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**Ecological Studies of**  
***Phoradendron serotinum* (Raf.) M.C. Johnston,**  
**American Christmas Mistletoe**

by

Annette Arcario Opler

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2003

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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**Annette Arcario Opler**

**Adviser: Dwight Kincaid, Ph.D.**

Mistletoe is the common name for approximately 1700 species of angiosperms, which share a common lifestyle. They are all parasitic or hemiparasitic and most are found in the crowns of trees. The study species, *Phoradendron serotinum* (Raf.) M.C. Johnston is found in the southeastern United States. It grows on at least 58 different species of hosts. Birds disperse the seeds.

Field and laboratory studies were undertaken to gain insight into the role played by *P. serotinum* in the community. Field studies were performed at two sites in Williamsburg, Virginia. The two study sites were separated by almost 10 km. Further, there was a clear host preference for *Gleditsia triacanthos* at one site and for *Carya cordiformis* at the other site. The concentration of mistletoe is equal at both sites. At Carter's Grove there were 39.85 bushes/km<sup>2</sup>, and at Capital Landing Road, there were 44.81 bushes/km<sup>2</sup>. Analysis of variance showed no difference between the mistletoe concentration at these two sites (P=0.84).

A series of laboratory bioassays demonstrated that *P. serotinum* produces secondary metabolites which exhibit anti-herbivore and allelopathic activities. The anti-herbivore activity was demonstrated against *Spodoptera eridania*, southern army worm, a

voracious and formidable herbivore. The allelopathic activity was demonstrated using soil from the study sites.

In addition, the study species is high in anti-oxidants. It is established that free radical production increases with stress. All canopy residents are exposed to high levels of UV light and in addition mistletoe is subject to water stress when the upper canopy of the host develops low water potential. Therefore, production of phytochemicals capable of neutralizing free radicals is an attribute to be expected from a vulnerable canopy resident.

The production of these defensive phytochemicals by the study species led to prescreening of mistletoe for anti-cancer activity. Although all prescreening assays have yielded positive results, none of the active compounds have been isolated to date.

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## **Chapter 1. *Phoradendron serotinum*: Nomenclature, Structure, and Niche**

Mistletoe is the common name for about 1700 species of flowering plants, which share a common lifestyle. All are members of the order Santalales. Kuijt and Cronquist recognized three families: Loranthaceae, the Viscaceae, and Eremolopidaceae (Cronquist, 1981; Kuijt, 1969). However, Nickrent (Watson, 2001) would increase the number to at least five based on nuclear S rDNA and chloroplast rbcL sequences. Santalaceae and Misodendronaceae should be added to the list. They are all parasites or hemiparasites. Most live in the crowns of trees but there are at least three genera of Loranthaceae (*Nuytsia*, *Atkinsonia*, and *Gaiadendron*) which are terrestrial root parasites. This condition is considered primitive (Kuijt, 1969). Others have evolved to parasitize Euphorbiaceae, Cactaceae, grasses, orchids, and even ferns.

Only members of the North American species of dwarf mistletoe belonging to genus *Arceuthobium*, are known to obtain photosynthetic products from their hosts (Calder, 1983). This genus connects to phloem as well as xylem.

Mistletoe species are often left out of popular accounts of the flora of the Eastern United States. Most people will be aware of the oak tree growing in their neighborhood but will not notice the mistletoe bush which sits in the crown of that same oak tree. Lack of recognition is even noted in ancient mythology. A goddess in Scandinavian mythology ignored mistletoe's woody branches and it proved fatal for her young son when an arrow, which was fashioned from a mistletoe branch, delivered a fatal wound (Frazer, 1959).

Mistletoe is distributed from temperate forests through equatorial rain forests on all continents with the exception of Antarctica, and many endemic species are found inhabiting islands. This life style survives in all but the extremes of climate.

All members of the genus *Phoradendron* are hemiparasites (Lichter and Berry, 1991). American Christmas mistletoe, *Phoradendron serotinum* (Raf.) M.C. Johnston, is typical mistletoe found growing as a small bush in the crown of its host (Figure 1.1). While the species finds its habitat in the crowns of trees, it is not an epiphyte, as it invades the branch upon which it is perched. Mori and Hecklau (2002) refer to this relationship between "all species of Loranthaceae and Viscaceae and their hosts as aerial hemi-endophytes." They occupy the niche of a hemi-parasite and the habitat of an aerial hemi-endophyte.

The hemiparasitic nature of the study species is clear from its very beginnings. The seed quickly turns green to provide the developing embryo with food required for growth. Initial growth involves securing its position on the host by means of a green holdfast, which seeks nutrients from the host's xylem (Figure 1.2). The organ credited with establishing the bridge between host and parasite is the haustorium. This organ is the sole contribution of the parasite to the connection between the two organisms. There are primary and secondary haustoria. The primary haustorium is always terminal and results from the growth of the radicle, while secondary haustoria are the result of lateral growth. Secondary haustoria are found in Santalales, Orobanchaceae, and Scrophulariaceae. The majority of the cells in the haustoria, both primary and secondary, are parenchymous (Figure 1.3). The structure quickly assumes the shape of the host substrate and serves several functions. This organ anchors the seeds to the branches of

the host and quickly invades the branches to seek out xylem. The haustoria then connect to the xylem of the host and channel water and mineral nutrients to the parasite (Kuijt, 1979).

Upon reaching maturity, healthy *P. serotinum* achieves a deep rich blue-green color and contains active chloroplasts. Its bushlike form and evergreen leaves is easily seen in barren deciduous trees in winter. It is more difficult, but not impossible, to identify this species in summer amidst mature foliage (Figures 1.4 and 1.5).

Perhaps the difficulty involved in retrieving specimens and the host promiscuity of the species led to its murky nomenclature. Taxonomists have changed the nomenclature at least eleven times since its initial incorrect identification in 1788 by Walter who misidentified a specimen as *Viscum album*, the European species that had not yet been brought to North America. Pursh in 1813 correctly recognized a specimen as a new species and gave it the name *Viscum flavescens*, an orthographic variant of a West Indian species *Viscum flavens* Swartz (Pursh, 1814). To Pursh's credit, he did not use the West Indian name, although he clearly states that he found that the specimen he examined in the United States had a strong resemblance to the West Indian specimen he had examined previously.

Four years later, in 1817, Rafinesque described the species as *Viscum leucarpum*. Rafinesque did not stop there and incorrectly identified a second specimen as yet another new species in 1820. He cited it using the epithet *Viscum serotinum* (Rafinesque, 1830).

Candolle in 1830 misspelled Rafinesque's original 1817 "*leucarpum*" as "*leucocarpum*." The result was *Viscum leucocarpum* (Candolle, 1830). In 1848 Nuttall suggested that *Phoradendron flavescens* would eliminate the confusion between

“*leucocarpum*” and “*leucarpum*” but never formally proposed the change and he was definitive that the specimens he had examined belonged in the genera *Phoradendron* and not that of *Viscum* (Nuttall, 1848).

In 1850 Engelman formally changed the name of the genus (Johnston and Reveal, 1989) to *Phoradendron* (Engelmann, 1850). This resulted in the citation *Phoradendron flavescens* Nutt. ex Engelm.

Sixty years passed and Patschofsky picked up on Candolle’s error, using *Phoradendron leucocarpum* (Reveal and Johnston, 1989) and Patschofsky added another name to the list. Finally, in 1957, Johnston argued that the name “*leucarpum* “ could be too easily confused with *leucocarpum* and the use of *Phoradendron serotinum* ensued.

Finally, the Committee for Spermatophyta reviewed the matter and an official change came in 1989 when *Phoradendron leucarpum* (Raf.) Rev. & M.C. Johnston became the official name (Reveal and Johnston, 1989). A total of 11 different names were awarded to the study species over a period of 203 years from 1788 to 1986 and all of these names can be found cited in the literature (Table 1.1).

It should be noted here that misspelling of the species epithet is not limited to Candolle's error of 1830, as Mabberley also misspells the species name “*Phoradendron leucarpon*” Reveal and M. Johnston in his much used and authoritative dictionary, *The Plant Book* (1998). However, this error does not seem to be used by other authors.

The three epithets predominantly encountered in databases such as Agricola, Medline, and Biosis are *Phoradendron leucarpum*, *Phoradendron flavescens*, and *Phoradendron serotinum*. Fortunately, a comprehensive bibliography has been posted on the Internet by the United States Department of Agriculture at the Mistletoe Center

(2003). This web site eliminates some tedious work searching under the list described in Table 1.1 below. The epithet *Phoradendron serotinum*, which was used by Gleason and Cronquist (1991) will be used here because it appeared to be the one used most often.

Table 1.1. Summary of Nomenclature Changes

Date	Author	Name
1788	Walter	<i>Viscum album</i> L. (Reveal and Johnston, 1989)
1813	Pursh	<i>Viscum flavescens</i> (Pursh, 1814)
1817	Rafinesque	<i>Viscum leucarpum</i> (Rafinesque, 1820)
1820	Rafinesque	<i>Viscum serotinum</i> (Rafinesque, 1830)
1830	Candolle	<i>Viscum leucocarpum</i> (Candolle, 1830)
1837	Candolle	<i>Viscum verticilliatum</i> NSP. (Audubon, 1837)
1848	Nuttall	<i>Phoradendron flavescens</i> (Nuttall, 1848)
1850	Engelmann	<i>Phoradendron flavescens</i> (Engelmann, 1850)
1911	Patschofsky	<i>Phoradendron leucocarpum</i> (Reveal and Johnston, 1989)
1957	Johnston	<i>Phoradendron serotinum</i> (Reveal and Johnston, 1989)
1986	Reveal and Johnston	<i>Phoradendron leucarpum</i> (Reveal and Johnston, 1989)
1998	Reveal and Johnston	<i>Phoradendron leucarpon</i> (Mabberley, 1998)

The taxonomic ambiguity does end with the epithet for the study species. Gleason and Cronquist (1991) put the genus *Phoradendron* into the family *Viscaceae*, and Kuijt (1969) also puts *Phoradendron* into the family *Viscaceae* based on the embryology. This identification has not been challenged in the recent literature.

Ambiguity with the nomenclature ends but more confusion lies ahead when considering the relationship of mistletoe to its host and to the community at large. In those mistletoe species which carry on extremely limited photosynthesis and are incapable of supporting their own growth and development (as with *Arceuthobium*), damage done to the host is not disputed.

In *P. serotinum* the connection between the host xylem and that of the mistletoe is established early as described above. Besides xylem to xylem contact, coordinated growth of the host and the parasite is required (Fisher and Calder, 1983). In order to accomplish this, *P. serotinum* maintains a cyclical seasonal growth cycle very similar to that of its host. The growth pattern is similar to the host even though the study species is an evergreen. The annual rings are easily distinguished in its branches (Figure 1.6). It should be noted that what appears to be phellem is actually epidermis (Kuijt, 1969).

The need to maintain a growth rate that parallels that of the host may reduce survival rates for seedlings that fail to land on a branch which is exposed to light. In recent experiments, Lopez de Buen and Francisco Ornelas show that *Psittacanthus schiedeana* survival was affected by canopy cover (Lopez de Buen and Francisco Ornelas, 2002). Without sufficient light, the parasites cannot maintain a reasonable growth rate. In light of this recent finding, the pattern of dispersal within the crown of the host appears to be light dependent. If a tree is in the middle of a pasture, we see

instances of the mistletoe being evenly distributed throughout the crown (Figure 1.7). If a tree is part of a small stand, then the mistletoe bushes seem to accumulate amidst the upper branches (Figure 1.8). However, the influence of frugivores may also explain this phenomenon. This spatial distribution could be entirely the production of bird behavior (Martinez del Rio et al., 1996). The relationship of bird vectors to mistletoe distribution will be discussed further below.

