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Bioprospecting of botanical insecticides: The case of ethanol extracts of *Magnolia schiedeana* Schltl. applied to a Tephritid, fruit fly *Anastrepha ludens* Loew

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Abstract
The present study was set out to determine the insecticidal properties of different vegetative structures of *Magnolia schiedeana* Schltl. (Magnoliaceae) against adults of *Anastrepha ludens* (Loew) (Diptera: Tephritidae). To evaluate the insecticidal effect, a feed bio-essay was conducted on adult individuals using ethanol extracts of vegetative structures of *M. schiedeana*. Only seed and sarcotesta extracts showed insecticidal activity on flies. Extracts of other plant organs (leaves, flowers, bark, follicles) showed no significant biological activity. These results suggest that the sarcotesta of *M. schiedeana* has secondary metabolites with potential for the development of an insecticide for the control of adults of *A. ludens*.

Keywords: bioprospecting, botanical insecticides, crude extracts, Magnoliaceae, Tephritidae.

1. Introduction
Highly diverse ecosystems are very suitable for bioprospecting, for interesting biological compounds can easily be found among their many species. Among these ecosystems is the tropical montane cloud forest (TMCF, also known as "cloud forest"), characterized by long periods of foggy weather conditions and a flora composed of Neotropical as well as Holarctic species [1]. Mexico's TMCF is known for its archipelago-type distribution, at an altitude between 1,000 and 3,000 m. The botanical richness of this ecosystem is unparalleled in the country. It concentrates the largest number of species per unit area, 6,790 vascular plant species, 1,625 genera and 238 families, including 2,361 endemic species [2]. The uses of these species are many and they include medicinal, ornamental, timber and food, of which local inhabitants benefit. However, in the absence of sustainable management programs, the conservation and proper utilization of these species is at risk. The TMCF in Mexico is highly disturbed and fragmented, and is currently in danger of extinction. Presently, 83 species are nearly extinct, 206 endangered, and 175 vulnerable [3].
In the case of the family Magnoliaceae, it comprises 220 species of deciduous and evergreen trees native to Asia and America. About 80 percent of these species are distributed in Southeast Asia, and the remaining 20 percent is distributed in America. Only two genera: *Magnolia* and *Liriodendron*, are present in America [4]. Species of the *Magnolia* genus are important in traditional and modern medicine in countries like China, Japan and Mexico, due to their biological and pharmacological effects in various organisms. These plants contain, for example, the biphenyls Magnolol and Honokiol, which intervene in the functions of the central nervous system, as well as the digestive, cardiovascular, skeletal-muscle and neurological systems [5]. So far, more than 255 different secondary metabolites have been isolated in the vegetative structures of different *Magnolia* species, chiefly among them alkaloids, flavonoids, lignans and terpenoids. The species with a greater number of isolated metabolites are *M. grandiflora* L., *M. kobus* DC, *M. obovata* Thunb., *M. officinalis* Rehder, and Wilson subsp. *biloba* and *M. salicifolia* (Siebold and Zucc) Maxim [6]. Biological activity has been registered following the application of these metabolites to insects, nematodes, fungi and bacteria [7], which suggests that *Magnolia* species have a potential for manipulating the biological

activities of these organisms, particularly as insecticides, as has been reported in the case of *M. dealbata* Zucc [8], *M. fargesii* Cheng [9] and *M. salicifolia* [10].

In Mexico there are 21 species of *Magnolia* associated to the TMCF. According to some authors [11], these species are representative and indicative of the state of conservation of this ecosystem. The *M. schiedeana* Schltl, a species endemic to the TMCF in the central portion of the watershed of the Gulf of Mexico [12], has been classified as endangered due to fragmentation of its natural habitat, as a result of changes in land use and the expansion of the urban area [13]. Due to its tolerance to shade, this species is associated to mature and advanced stages of forest succession, and needs a mature forest to get established. Moreover, this plant has a limited production of seeds, since it's very specific reproduction system is associated only with two beetles *Cyclocephala jalapensis* Casey (Scarabaeidae: Dynastinae) and *Myrmecocephalus sp* (Staphylinidae) [14].

