Tam (1985) who observed that α -tomatine, a glycoalkaloid present in solanaceous plants, exerted an antibiotic effect on C. capitata larvae. Increased concentrations of α -tomatine resulted in decreased larval survival, lower pupal weights, an extended pupation period and a prolonged period of adult emergence. Likewise, Steffens and Schmutterer (1982) found that C. capitata were sensitive to crude methanolic neem seed kernel extract incorporated in an artificial larval diet. Not only did the extract cause a prolonged larval period, high mortality of pupae descending from surviving larvae and reduced pupal size but the chemical also affected the quality of surviving adults significantly. Prolonged developmental period and lower pupation of the medfly suggest that A. squamosa seed extracts influenced physiological processes of the insect rendering it sensitive to the chemical during early development. Reese and Beck (1976a, b) found that survival

and growth of the black cutworm, Agrotis ipsilon Hufnagel, was affected by a variety of plant chemicals which had different effects on ingestion, assimilation and/or conversion of food. When incorporated with the diet, L-Dopa inhibited growth by reducing food assimilation and efficiency of conversion of assimilated food into insect tissue but had no effect on amount ingested. In the light of these findings, it is very likely that A. squamosa seed extracts have an anti-nutritional effect which affected food consumption and utilization by the medfly larvae. The effect of the extracts on the insect's food ingestion and utilization needs to be studied to determine if the delay in larval development and mortality are due to the influence of the chemical on nutritional parameters.

A lower developmental rate of C. capitata larvae would increase mortality through longer exposure to parasites, predators, pathogens and adverse physical factors, even though the extract may not be immediately toxic to the insect. Prolonged developmental period can also disrupt the life cycle of the insect which consequently would be extended enough to put it out of synchrony with seasonal changes in weather and in host plant availability.

V. SUMMARY AND CONCLUSION

Insecticidal activity and biological properties of A. squamosa seed extracts against C. capitata were evaluated in the laboratory. Fractionation of crude extracts and purification of active components were done with solvent partitioning, column and thin layer chromatographies. Extracts were effective against the medfly from the egg to the adult stages. Extract bioassays with respect to medflies clearly demonstrated that A. squamosa contained a factor toxic to the insect. Assays successfully guided the fractionation of the extract to yield 1 or 2 active insecticidal compounds. Insect active fraction was partially characterized based on its physical and chemical properties. It was miscible with acetone, chloroform, diethyl ether, methylene chloride, benzene and xylene but insoluble in dilute acid, dilute alkali, ethanol, methanol and water. High pressure liquid chromatographic analysis of the fraction showed four peaks with the first peak as the major component.

Toxic effects of the extracts were determined on newly-emerged adults and third instar larvae. Adults were topically applied with different concentrations of the test compounds. Male flies were generally more susceptible to the toxic effect of the extracts than females. Malathion was:

1625X and 1649X more toxic than EtOH extract to males and females; 2362X and 2331X more toxic than CHCl₃ extract to males and females; and 1312X and 1636X more toxic than SPE to males and females. The insect active fraction was 2.55×10^{-3} and 2.07×10^{-3} as toxic as malathion to male and female medflies, respectively. Toxicity of the extracts on third instar larvae was assessed by dipping the insects in various concentrations of the plant material. Pupation and adult emergence were not affected by dipping the larvae in chloroform extract. Likewise, semi-purified extract did not influence emergence but significantly reduced pupation when larvae were dipped in 25% of the extract. When survivors were allowed to continue development to the adult stage, no morphogenetic effect was observed in the ensuing pupae and emerging adults.

Topical application of sublethal doses of A. squamosa seed extracts caused no abnormal behavior among the flies. Insects were able to feed, undergo courtship behavior, mate and oviposit eggs similar to controls. Chloroform extract did not cause any significant effect on adult longevity and the number of eggs laid. However, a sterilant effect was observed when females treated with > 8 ug chloroform extract per insect and mated with untreated males. Similarly, semi-purified extract treatment did not affect longevity but significantly reduced egg hatching when females treated with

9 ug per insect were mated with treated males. Although both chloroform and semi-purified extract treatments caused a significant reduction in hatchability, egg production of treated flies did not differ from controls.

Ovicidal activity of the extracts was further studied by exposing newly-laid eggs to different concentrations of the test compounds. Hatchability decreased as concentration of the extract increased. Significantly less eggs hatched when dipped in > 0.313% ethanol, chloroform and semi-purified extracts. Microscopic examination of unhatched eggs showed dead embryos suggesting that the ovicidal action seemed to occur late in embryogenesis.

Measurement of the ovarian length of treated flies revealed that A. squamosa seed extracts affected oocyte development. Chloroform and semi-purified extract treatments significantly inhibited ovarian growth at > 5 ug and > 2.5 ug per insect, respectively. However, treated flies were able to recover from the effect suggesting that growth recovery is possibly due to degradation and subsequent loss of A. squamosa extract activity over a period of time.

The repellent effect of the extracts was demonstrated when flies were offered papaya sections treated with different concentrations of the compound. Significantly, fewer flies were attracted to papayas treated with > 0.78%

chloroform extract compared to the controls. However, the extract did not affect the number of eggs laid by the females. In the same manner, flies were significantly repelled from settling on papayas treated with > 0.39% semi-purified extract. Unlike chloroform extract, however, semi-purified extract caused a significant reduction in the number of eggs laid at > 0.78% treatment. Results suggest that semi-purified extract is both a repellent and an oviposition deterrent. Oviposition was deterred as the medfly laid one-fifth the number of eggs on papaya sections treated with > 0.78% extract as on controls treated with acetone only.

Chronic exposure to A. squamosa seed extract treated diets showed that survival and developmental characteristics of C. capitata were variously affected. Chloroform extract significantly reduced pupation at 0.625%. It also retarded larval growth at > 0.312% and caused 28% mortality at 0.625% compared with the controls. The extract did not affect pupal size, adult emergence and the length of time between pupation and eclosion. Feeding the medflies with semi-purified extract treated diet had no effect on pupation, pupal size, emergence and the duration between pupation and emergence. However, the extract significantly prolonged the larval stage at > 0.078%.

Delays in development occurred only during the larval growth, with no treatment effect on length of pupal period.

Evaluation of the insecticidal activity and biological property of A. squamosa seed extracts against C. capitata has provided interesting tools which require further investigation. The effect of the extracts on medfly embryogenesis is an important aspect of insect development which needs to be examined in details. If the extract interferes with some determinative information necessary for embryogenesis, it would be worthwhile to determine at which stage in the process it occurs. Further studies are necessary to elucidate the roles and interactions of the extracts in C. capitata reproduction.

The exact mode of action of A. squamosa seed extracts on C. capitata is unknown, but presumably it acts as a contact and stomach poison. Regardless of the mode of action, the sterilant effect and the inhibition of ovarian growth make these compounds good candidates for physiological research. Furthermore, the potent insecticidal activity of these chemicals against different stages of the medfly would justify studies with additional insect species and exploration of its potential for development. Experiments with other insect species are needed to discover whether the apparent effects of the extracts on the medfly is representative of the responses of insects in general. The

toxic and growth regulating effects of the extracts against the medfly both when contacted and ingested should make these compounds a valuable addition to present control methods, if development can be accomplished.

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