The recent hypothesis, which defines mistletoe as a keystone resource, has brought renewed interest in many species of the parasite. In his article entitled "Mistletoe, a Keystone Species," Watson (2001) identifies mutualistic relationships between mistletoe and birds, between mistletoe and mammals, and even between mistletoe and one species of Amazonian fish, which during high flood season relies on mistletoe. In total, he lists 97 species, which rely on mistletoe for their livelihood. He cautions that this list is far from complete. Many of these species are reliant on the berries in times of scarcity.

It should be noted here that Paine's original definition of a keystone species relied on maintenance of a high position in the food chain on the part of the keystone species in question (Paine, 1968). Paine demonstrated his working definition of keystone species experimentally with starfish in the rocky intertidal zone. Paine later modifies this definition. In "Challenges in the Quest for Keystones" Paine and co-authors (Power et al., 1990) have broadened the working definition of a keystone species to include a species that, while not abundant, has a disproportionately widespread effect on other species. They quantify their definition but fail to demonstrate the validity of their equations experimentally. They acknowledge that mutualists such as seed dispersers may

also be keystone species (Power et al. 1996). Watson clearly rejects Paine's early definition and adopts Paine's later interpretation (Watson, 2001).

Finally, a third view of the value of keystone species comes from Bertness (Callaway and Bertness, 1994), who points out that under extreme conditions, such as in a desert or in a rocky tidal environment, mutualism may synergistically contribute to the stability of the community. This synergistic contribution to stability may be more widespread and more enduring than that of any individual keystone species. Bertness also demonstrates his hypothesis experimentally (Bruno et al., 2003; Callaway and Bertness, 1994).

Two species of mistletoe, which have been characterized as keystone species, find their habitats in semi-arid and desert conditions, *Phoradendron macrophyllum* and *Tristerix aphyllus*, contribute food and water to the survival of frugivores (Aukema and Martinez del Rio, 2002; Watson, 2001). The berries, which are rich in nitrogen, mature when food is scarce. There is a strong case made for the inability of several species to survive without this source of nitrogen when food is so scarce. These species in turn affect the community (Aukema and Martinez del Rio, 2002b).

There is also evidence that mistletoe makes a positive contribution to the survival of the host in which it finds its niche. Consumer pressure must be great in the crown of the host. Herbivores often visit the crowns of trees in uninterrupted eating frenzies. However, we will present anti-insect feedant data to support the hypothesis that mistletoe may interfere with such frenzied attacks.

Mistletoe is known to live undisturbed with at least 100 species of fungi (Watson, 2001). We have collected mistletoe from one host tree in which the leaves from five

different bushes, were covered with epiphytic hyphae, while the leaves of the host remained unblemished (Figure 1.9). The mistletoe bushes on this tree are by far the most productive mistletoe seed producers that have been harvested during the course of these studies. There is nothing in the literature that begins to answer the contradiction in heavy fungal infestation and high seed production.

To further elucidate the role of the species in the community, data will be provided which demonstrates that mistletoe contributes allelopathic compounds to the soil. These allelopathic compounds could potentially limit the growth of trees that might compete with the host and the secondary metabolites will also inhibit understory vegetation.

Another striking effect of mistletoe is its ability to act as a windbreak in the difficult winter months. Some species of birds like to build nests in the mistletoe bushes themselves or in the witch's brooms, which are produced by the host tree in response to the parasite (Aukema and Martinez del Rio, 2002b).

In addition to the above contributions to its community, mistletoe attracts birds to some host trees, which in turn rely on these birds for seed dispersal and to rid them of herbivores which might prove fatal (Milius, 2002).



**Figure 1.1. A small mistletoe bush growing in the crown of a tree against the winter sky.**



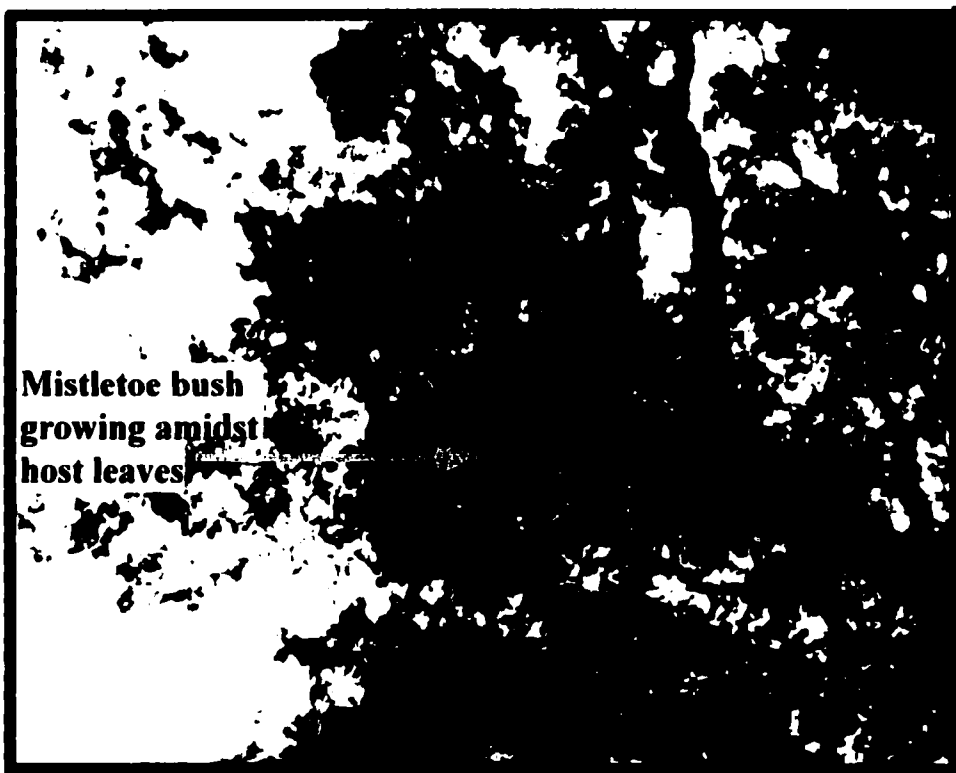
**Figure 1.2.** *P. serotinum* seedling germinating on *Quercus virginiana* in greenhouse.



**Figure 1.3. Haustoria emerging from a germinating seed grown on agar.**



**Figure 1.4. Mistletoe is easily spotted in a barren winter host.**



**Figure 1.5.** Although obscured, the mistletoe bush is still visible through host leaves in summer. This is due to the rich blue green color of the leaves of the mistletoe bush, which appear much darker than those of its host.



**Figure 1.6. Epidermis surrounds the distinctive annual rings in a mistletoe stem.**



**Figure 1.7. Mistletoe invades the lower branches of a host which is exposed on four sides.**



**Figure 1.8. Mistletoe accumulates in only the upper branches of a tree which is closely surrounded by other trees.**



**Figure 1.9.** Fungal hyphae growing on extremely productive *P. serotinum*, which was in turn parasitizing, *Carya cordiformis*

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## **Chapter 2. Distribution of Mistletoe**

### **Introduction**

The study species, *P. serotinum* ranges from southern New Jersey, Ohio, Indiana, and Montana on the north, and to Florida and Texas on the south (Gleason and Cronquist, 1991). The northernmost limit may vary. Chrysler (1933) reports having seen mistletoe eight miles south of New Brunswick, New Jersey. There is also a report that it has extended as far north as Long Island (Spooner 1983). The range is consistent with 180 frost-free days per year. Protective factors such as proximity to large bodies of water will moderate microclimate, allowing *P. serotinum* to exist in places that do not have 180 frost-free days per year (Spooner, 1983). It is this latter supposition which explains the rare occurrences in Long Island and along rivers in Pennsylvania and Ohio where there is an obvious absence of 180 frost-free days.

Bird vectors spread seeds from the study species of mistletoe (Figure 2.1). Cedar waxwings, bluebirds, robins, thrushes, and starlings are among the species that have been implicated in spreading the seeds (Hawksworth and Scharpf, 1974). The Cedar waxwing, *Bombycilla cedrorum*, is a migratory species, which travels in a group of a hundred or more birds. This species relies heavily on berries for survival in general and on *P. serotinum* in particular during the winter months (Eleuterius, 1976). The seed is nutritious providing both calories and protein. The seed reportedly contains 5.25% protein and 4.39 kc/g (McPherson, 1987). More will be said below, about the importance of mistletoe in the community.

After passing through the gut of the bird, the seed of *P. serotinum* sticks to the branches of trees. It attaches to its host at first because there is a proteinaceous glue-like

substance which remains on the seed even after travelling through the bird's digestive tract (Martinez del Rio et al., 1996). The study species of mistletoe contains a great deal of lectin in the seeds and some of the lectin probably remains on the surface of the seed. The seeds are green and capable of photosynthesis. Soon after the seed adheres to the branch it germinates given enough moisture. Without delay, the haustoria emerge, as described in Chapter 1.

Tidewater Virginia was chosen as the study site because of verbal reports of an abundance of mistletoe in the area, which proved to be correct. Our studies were conducted along Old Country Road and Route 60 in four counties: York County, James City County, Williamsburg County, and Newport News County. These counties form part of a peninsula. The entire peninsula is bound by the York River on the southwestern side and by the James River on the northeastern side (Figure 2.2). Both roads, Old Country Road and Route 60 are closer to the James River than they are to the York River, with Old Country Road running just south of Route 60 (Figure 2.3).

In addition, studies were carried out at Carter's Grove, which is a five hundred acre site on Route 60. The peninsula narrows around this site. The site includes an old plantation with a restored manor and slave quarters, an archaeology museum, a barn, several pastures where oxen and horses graze, an apple orchard, and the remnants of an early English settlement, Wollenstenholme Towne (Figures 2.4 and 2.5). This settlement predates Jamestown. The major relics of the settlement, which are housed in the museum were recovered from excavated gravesites, adjacent to the James River.

Some of the historical documents give indications of climatic conditions and failed attempts to raise crops but no mention is made of mistletoe. Mistletoe was not

unknown to these early English settlers. *Viscum album* is found all over England. However, there is no mention of mistletoe in the recordings of these early European immigrants. Therefore, it was not considered useful nor was it considered a pest.

Thomas Jefferson, who was considered extremely learned in the sciences, outlines some common flora and agricultural crops in his writings, but no mention was found of the study species in documents housed in Williamsburg. It is highly unlikely, given his meticulous notes about agricultural matters that mistletoe invaded any of the orchards with any seriousness during his lifetime.

### **Materials and Methods**

Counts of mistletoe plants were done during the winter and early spring between 1999 and 2002. At Carter's Grove all footpaths were walked and investigated for mistletoe presence. Binoculars were used to differentiate between mistletoe, clumps of leaves, and birds' nests. However, the characteristic shape of *P. serotinum* makes it easy to spot from the ground as discussed above. If mistletoe was observed and the host was part of a larger stand, then a walk into the adjacent woods to scan a 30 m circumference around the infected tree was conducted. In this way, the presence or absence of neighboring infections could be confirmed.