In view of the high risk of extinction of *M. schiedeana* due to exposure the continuous expansion of grasslands and the urban sprawl over the TMCF, sustainable management and habitat conservation programs are needed to protect the populations. One of the options at hand to finance conservation programs for endangered species is bioprospecting [15].

Bioprospecting of plants has a great potential for biodiversity protection through the sustainable management of biotic resources [16]. It is based on the identification of the properties of vegetative structures plant and their possible applications to, among other things, the pharmaceutical, biotechnology, food, and bioremediation industries [17]. As a result of their prolonged interaction with insects through their evolutionary process, plants contain a wealth of insecticide products that can be used to combat pests. Every year, about 70 percent of the fruit produced in Mexico is lost to the Mexican fruit fly (*Anastrepha ludens* Loew) either through direct damage to fruits or as a result of the quarantine barriers imposed to their commercialization [18]. Control of this pest has so far relied on the application to fruits of massive quantities of chemical insecticides with very high action spectrum compounds, such as Malathion and Spinosad [GF 120], an unsustainable practice whose effectiveness is likely to be compromised by the development of insecticide resistance by pests. It is therefore necessary to turn to insecticides derived from plant extracts, which are compatible with the natural environment [19].

The aim of this study was to determine the insecticidal properties of eight vegetative structures of *M. schiedeana* against adults of *A. ludens*.

2. Materials and methods

2.1 Collection of plant material

The vegetative structures of *M. schiedeana* Schltl. (Magnoliaceae) were obtained from trees located at the Volcano of Acatlán, Veracruz, Mexico (19° 41' 0.8" N and 96º 51' 14" W) at 1,998 masl. The average temperature in this sites is 20 °C and the average annual rainfall 1,570 millimetres. Of the existing 23 mature trees, 10 were randomly selected for the study. Samples were collected during 2013 according to plant phenology. The following structures were sampled: leaves (mature in February and young in April), flowers (May), bark fragments (June), and polyfollicles and seeds (July and August). The biological material was taken to the Instituto de Biotecnología y Ecología Aplicada (INBIOTECA) of the Veracruzana University in Xalapa, Veracruz, Mexico, where every structure collected was rinsed with distilled water and placed in paper bags for drying in a vacuum oven at 35-40 °C for 96 hours. To separate the polyfollicle, seeds were allowed to dry at room temperature for eight days. The sarcotesta was separated from fresh seeds and dried at room temperature. Each mature tree of *M. schiedeana* has in average 6 ± 3.7 polyfollicles with approximately 25 seeds, therefore were available for the experiment a limited number of seeds.

2.2 Insects

Laboratory Mexican fruit flies, *Anastrepha ludens* (Loew) (Diptera: Tephritidae) were used for the experiment. They are mass-produced at the MoscaFrut breeding plant located in Metapa de Domínguez, Chiapas, Mexico. Samples in pupal stage were sent by air cargo to the Inbioteca's Laboratory for Invertebrates. There they were kept in cages made of wood and cotton mesh measuring 9,000 cubic centimeters until the adult stage was reached. There were approximately 500 flies per cage. They were provided with purified water and food (table sugar) *ad libitum*. A light regime of 12:12 hours of light, and a temperature and relative humidity of 25 ± 1 °C and 70 ± 10 percent respectively, were maintained in the laboratory.

2.3 Preparation of crude extracts at the proportion of 1:5 p v^{-1}

Each vegetative structure was pulverized separately in an industrial Waring Commercial Blender (model 51BL31), except for the sarcotesta, which was ground in a mortar. A sample of 50 grams dry weight of powder was taken and macerated with 250 milliliters of ethanol at 95 percent (ratio of 1:5 w v^{-1}) for at least one week at cold temperature (4 °C), and then the solvent was decanted. The total solvent volume was reduced to 10 milliliters at a vacuum of 56 centimeters Hg^{-1} using a rotary evaporator (Buchi, Model R-210, 40 °C). Crude ethanol extracts were kept refrigerated at 4 °C until evaluation.