The number of mistletoe bushes in each tree was counted. Each bush was divided into one of three size classes. The largest class was between 0.75 and  $1.0 \pm 0.2$  meters in diameter. The mid-range class was between 0.75 to  $0.5 \pm 0.2$  meters in diameter. The smallest class was less than  $0.5 \pm 0.1$  meters. Species and DBH of each host tree were recorded. Basal area was computed from DBH. Height was measured using an Abney Level. Core samples were taken from ten host trees, which were *Carya cordiformis*, and

three controls, which had no obvious infections. The controls were also *Carya cordiformis*. Temperature and relative humidity measurements were done at both the Carter's Grove and Capital Landing Road sites in areas where trees were heavily infected and in areas where mistletoe was not found.

The Global Positioning System Trimble Geo Explorer II was used to identify the position of host trees at both sites in the study. Cartesian coordinates were taken using Universal Transverse Mercator Map Projection (UTM Grid). This system consists of 60 north-south zones with 6 degrees of longitude in each (Jones, 1997). It was used because the maps chosen for the project are compatible with this system and because it provided more precise measurements than Decimal Degrees on the Geo Explorer II.

As a second GPS was not available to correct for errors in real time and imprecise readings, which occur due to weather conditions and/or Defense Department interference, coordinate positions were only taken on extremely clear days and only after at least three satellites were located. Defense Department interference was a continuous problem. It is clear from the maps that proximity to Defense Department sites, which include the Armed Forces Experimental Station, Camp Perry Naval Base, Fort Eustis Military Station, and the U.S. Naval Supply Station, are an impediment to GPS usage (Figure 2.6).

For all the above reasons, all points were checked in reference to distance from known positions on the maps. The Carter's Grove Manor and relevant intersections along Capital Landing Road and Route 60 were used as reference points. If GPS determined positions were not logical on a clear day, then the coordinates were ignored and the data acquisition was repeated. Fortunately, the deliberate governmental

scrambling was very obvious and it was possible with repeated measure to acquire accurate data.

The coordinates had to be entered by approximating the position on the map directly, as the computer program Arc View 3.2 does not enter the positions from raw data into UTM directly. This technique may have resulted in some inaccuracy,  $\pm 10$  m on each of the maps. These minor inaccuracies do not diminish the importance of spatial analysis especially when dealing with a parasite which has many of the characteristics of an epidemic. It should be remembered that in England, Snow connected cholera to the Southwark and Vauxhall water pumps 32 years before the disease was connected to *Vibrio cholerae* simply by mapping spatial distribution and identifying clusters in proximity to water pumps (Gerstman, 1998).

The points were then displayed using Arc View GIS 3.2 and Spatial Analyst 2.1 on four United States Geological Survey maps, which were combined in the projection (ESRI, 1992-1997). Those used were:

1. Hog Island Quadrangle, from  $76^{\circ} 45'$  to  $76^{\circ} 37' 30''$  longitude and from  $37^{\circ} 15'$  to  $37^{\circ} 07' 30''$  latitude (USGS 1984a);
2. 2. Yorktown Quadrangle, from  $76^{\circ} 37' 30''$  to  $76^{\circ} 30'$  longitude and from  $37^{\circ} 15''$  to  $37^{\circ} 07' 30''$  latitude (USGS, 1984b);
3. 3. Williamsburg Quadrangle, from  $76^{\circ} 45'$  to  $76^{\circ} 37' 30''$  longitude and from  $37^{\circ} 22' 30''$  to  $37^{\circ} 15'$  latitude;
4. 4. Newport News Quadrangle (USGS 1984b). The DRGs are georeferenced to the Universal Transverse Mercator (UTM) grid on these maps. All four quadrangles are in Zone 18 on the UTM Grid.

ESRI data was also used to create the maps (ESRI, 1999). The ESRI data was reprojected in Universal Transverse Mercator and used to create map layers. These layers were then used to update Highways and Roads on the three 1984 7.5' USGS Quadrangles described above. These USGS maps have seams with data distributed at the edge of the map and cannot be displayed in Arc View at the same time. These seams do not distort distance or interfere with measurements (Figures 2.7a and 2.7b). The maps were displayed in separate layouts in Arc View and when possible the final layout was done using Canvas (Canvas, 1998). However, Canvas does distort the original projection so all measurements were done in Arc View. Canvas projections were used only to elucidate text and never for measurements.

To study total frequency distribution of mistletoe per host at Carter's Grove and Capital Landing Road, descriptive statistics and histograms were done using Statview (SAS).

To compare the spatial distribution of mistletoe at the Carter's Grove Site to that of the Capital Landing Site, a randomization experiment was designed. In this design a circle was drawn around each site. The diameter of each circle was just large enough to encompass all host trees. A square quadrat was then layered over each circle using the symbol tool from Arc View. The diameter of the circle was used to define the dimensions of the quadrats (Figure 2.8). The number of mistletoe bushes in each of the two quadrats was determined and the area of each quadrat was measured using the Arc View measuring tool. An ANOVA was done using JMP (SAS) to compare the concentration of mistletoe at the Carter's Grove site to that at the Capital Landing Road site.

Using the quadrats described above a grid was drawn over each site, the Capital Landing site and the Carter's Grove site. These grids were drawn for the sole purpose of comparing the concentration of mistletoe per unit area at the Carter's Grove Site to the concentration of mistletoe at the Capital Landing Road Site. There were 484 subdivisions in the grid over the Capital Landing site and 1764 subdivisions in the grid over the Carter's Grove site (Figure 2.8). These measurements had to be done at the same magnification because the grids would not convert to shape files. It was then possible using an Avenue Script (Figure 2.9) to randomly choose a set of subdivisions. The density of hosts and mistletoe bushes were computed and recorded for the randomly chosen set of subdivisions from the Carter's Grove site and independently for the randomly chosen set of subdivision from the Capital Landing Site.. An ANOVA was done in JMP to compare the distribution at both sites.

## **Results**

There were a total of 829 *P. serotinum* bushes counted on 54 host trees. The trees were all within a ten kilometer radius. The maximum number of mistletoe bushes/host tree was 85. They were distributed logarithmically (Figure 2.10). The mean number of bushes/ host tree was 15.31 (Tables 2.1 and 2.2).

At Carter's Grove the mistletoe ran in a north south direction from the James River to Route 60 (Figure 2.10). The line of infected trees continued north on Route 60. Most of the mistletoe was concentrated within 300 meters of the James River. The distribution at the Carter's Grove Site maintained the logarithmic distribution with one tree being more heavily infected than all the rest (Figure 2.11a) and the host trees were distributed in a north south direction (Figure 2.12). At the Capital Landing Road Site the

infection site is not clearly oriented in a north south direction (Figure 2.13). As was mentioned previously, this latter site is younger and it is possible that with time a more north south direction will prevail here too. It is also possible that with time the distribution at the Capital Landing Road site will also be more logarithmic (Figure 2.11b).

At the Carter's Grove Site the mean number of bushes per host was 15.56 and at the Capital Landing Road Site the mean number of mistletoe bushes per host tree was 14.00. The maximum at Carter's Grove was 85 bushes per host and at the Capital Landing Road Site it was 40 bushes per host. Carter's Grove had a total of 607 bushes, while Capital Landing Road had a total of 196 mistletoe plants per host.

**Table 2.1. Descriptive Statistics for Carter's Grove and Capital Landing Road Sites.**

	<b>Carter's Grove</b>	<b>Capital Landing Road</b>	<b>Total</b>
<b>Mean bushes/host</b>	<b>15.56</b>	<b>14.000</b>	<b>15.31</b>
<b>Standard Deviation</b>	<b>17.38</b>	<b>12.818</b>	<b>17.03</b>
<b>Standard Error</b>	<b>2.78</b>	<b>3.426</b>	<b>2.254</b>
<b>Count</b>	<b>39</b>	<b>14</b>	<b>56</b>
<b>Total number of mistletoe bushes</b>	<b>607</b>	<b>196</b>	<b>816</b>

**Table 2.2. Quantiles for both sites together.**

<b>Quantiles</b>		
100.00%	<b>maximum</b>	<b>85</b>
90.00%		<b>40</b>
75.00%	<b>quartile</b>	<b>22</b>
50.00%	<b>median</b>	<b>8.5</b>
25.00%	<b>quartile</b>	<b>3</b>
10.00%		<b>2</b>
2.50%		<b>1</b>
0.50%		<b>1</b>
0.00%	<b>minimum</b>	<b>1</b>

Using the quadrats drawn in Arc View, as described above, the quadrat at Carter's Grove measured 15.25 km<sup>2</sup>, 3.9 km by 3.9. The quadrat at the Capital Landing Road Site measured 4.23 km<sup>2</sup>, 2.06 km by 2.06 km. The grid placed over the Capital Landing quadrat contained 484 subdivisions and the grid placed over the Carter's Grove quadrat contained 1764 subdivisions. Each subdivision in the Carter's Grove Site measured 8.7 km<sup>2</sup> and the grid at the Capital Landing site was drawn so that each subdivision there also measured 8.7 km<sup>2</sup>.

All host trees infected with mistletoe were included in the spatial distribution grids. There are 39.85 mistletoe bushes/ km<sup>2</sup> and 2.62 hosts/ km<sup>2</sup> at Carter's Grove and there are 46.3 bushes/km<sup>2</sup> and 3.31 hosts/km<sup>2</sup> at the Capital Landing Site. The frequency distribution at both Carter's Grove and Capital Landing Road are parallel (Figures 2.11a and 2.11b).

**Table 2.3 Spatial distribution at Carter's Grove and Capital Landing Road.**

<b>Variable measured</b>	<b>Carter's Grove</b>	<b>Capital Landing Road</b>
Size of larger quadrat	15.25 km <sup>2</sup>	4.23 km <sup>2</sup>
Number of quadrats	1764	484
Size of each subdivision	8.7 km <sup>2</sup>	8.7 km <sup>2</sup>
hosts/km <sup>2</sup>	2.84	3.31
bushes/km <sup>2</sup>	39.85	44.81
mean height of host m	20.4	15.9
mean basal area of host	5161 cm <sup>2</sup>	1585 cm <sup>2</sup>

Using the data derived from the randomization experiment circle described above, two ANOVAs were done to compare spatial distribution at Capital Landing Road and at Carter's Grove. The first ANOVA was done with 10% of all subdivision picked randomly (Table 2.4). The second ANOVA was done picking only those subdivision containing mistletoe bushes (Table 2.5). The number of mistletoe bushes/ quadrat, shows no statistically significant difference between the two sites. With an R square of 0.000031 and  $P = 0.789$  (DF 1, 2282), there is no difference between the two sites (Tables 2.4 and 2.5).