2.4 Preparation of crude extracts at the proportion of 2:10 v p^{-1}

To double the concentration of the extracts with higher adjusted mortality (seed with sarcotesta and separate sarcotesta), the proportion was changed to 2:10 p v^{-1} (100 grams dry weight of powder in 500 milliliters of ethanol at 95 percent). Extracts were reduced by rotary evaporation with the same procedure to 12 milliliters, to obtain three dilutions (0.1, 0.01 and 0.001 mg mL^{-1}). The extract obtained from the first dilution (0.1 mg mL^{-1}) was taken as reference, then one milliliter of extract was gauged at 10 milliliter for the second dilution (0.01 mg mL^{-1}), and for the third one, one milliliter was taken from the extract and gauged at 100 milliliter capacity (0.001 mg mL^{-1}).

2.5 Treatments and experimental procedure

In each cage, 50 Mexican fruit flies (25 females and 25 males, all between 10 and 20 days old) were placed. The water was placed in a container with cotton to prevent flies from drowning. To ensure a sufficient intake of the treatment mixture, flies were left without food for 24 hours before the mixture was applied. The treatment mixture consisted of 1 gram of sugar mixed with two milliliters of the extract. A specific plant vegetative structure extract (1: 5 p v^{-1}) was evaluated in each experiment. In order to reduce the risk of adherence of the flies to the sugar, the extract was applied to 0.05 milligrams of cotton. A total of eight experiments with five replicates each in two different cohorts of flies were carried out.

As a positive control, an ethanol extract of crude *Chrysanthemum grandiflorum* Kitam (Asteraceae) was used given its content of Pyrethrin, known for its insecticide

properties [20]. Chrysanthemums were bought locally at the San José market, in the city of Xalapa, and the same extraction method described above at a proportion of 1:5p v-1 was followed. For negative control, one gram of sugar was mixed with two milliliters of ethanol at 95 percent. The number of survivors of Mexican fruit flies per cage was recorded during five consecutive days.

2.6 Statistical analysis

A completely randomized experimental design was used. Natural mortality was corrected with Abbott's formula (1925) [21] to determine the efficacy of treatments; CM (%) = (1- (X-Y) / X × 100). Where CM is the corrected mortality, X is the number of control individual survivors, and Y the number of surviving individuals from treatment.

The corrected mortality (CM) data of the treatments of each experiment were analysed by means of a nonparametric test (Kruskal-Wallis) with JMP 7.0.1 software [22]. Later, an analysis of the survival of flies exposed to extracts with higher CM was performed according to the Kaplan-Meier method, with the same software.

3. Results and discussion

The Abbott index indicated more effectiveness in the sarcotesta and seed with sarcotesta extracts in the proportion of 1:5 pv^{-1}, which resulted in an increased mortality in fruit flies (36.8 ± 16.4 and 35.5 ± 20.1 percent, respectively) (Chi-square = 21.25, GL = 7; P <0.003) (Table 1A). A similar effect was observed in crude extracts of sarcotesta of *M. dealbata*, which indicates that the biologically active substance is similar in both species and is present in the sarcotesta, possibly with a protective function against insect seed predators [8].

Table 1: Abbott index of *A. ludens* exposed to ethanolic extracts of vegetative structures in *M. schiedeana* in A: proportion 1:5 p v^{-1} and B: proportion 2:10 p v^{-1} with three dilutions (0.1, 0.01 y 0.001 mg mL^{-1}). Active extracts are presented in bold. Mean±SD.