**Table 2.4. Analysis of variance comparing the concentration of mistletoe bushes per unit area at Carter's Grove to that at Capital Landing Road (R square = 0.000031). This test is done using all subdivisions including those which contain no mistletoe bushes.**

<b>Source</b>	<b>DF</b>	<b>Sum of Squares</b>	<b>Mean Square</b>	<b>F Ratio</b>	<b>P</b>
	2	1.084	1.084	0.0717	0.7889
<b>Error</b>	2282	34507.005	15.1214		
<b>C. Total</b>	2283	34508.089			

**Table 2.5. Analysis of variance comparing the concentration of mistletoe bushes per unit area at Carter's Grove to that at Capital Landing Road (R square = 0.000968). This test is done only on those subdivisions which contain mistletoe bushes.**

<b>Source</b>	<b>DF</b>	<b>Sum of Squares</b>	<b>Mean Square</b>	<b>F Ratio</b>	<b>P</b>
	<b>1</b>	<b>15.927</b>	<b>15.927</b>	<b>0.0320</b>	<b>0.8592</b>
<b>Error</b>	<b>33</b>	<b>16441.616</b>	<b>498.231</b>		
<b>C. Total</b>	<b>34</b>	<b>16457.543</b>			

Further, it should be noted that the host trees at Carter's Grove were taller than the host trees at Capital Landing Road. The mean height at Carter's Grove was 20.4 m while that at Capital Landing Road was 15.9 m high. These differences are not dramatic but the difference in mean basal area was much greater at Carter's Grove. The mean basal area at the Capital Landing Road site was 71.2 cm<sup>2</sup> while the mean basal area at the Carter's Grove site was 2357 cm<sup>2</sup> (Table 2.6)

The effects of height, DBH, and basal area on mistletoe concentration were examined using logistic fit of mistletoe concentration by height, DBH, and basal area (Figures 1.11 and 1.12). Dividing the host trees into two groups those with three or more mistletoe bushes and those with less than three mistletoe bushes showed significant differences.

**Table 2.6 Comparison of DBH, basal area and height at Carter's Grove and Capital Landing Road.**

		<b>Carter's Grove N=40</b>	<b>Capital Landing Road N=14</b>	<b>Both sites combined N=54</b>
<b>Basal Area</b>	<b>mean</b>	7172 cm <sup>2</sup>	2099 cm <sup>2</sup>	5857 cm <sup>2</sup>
	<b>minimum</b>	1182 cm <sup>2</sup>	38 cm <sup>2</sup>	38 cm <sup>2</sup>
	<b>maximum</b>	49662 cm <sup>2</sup>	8171 cm <sup>2</sup>	49662 cm <sup>2</sup>
	<b>total</b>	283,235.0 cm <sup>2</sup>	33,048.8 cm <sup>2</sup>	316286.8 cm <sup>2</sup>
<b>DBH</b>	<b>mean</b>	77.4 cm	40 cm	67.5 cm
	<b>minimum</b>	38.8 cm	22 cm	22.0 cm
	<b>maximum</b>	135 cm	92.1 cm	135.8 cm
<b>Height</b>	<b>mean</b>	20.1 m	17.7 m	19.5 m
	<b>minimum</b>	11.1 m	8.7 m	8.7 m
	<b>maximum</b>	30.5 m	29.0 m	30.5 m

The size of the tree appeared more important when dividing infected trees into those with four or more bushes. Using this technique all trees with four or more bushes were likely to have a larger DBH (Figure 2.14). The same is true for height (Figure 2.15) and basal area (Figure 2.16).

Old Country Road runs south of Route 60 just parallel to it and considerably closer to the James River (Figure 2.3). The first observations on Old Country Road were done on foot in the early spring before car access was allowed. The road is not accessible in the winter months. However, an extensive search for mistletoe on this road was not done until the last two weeks of April 2002. The leaves of the host trees while not fully developed may have obscured minor infection sites but even the presence of a bush in the smallest class ( $0.5 \pm 0.1$  m) above 0.20 meters would have been visible between the leaves. The young deciduous leaves are a light green and the mistletoe leaves are a much darker bluish green. In addition the shape of the mistletoe leaf is very different from its hosts. Despite three surveys we were unable to observe any infection sites along this road.

We also failed to observe any mistletoe further south on Route 60. This interstate quickly narrows after Carter's Grove, becoming a two-lane road with virtually no shoulder. The canopy is often closed overhead along this section of road.

We found no meaningful differences in temperature or humidity at any of the sites investigated. Unfortunately, recording equipment could not be left on site so all measurements were discrete and although they were done at the same time of the day, these measurements could not be done simultaneously. Furthermore, the only measurements that would be indicative of the mistletoe microclimate would have had to

have been taken in the crowns of the trees and would probably have had to include 60 second recordings (Parker, 1995); such equipment intensive techniques were beyond the scope of this project.

### **Conclusion**

The similarity in the concentration of mistletoe per unit area at the Carter's Grove Site, 39.8 bushes/km<sup>2</sup> and the Capital Landing Road site, 46.3 bushes/km<sup>2</sup> came as a surprise. Once the first map was completed using the UTM coordinates, the clustering was obvious at the two sites (Figure 2.13). The quantitative similarity is not clear until the quadrats are drawn and the ANOVA completed. If 99.997% of the difference in mistletoe density between the two sites can be explained by chance, the density of mistletoe bushes at both sites is the same. This similarity is there despite a pronounced difference in host species, which will be discussed in Chapter 3. There is also a remarkable difference in the age of the trees at the Capital Landing site and the Carter's Grove site. The oldest and the most heavily infected tree at the Capital Landing site was only 35 years old while the age of the most heavily infected tree at Carter's Grove was well over 100 years. The age of the trees was confirmed by the owner of the property at the Capital Landing site and by the chief Horticulturalist at the Carter's Grove site.

Whether this has to do with natural immunity on the part of specific hosts or some other environmental factor is unclear at this time. However, light may well play a roll here. The trees at Capital Landing are considerably smaller than the trees at Carter's Grove because of age and species. Therefore, a greater percentage of mistletoe seeds

would be exposed to more light from germination. In addition, bird behavior is probably in part responsible for density.

There are at least three possible causes for the lack of mistletoe on Old Country Road and the abundance of mistletoe on Route 60. It could be environmental, a genetic difference in the hosts available, or perhaps the vector does not frequent this route after having eaten. The birds, which frequent the trees on Route 60 also frequent the trees on Old Country Road and may even be perching on members of the genus *Carya*, *Acer*, and *Quercus*. However, are they perching on these trees after having eaten large quantities of mistletoe berries?

There were no significant differences in humidity or temperature along either of these roads. Again however, the only meaningful measurements of humidity and temperature differences would have required measurements every ten seconds in the canopy (Parker, 1995). As mentioned earlier, the equipment and labor necessary for such an undertaking are beyond the scope of this work.

There is mention in the literature that some species of mistletoe do better in a disturbed area. This might explain the lack of mistletoe growth on Old Country Road. The road is a narrow, single lane dirt path. While small SUVs are allowed on the road, buses and larger trucks are prohibited. The canopy closes overhead along most of the road. Route 60 in the heavily infected areas, does not narrow to less than a two-lane road with a shoulder on one side of the road. The canopy never closes across the road. To further support this argument, south on Route 60 the road narrows considerably and the canopy closes in overhead and again there is no visible sign of mistletoe.

Thus, there are two sites in which the mistletoe is abundant and two sites, in close proximity to Carter's Grove and Capital Landing Road and along Route 60 with equal potential hosts of the same species, yet there is an absence of *P. serotinum*. Again, the only difference observed during these studies, was the lack of a break in the canopy.

There are two potential explanations from the literature for the observed distribution. One is genetic. Genetic differences have been found by molecular ecologists among three races of dwarf mistletoe. *Arceuthobium americanum* infects three hosts, *Pinus banksiana* in western Canada, *Pinus contorta* in the western United States and *Pinus contorta* in the western United States and Canada. The races were defined using amplified fragment length polymorphism analysis. Each of these races infects a different host (Ford and Jerome, 2002). Norton and Carpenter postulate that host abundance has a key role in eventual speciation (Carpenter and Norton, 1998). This latter explanation only works if there are no hosts available in a specific region. Given the proximity of the two sites at which this work was done and given that a variety of hosts were available at both sights this explanation fails to respond to the data.

A second explanation comes from the vector dispersal of the mistletoe seeds. This theory is consistent with our data. Martinez del Rio and coworkers (Martinez del Rio et al., 1996; Aukema and Martinez del Rio, 2002a; Aukema and Martinez del Rio 2002b) carried out several experiments in which they measured seed rain as deposited by birds. The pattern of the rain was enough to explain the variations. These authors go on to say that the fact that the tallest trees are infected is consistent with bird behavior. The data presented above agrees with this conclusion. The R square was greater for height and mistletoe concentration than it was for DBH and mistletoe concentration. In addition

the fact that some trees have extremely high concentration of mistletoe, while others have very little or none is also consistent with bird behavior. It should be noted here that the data presented above is very consistent with the data published by Aukema and Martinez (2002 a and b) del Rio, even to the superinfected host at one end of the histogram (Figure 2.14). It is remarkable that data obtained in the desert is consistent with data obtained in a temperate forest.

Martinez del Rio et al. (1996) postulates that while mistletoe has the pattern of a metapopulation, Overton's equation does not work unless it is modified to take into account the existence of a vector (Overton, 1994). However, he cautions that the equation must be modified even further to account for bird behavior (Martinez del Rio et al, 1996; Aukema and Martinez del Rio, 2002b).

In a study similar to the one presented here, the spatial distribution of *Plicosephalus curviflorus* was studied on an *Acacia tortillis* host. In this study Donohue (1995) found that mistletoe followed an infectious disease model with acacia habit and density being a possible correlation to mistletoe intensity. The seeds of *P. curviflorus* are also carried by a bird vector. It was found that *Acacia* that assumed a bush-like habit had fewer parasites per host than those acacias that were tall and tree like. The author also noted that the acacias that were bush-like were in close proximity to other trees and bushes. All of this is in agreement with the data described above. It is also logical to draw the same conclusions as Martinez del Rio and coworkers. Much of the distribution is most likely due to seed dispersal on the part of birds.

Therefore, the data as described above supports Martinez del Rio's hypothesis that the distribution of mistletoe is primarily the result of bird vector activity (Aukema and Martinez del Rio, 2002a; Aukema and Martinez del Rio, 2002b).



**Figure 2.1. Bird vectors spread the seeds of mistletoe. Depicted here are Cedar waxwings, a migratory visitor to mistletoe bushes in the middle of winter.**

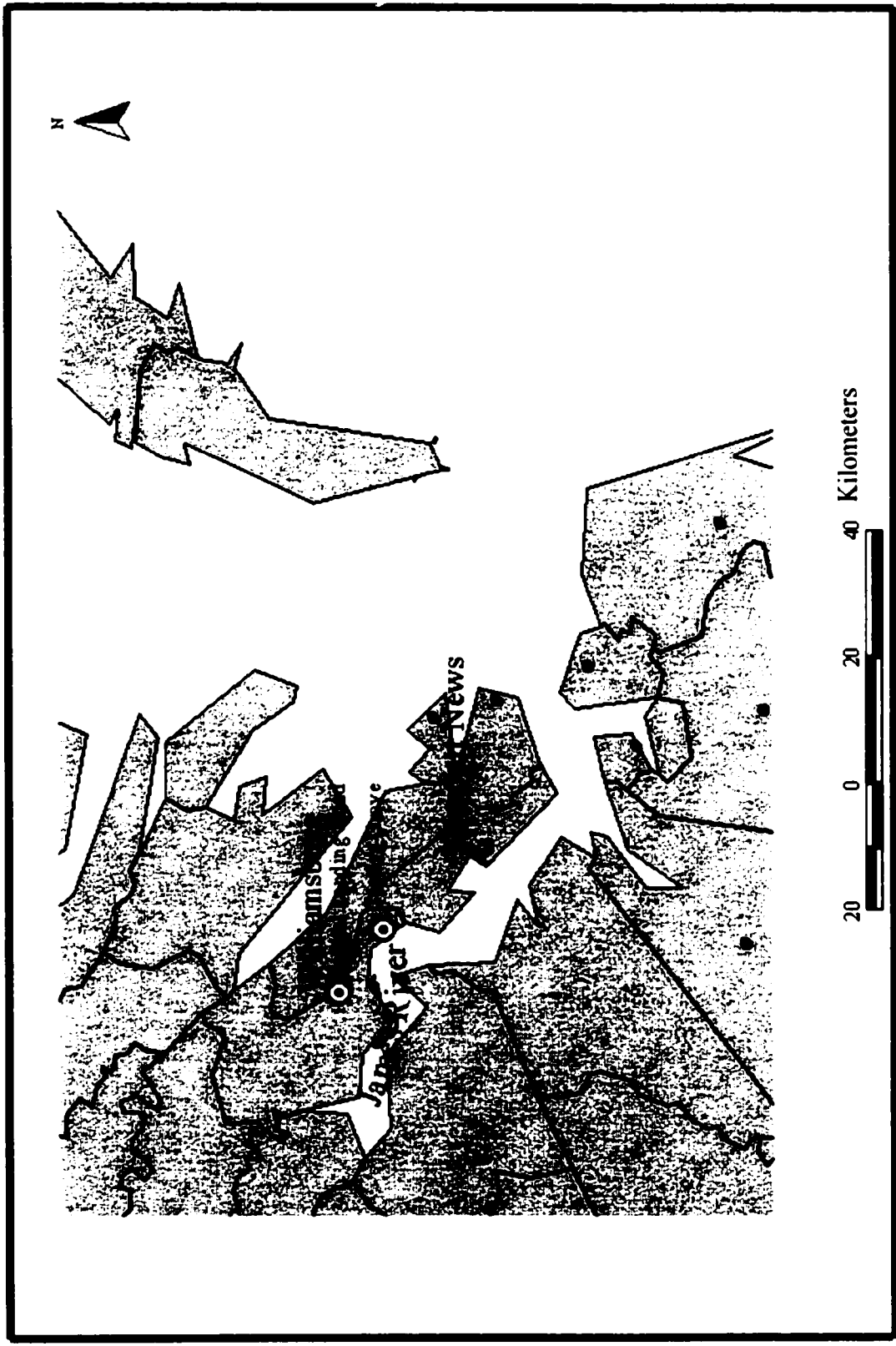


Figure 2.2. The two study sites, Carter's Grove and Capital Landing Road are both found on the Tidewater Virginia peninsula.

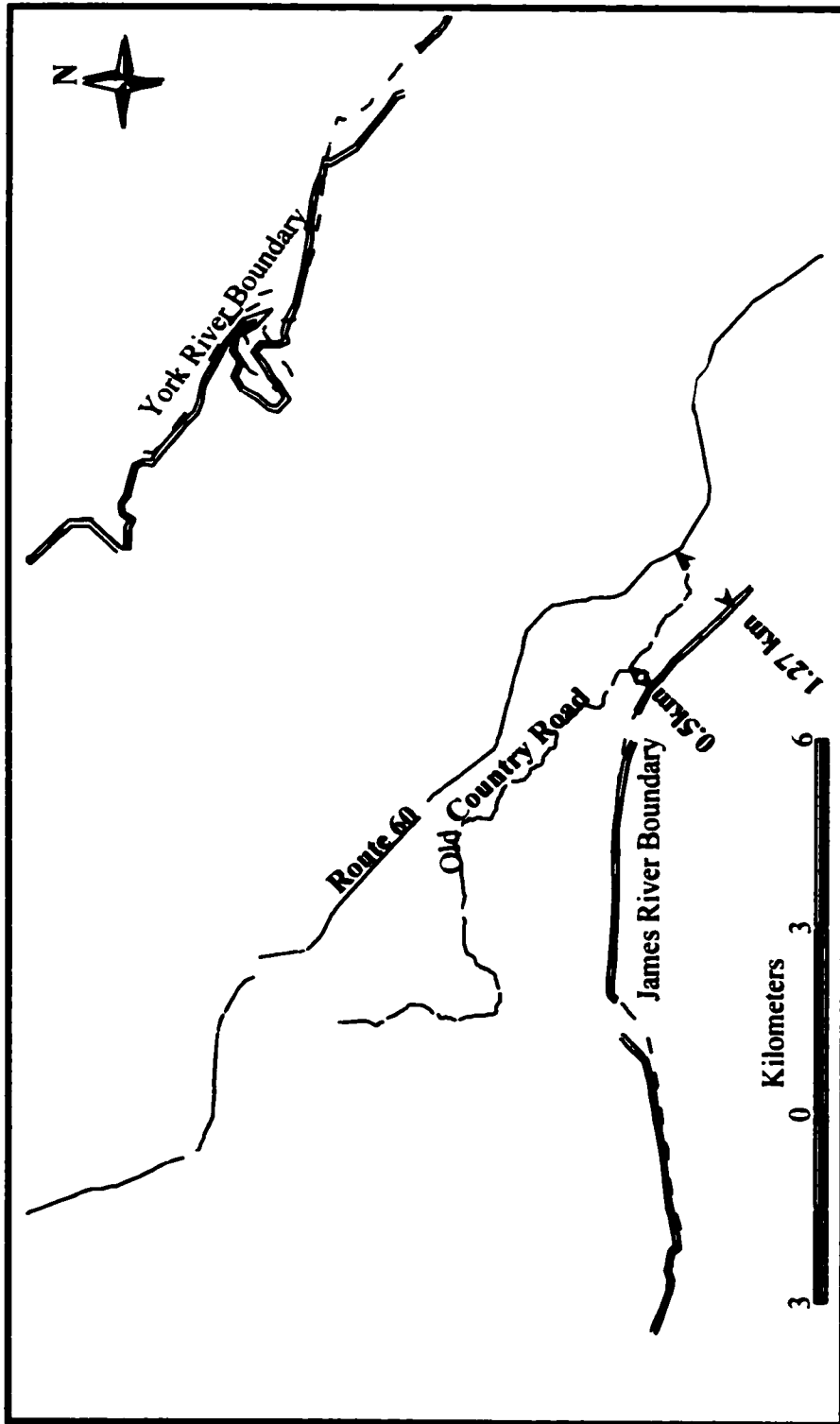
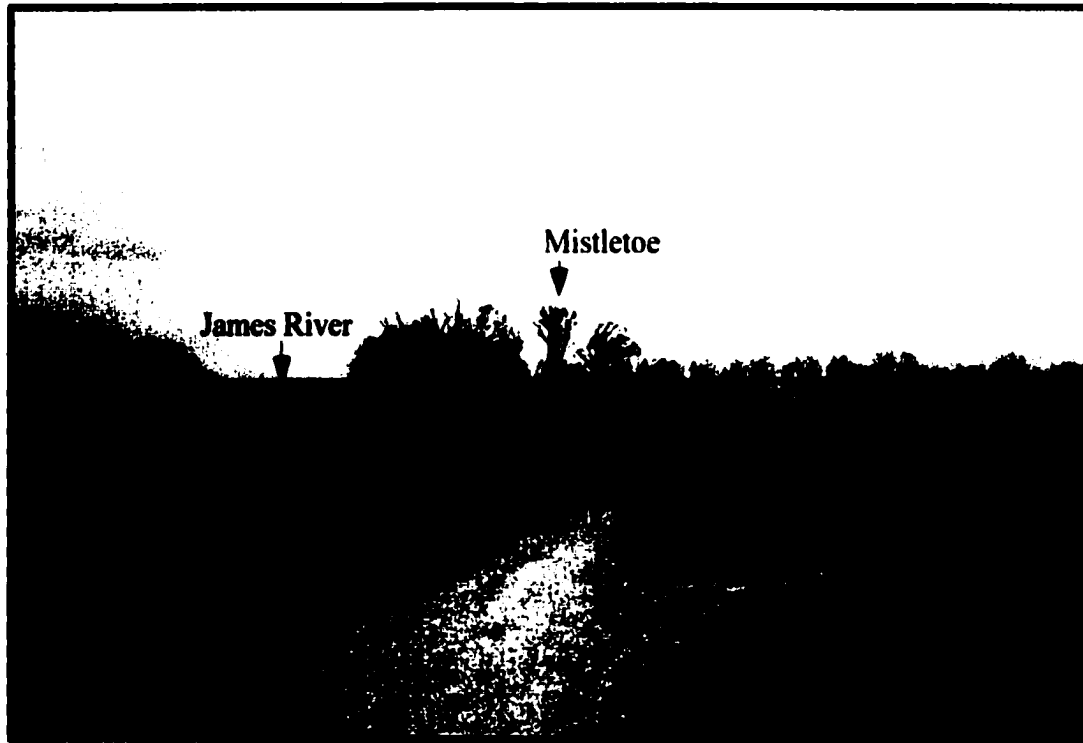


Figure 2.3. Old Country Road and Route 60 are closer to James River than to the York River.



**Figure 2.4. A typical Carter's Grove scene with a reconstructed colonial remnant and expansive open grounds.**



**Figure 2.5.** Carter's Grove with extensive pastures and a tree heavily laden with mistletoe in the background. This tree is close to the James River.

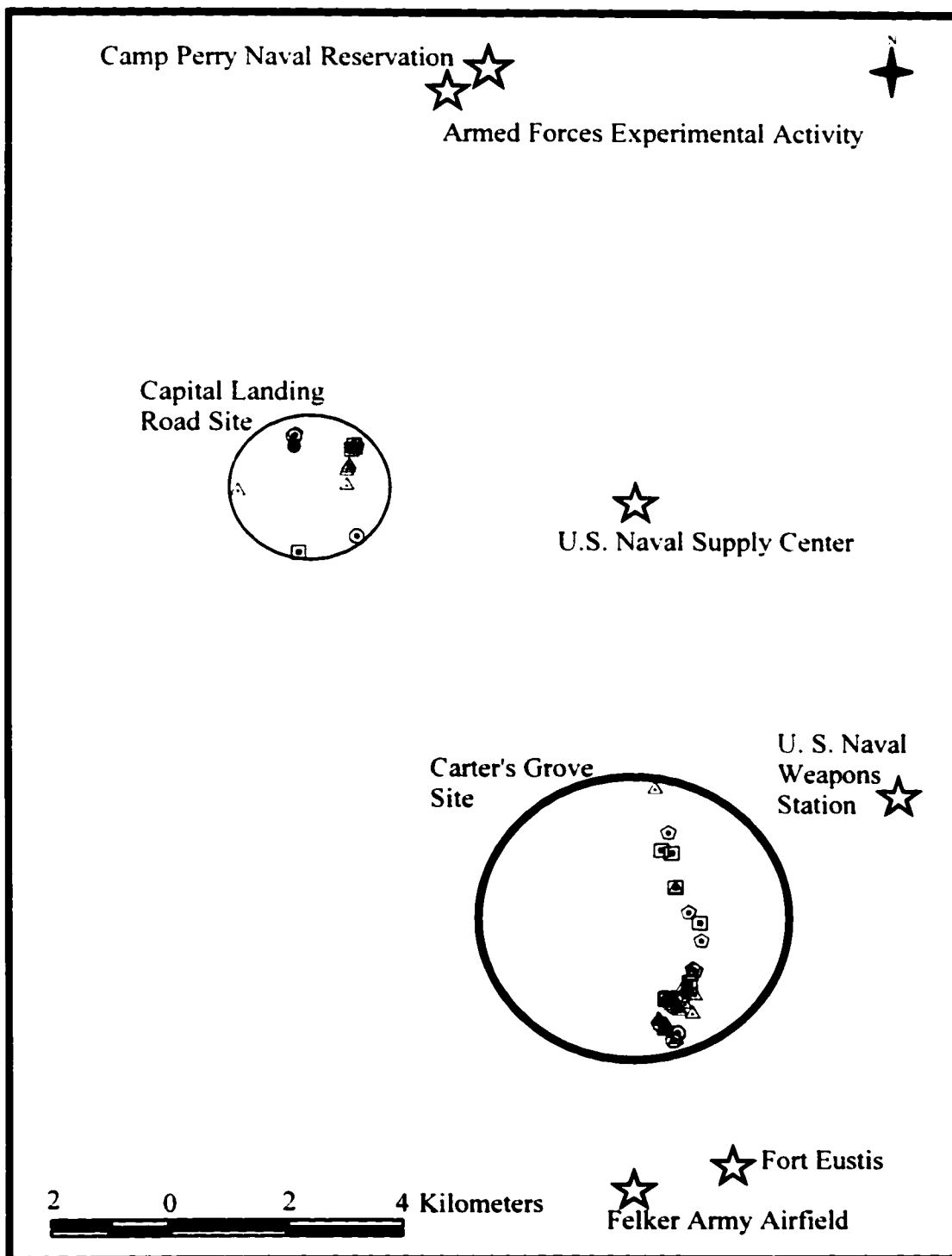


Figure 2.6. United States military installations (positions taken from USGS 7.5'Quad map) were probably responsible for poor GPS readings necessitating corrections.