A: Treatments to 1:5 p v^{-1}	**Concentration (gr/ml)**	**Abbott index (%)**
Leaves mature	1.75 ± 0.6	30.8 ± 15.1
Leaves young	3.78 ± 3.2	31.2 ± 22.9
Flowers	0.46 ± 0	0.08 ± 9
Bark	4.31 ± 0.3	0.54 ± 3.3
Polyfollicles	3.66 ± 0	5.26 ± 17
Seed with sarcotesta	1.73 ± 0.7	**35.5** ± **20.1**
Seed without sarcotesta	0.76 ± 0	12.6 ± 29.2
Sarcotesta	0.62 ± 0	**36.8** ± **16.4**
C. grandiflorum	1.38 ± 0.4	97.6 ± 2.6
B: Treatments to 2:10 p v^{-1}		
Seed with sarcotesta 0.1 mg mL^{-1}	5.27 ± 1.7	**59.3** ± **34.2**
Seed with sarcotesta 0.01 mg mL^{-1}	0.52 ± 0.1	62.13 ± 30.8
Seed with sarcotesta 0.001 mg mL^{-1}	0.052 ± 0	9.86 ± 22.4
Sarcotesta 0.1 mg mL^{-1}	2.02 ± 0	**64.7** ± **14.8**
Sarcotesta 0.01 mg mL^{-1}	0.202 ± 0	53.7 ± 12.1
Sarcotesta 0.001 mg mL^{-1}	0.0202 ± 0	13.4 ± 11.2
C. grandiflorum	1.65 ± 0.6	99.9 ± 0

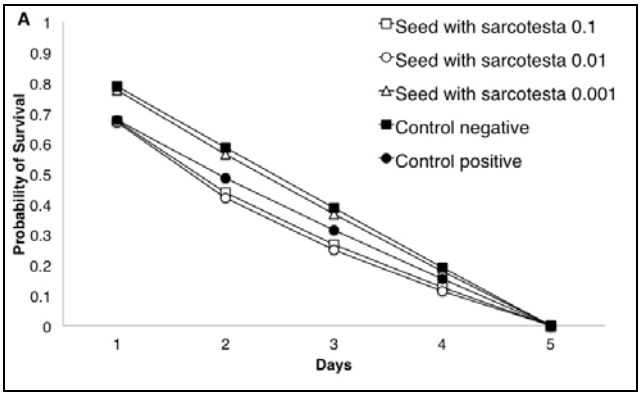
Secondary metabolites have also been isolated in seeds of other species of *Magnolia*, these include neolignans (magnolol and honokiol), lignans (yangambine and syringaresinol) phenylpropanoids (coniferine, syringine), flavonoids (rutin) and sesquiterpenes (costunolid), but their activity has been associated mainly with pharmacological applications [6], rather than with insecticides.

The extracts from the leaves, flowers, bark, polyfollicles and seeds without sarcotesta of *M. schiedeana* generated lower mortality among adult *A. ludens* individuals (Table 1A). However, other species of *Magnolia* have been proven to have an insecticidal effect. Miyazawa *et al.* (1994) [9] reported that the lignan, (+) - epimagnoline A, obtained from flower buds of *M. fargesii* inhibits the growth of larvae of *Drosophila melanogaster* Meigen. Other studies report that the geraniol and nerol obtained from bark and the trans-anethole, methyl eugenol and iso-methyl eugenol obtained from leaves, flowers and polyfollicles of *M. salicifolia*, showed 100 percent mortality in *Aedes aegypti* L. (4 instar) in a concentration range of 20-100 parts per million after 24 hours [10]. It may be that the compounds in these vegetative structures are effective on holometabolous insects at their immature stages, when their digestive metabolism differs from that of adults [23].

At a proportion of 2:10 p v^{-1}, an Abbott index of 64.7 ± 14.8 percent was obtained from sarcotesta, and 59.3 ± 34.2 percent from seed with sarcotesta at a dilution of 0.1 mg mL^{-1}, with a significant difference between dilutions (P <0.0001) (Table 1B). In this study, increasing the proportion of the active extracts allowed for greater biological activity [24]. The highest mortality (Abbott index) was found at the dilution of 0.1 mg mL^{-1}, which corresponds to the minimum lethal concentration for both crude ethanol extracts.