Figure 2.7a

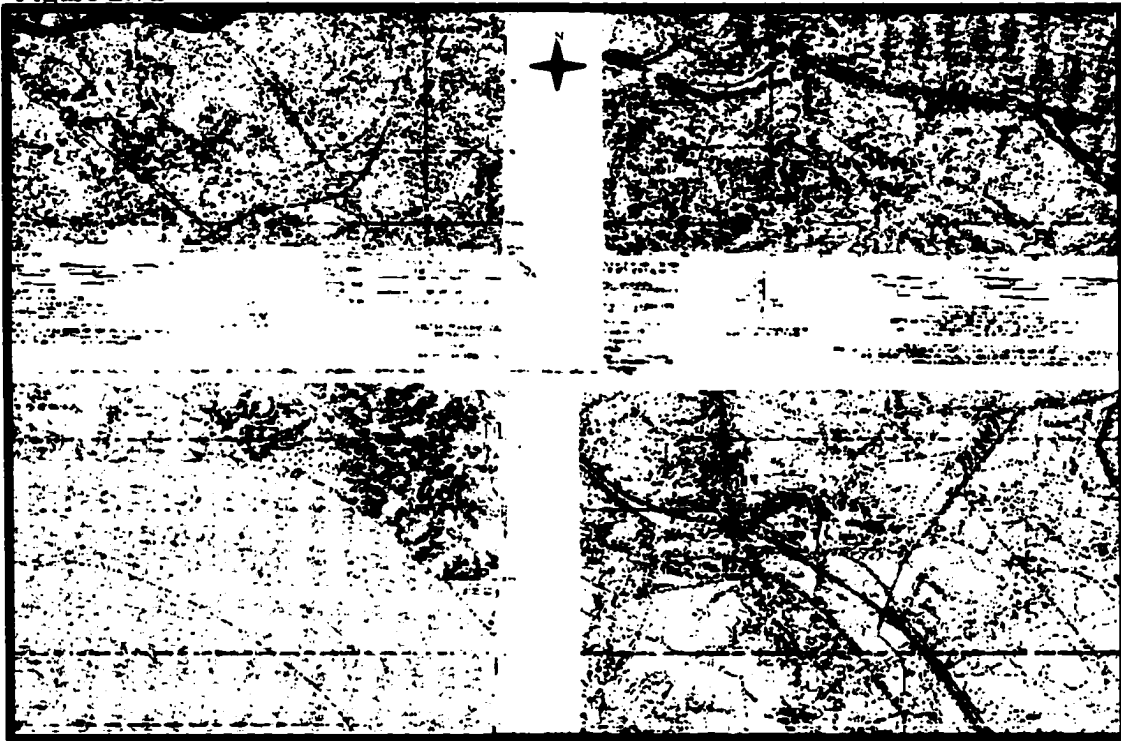
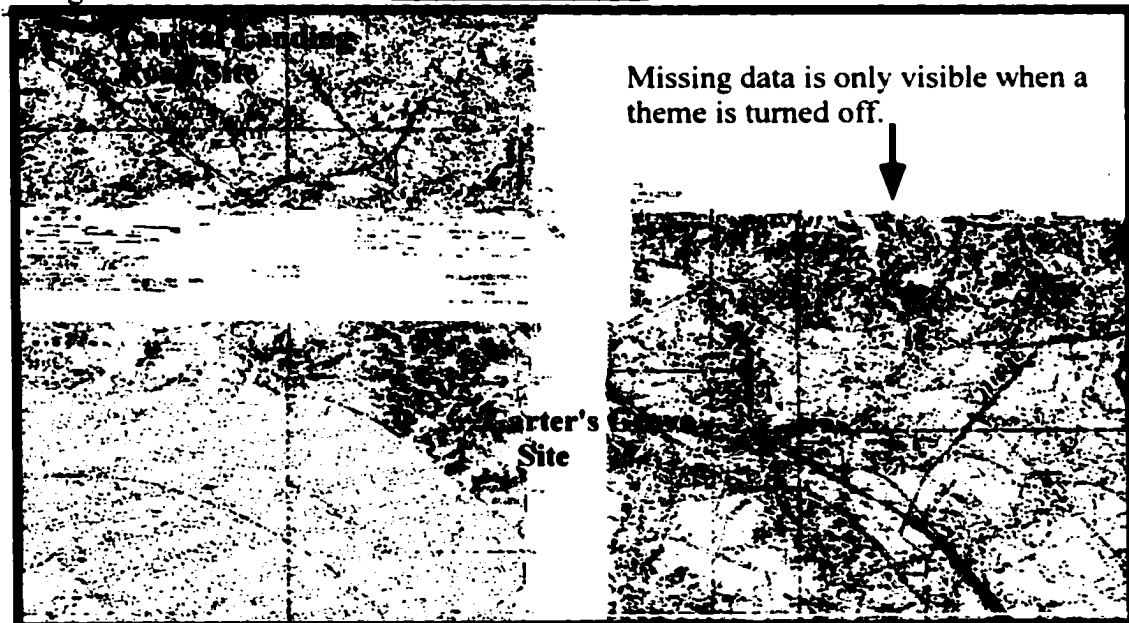
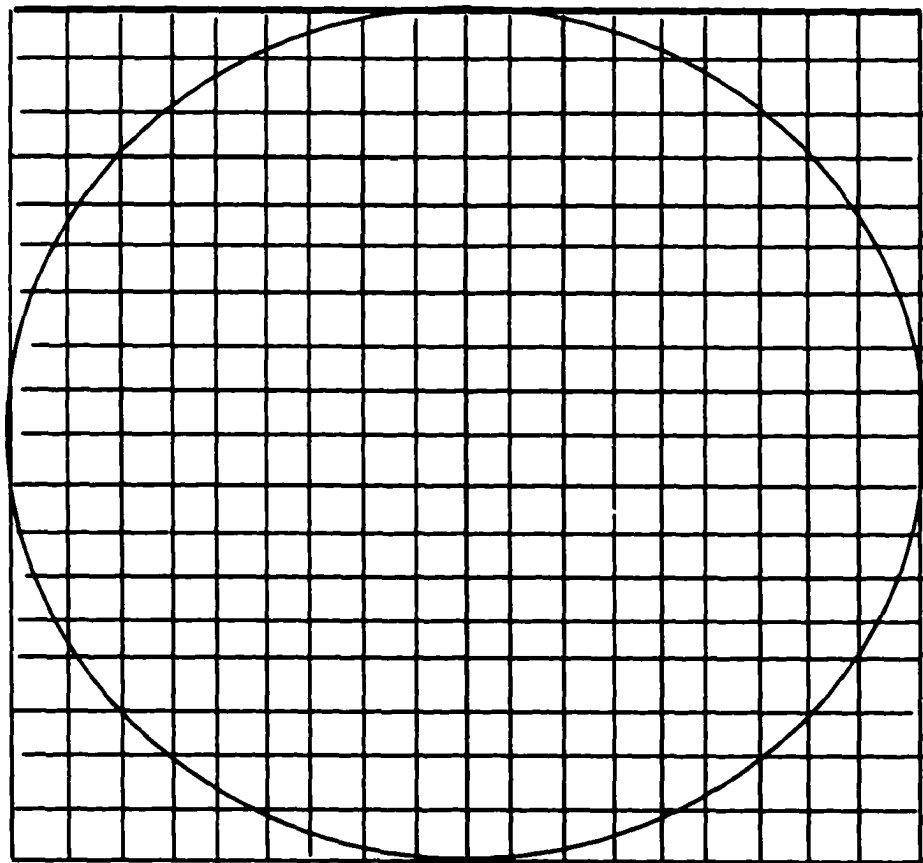


Figure 2.7b.

1 0 1 2 Kilometers



Figures 2.7a and 2.7b. Data at the edge of the map makes it difficult to visualize important areas when they fall in a seam.



**Figure 2.8.** A circle was drawn around the positions of all the hosts at each site and a quadrat was superimposed on the circle. Each quadrat was subdivided by a grid. Each box was randomly chosen using an Avenue Script.

Figure 2.9. Avenue Script for Randomization Experiment

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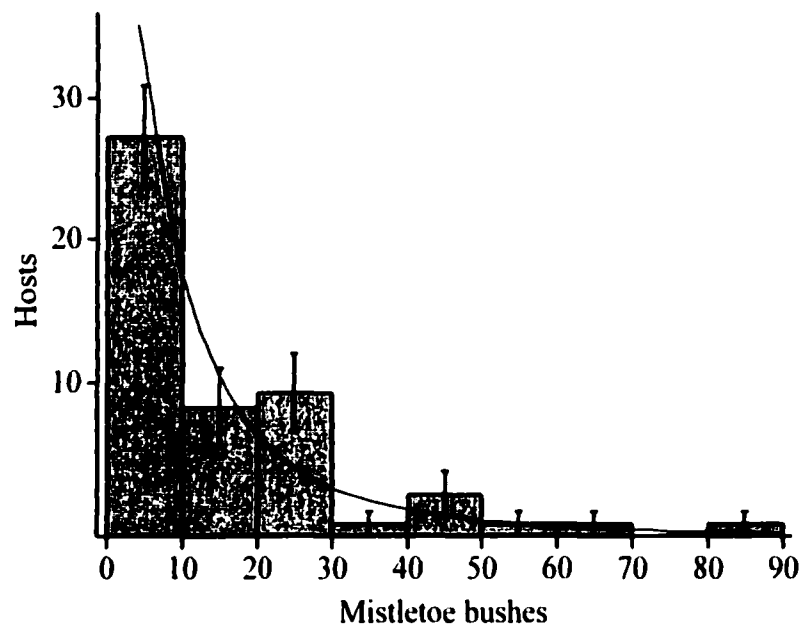
theView = av.GetActiveDoc
theDisplay = theView.GetDisplay
ThemeList = av.GetActiveDoc.GetActiveThemes
if (ThemeList.Count <> 1) Then
  msgbox.Error("You must have one and only one active theme"."Select Random Sample is
aborting")
  exit
end
theTheme = ThemeList.Get(0)
theFtab = theTheme.GetFtab
theBitMap = theFtab.GetSelection
theBitMap.ClearAll
TotalRecs = theFtab.GetNumRecords
PercentSelect = MsgBox.Input("Enter the percentage of Total Population:","Select Ran-
dom Sample"."10")

if ((PercentSelect = NIL) or (PercentSelect.IsNumber.Not)) then exit end
if((PercentSelect.AsNumber > 99) or (PercentSelect.AsNumber < 1)) then
  MsgBox.Error("You must enter a number between 0 and 100"."Select Random Sample is
aborting")
  exit
end
selectnumber = (PercentSelect.AsNumber/100 * TotalRecs).Truncate
if ( MsgBox.YesNo("Do you require an exact sample percentage?","Select Random
Sample".TRUE) ) then
  setbits = 0
  while (setbits < selectnumber )
    newnumber = Number.MakeRandom ( 0, totalrecs)

    theBitMap.Set(newnumber)
    setbits = theBitmap.Count
  end
else
  for each n in 1..selectnumber
    newnumber = Number.MakeRandom ( 0, totalrecs)

```

Figure 2.10. Log normal distribution of total mistletoe bushes per host tree has a mean of 15.32 and an N of 54 host trees containing 827 mistletoe bushes. (KSL Goodness of Fit Test D, 0.104256 and P = 0.1476).



**Figure 2.11.** Log normal distribution of mistletoe bushes per host tree at Carter's Grove has a mean of 16.95 and an N of 40 host trees containing 678 mistletoe bushes. (KSL Goodness of Fit Test D, 0.088719 and P = 0.1500.

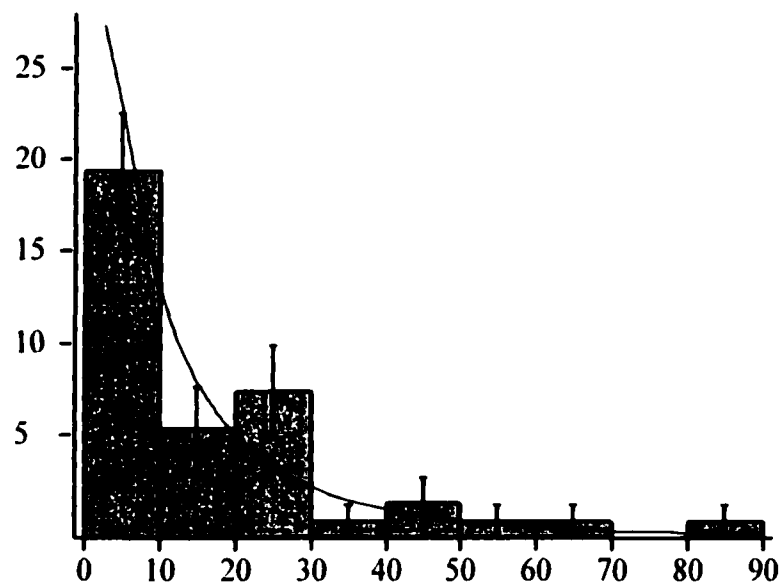
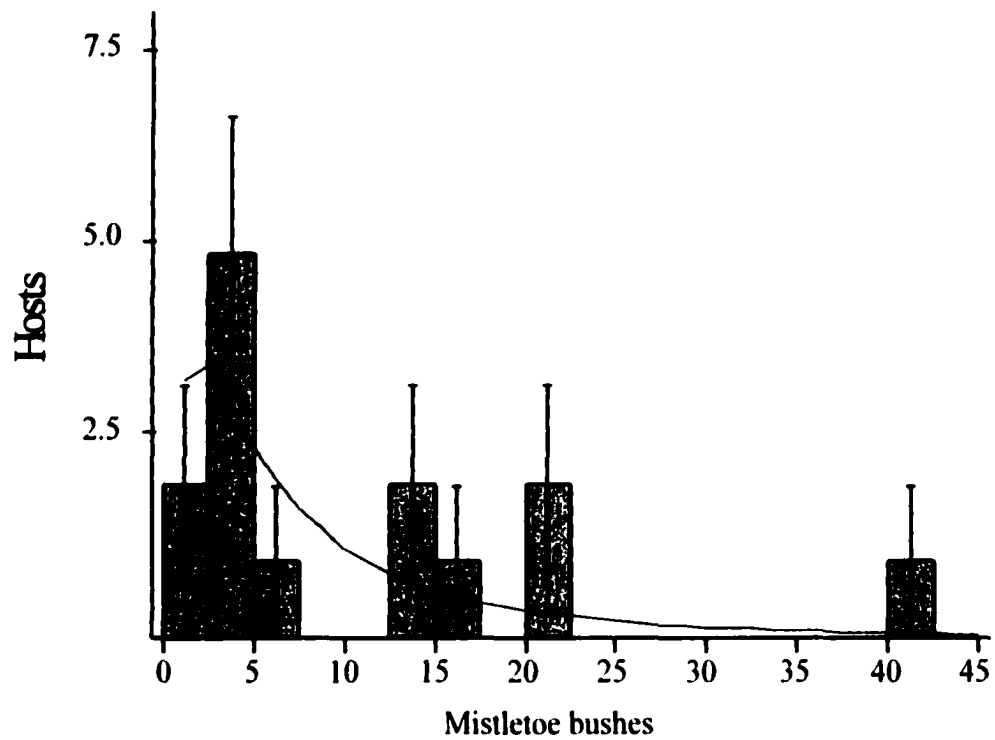


Figure 2.12. Log normal distribution of mistletoe bushes per host tree at Capital Landing Road has a mean of 10.64 and an N of 14 host trees containing 159 mistletoe bushes. (KSL Goodness of Fit Test D, 0.185153 and P= 0.1500.



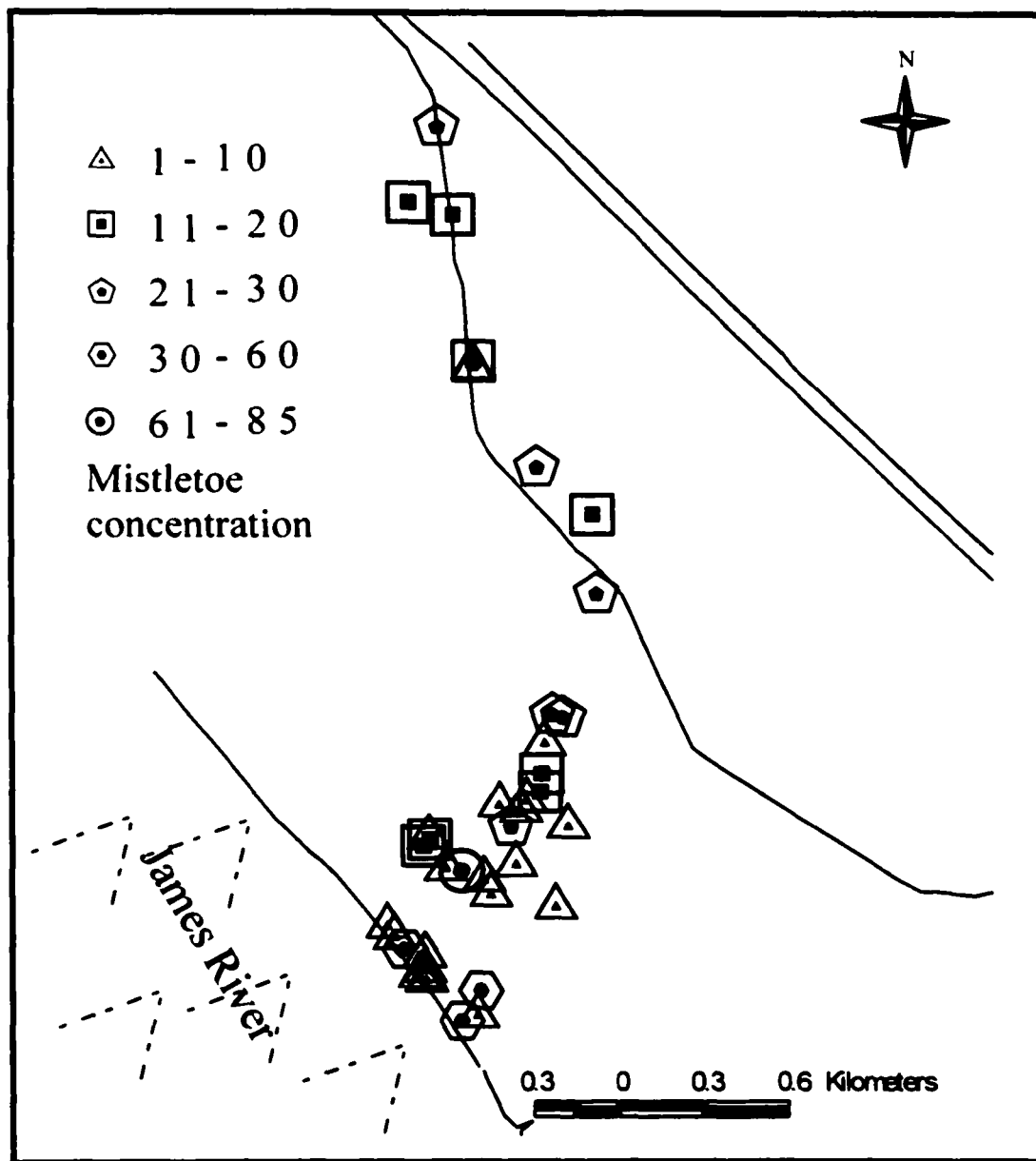


Figure 2.13. Mistletoe concentration at Carter's Grove is oriented in a north-south direction.

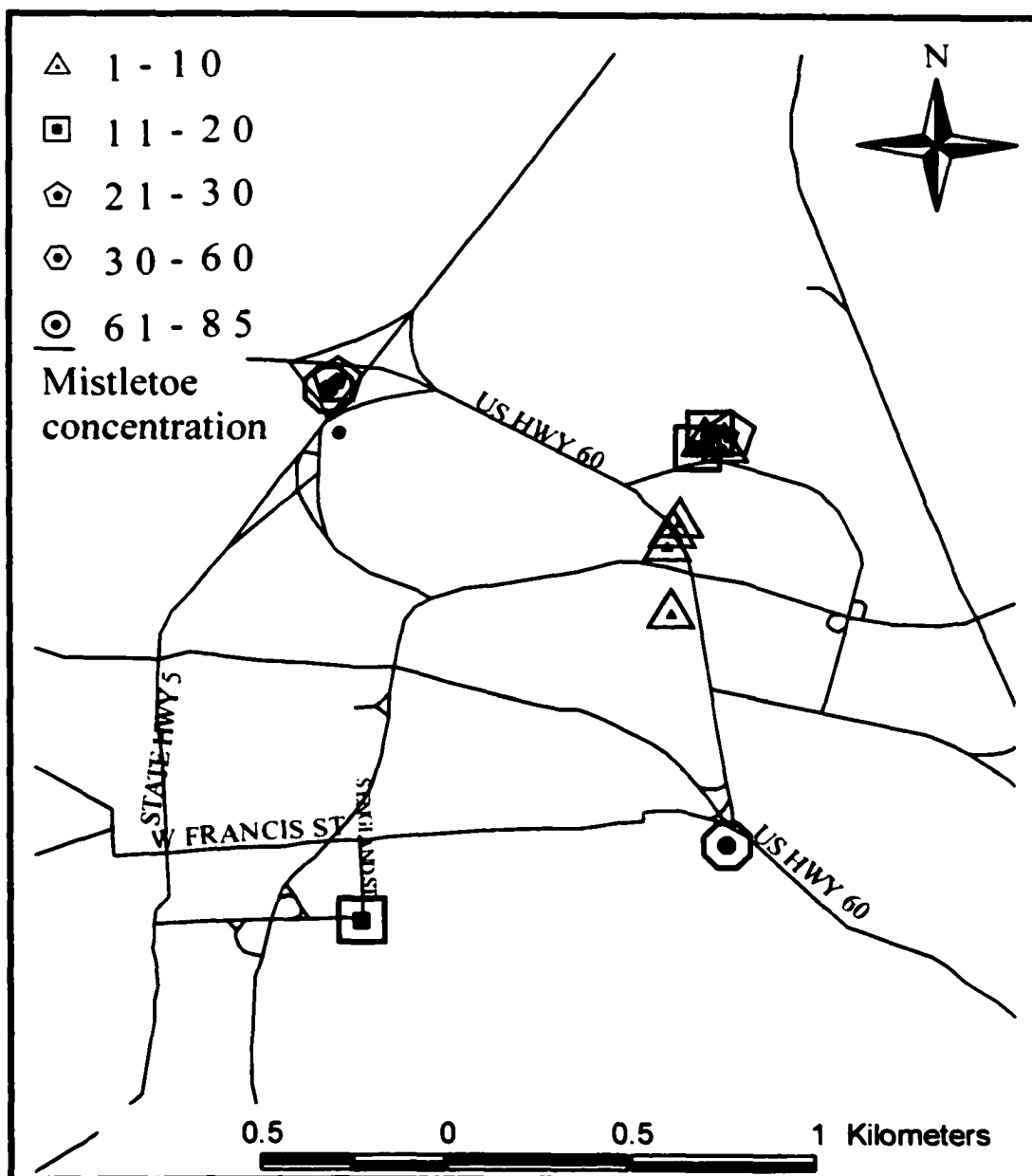


Figure 2.14. Mistletoe distribution at Capital Landing Road is not oriented in a north-south direction.

Figure 2.15. The effect of DBH on the number of mistletoe bushes per host tree. Nominal logistic regression ( $P = 0.0003$ ).

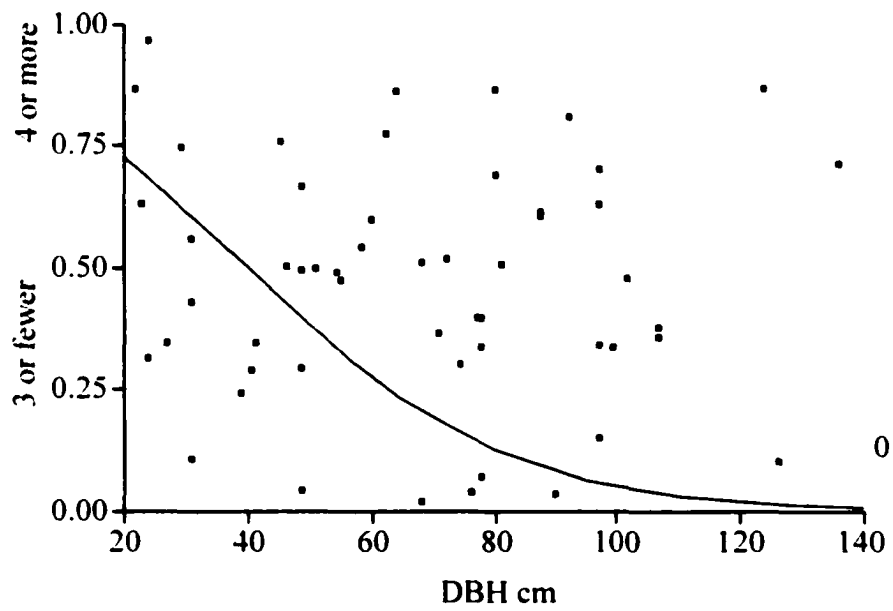


Figure 2.16. The effect of height on the number of mistletoe bushes per host tree. Nominal logistic regress ( $P = 0.017$ ).

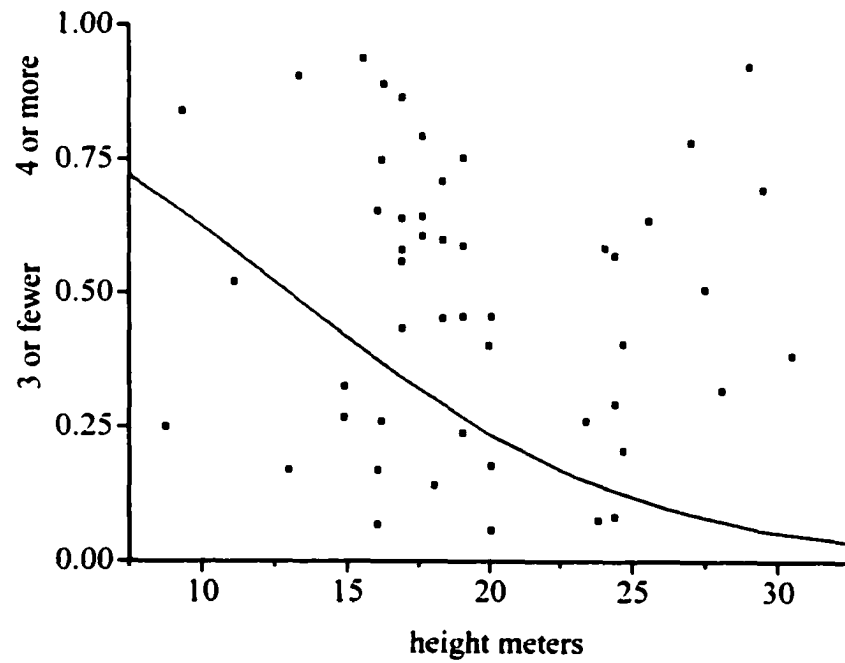
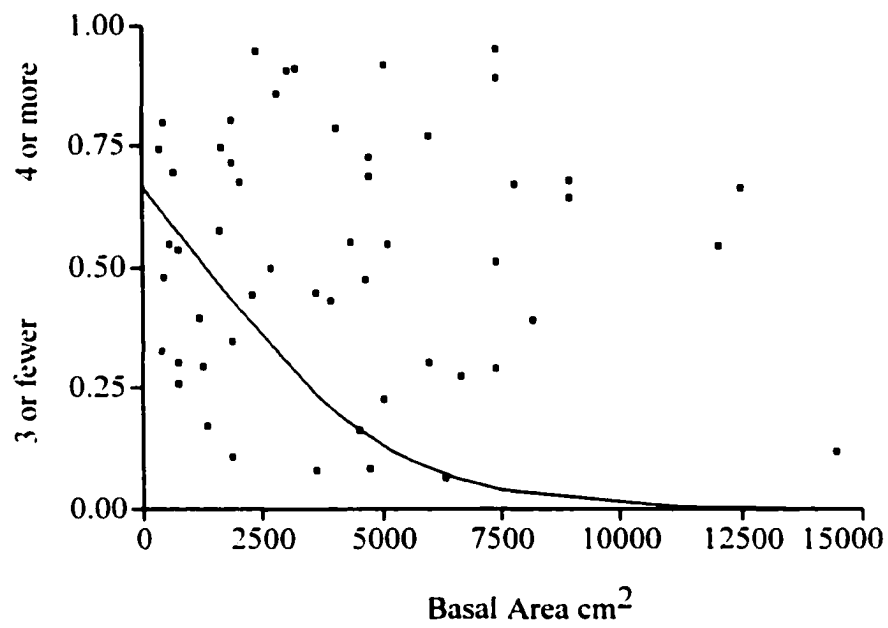


Figure 2.17. The effect of basal area on the number of mistletoe bushes per host tree. Nominal logistic regression ( $P = 0.0003$ ).



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**USGS (1984b)** Yorktown Quadrangle, 7.5 Minute edn. United States Geological Survey, Department of the Interior.

### **Chapter 3. Regional Host Preferences.**

#### **Introduction**

There are about 3000 species of parasitic plants in about 16 different families. Most of these species have been ignored in the literature. However, some of them infest economically important crops throughout the world. It is the latter quality that spurred new interest in these symbionts (Musselman and Press, 1995).

The habit of these parasites is diverse. There are trees among the Santalaceae, vines among *Cuscuta* and *Cassytha*, herbs among the Scrophulariaceae, and shrubs among the mistletoe. The majority of parasitic angiosperms are root parasites, but the mistletoes, with the exception of few species, find their homes in the crowns of trees where their haustoria connect to the stems of their hosts. Kuijt (1969) reports that Steyermark found a member of *Phoradendron* growing just above soil level and he suggests that it might have been engaged in root parasitism. It is not unusual to find the study species germinating on mature members of its own kind. Nuttall points out that *Phora* and *dedron* are Greek for thief and tree respectively but again exceptions occur (Nuttall, 1848).