The Kaplan-Meier survival analysis showed that flies exposed to an ethanol extract of seed with sarcotesta in two dilutions (0.1 and 0.01 mg mL^{-1}) died mostly over the first 3 days, as opposed to the control with *C. grandiflorum* (Log- Rank, Chi-square = 159.56, GL = 4; P <0.0001) (Figure 1A). This indicates that the efficiency of the seed with sarcotesta extract is higher than that of *C. grandiflorum* in the early days, and has a good potential for obtaining substances with insecticidal properties to control of *A. ludens*.

The sarcotesta extract was less effective than that of *C. grandiflorum* (Log-Rank, Chi-square = 895.05; DF = 4; P <0.0001) (Figure 1B). This result contrast with that of Flores-Estévez *et al.* (2013) [8] in *M. dealbata*. These authors found no differences in biological activity between extract of chrysanthemum and the extract of sarcotesta of *M. dealbata*. This difference may be because the concentration of active ingredients with insecticidal properties in the seeds and sarcotesta of *M. schiedeana* is lower. Differences in the life history of these species of plants may explain the contrasting result. For example, *M. schiedeana* is evergreen, while *M. dealbata* is deciduous. It may indicate that plant resources are assigned in a different way [25]. Additionally, the seeds are larger in *M. dealbata* and produced in larger numbers every year [26, 27].



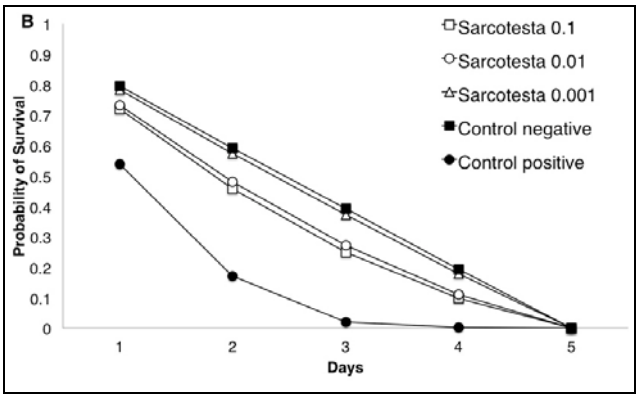


Fig 1: Kaplan-Meier survival plot for *A. ludens* exposed to ethanolic extracts of A) seed with sarcotesta and B) sarcotesta in three dilutions (mg mL^{-1}) of *M. schiedeana* during 5 days.

It has been demonstrated that different species of the genus *Magnolia* have a different production of secondary metabolites. More than 40 metabolites have been isolated in *M. salicifolia* and *M. obovata* Thunb., while in *M. ashei* Weath., *M. sprengeri* Pamp., and *M. thompsoniana* de Vos, only one has been found [6]. However, so far no chemical studies have been conducted on the secondary metabolites present in the vegetative structures of certain species. One of them is *M. schiedeana*, whose possible applications have been explored in the present study.

Our results show that *M. schiedeana* has a potential for developing a bioinsecticide for the control of certain tephritids that are regarded as pests. To evaluate the extent of the insecticidal properties of the species, further studies with isolated compounds of *M. schiedeana* seeds, including the sarcotesta, are necessary.

4. Conclusion

The evaluation of the vegetative structures of *M. schiedeana* indicates that the seeds contain compounds with insecticidal properties for adult individuals of *A. ludens*. Sarcotesta extracts in concentration of 0.1 mg mL^{-1} (minimum lethal concentration at the proportion of 2:10 p v^{-1}) caused 64 percent mortality over five days. A mean mortality was observed with other extracts: seeds with sarcotesta 59 percent, mature leaves 30 percent, and young leaves 31 percent mortality in five days.

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