The two species of mistletoe found in the eastern continental United States are *P. serotinum* and *Arceuthobium pusillum* Peck. The latter species of mistletoe parasitizes both black spruce and white spruce in its range from southern Canada into northern New Jersey and Pennsylvania. In two states, at least, Pennsylvania and New Jersey, the ranges for the two species, *A. pusillum* and *P. serotinum*, overlap in the Eastern United States (Gleason and Cronquist, 1991; Stalter, 2003).

There are no reports in the literature of *P. serotinum* parasitizing spruce nor are there any reports that *A. pusillum* parasitizes angiosperms. However, given the promiscuity of *P. serotinum* in relation to host species, the possibility of competition cannot be ruled out.

Some authors consider *Phoradendron* and *Arceuthobium* pests (Hawksworth, 1983). While members of the genus *Phoradendron* do not parasitize photosynthate, members of the genus *Arceuthobium* do. However, most of the damage comes from the growth irregularity in trees, which accompanies the *Arceuthobium* infection. Because of this, members of the *Arceuthobium* cause major destruction in softwood crops in Canada and the United States, where 90% of the trees in a stand of some species of pine are infected (Hawksworth, 1983; Knutson, 1983).

Not only do these parasites limit the reproductive ability of the host, but they also constitute a danger to the germination of surviving seedlings because the branches of the trees that they kill litter the forest floor (Wanner and Tinnin, 1989).

Kuijt (1969) lists the following criteria for applying the term parasite to a higher plant:

1. The presence of a natural and specialized physiological bridge, the haustorium.
2. The haustorium must be composed in part of living tissue.
3. A flow of nutrients and water from the host to the parasite must take place through this haustorium.

It should be noted that since Kuijt established this definition for a parasitic plant, it has come to light that some species of plants derive nutrients from other plants through mycorrhizal connections. Kuijt's criteria exclude these once removed parasites.

Both species of mistletoe, *Arceuthobium* and *Phoradendron* conform to Kuijt's criterion. The latter is known to be more independent in that it carries on photosynthesis more extensively (Marshall et al., 1994). *Phoradendron* does not extract photosynthate from its host, and its rate of photosynthesis is extremely slow. The photosynthetic rates of the mistletoes, in general, are among the slowest ever measured for C3 plants (Stewart and Press, 1990). Mistletoe was germinated on *Quercus virginiana* in a greenhouse during the course of these studies. However, these efforts were abandoned because of the slow growth rate of *P. serotinum*. The only successful seedling to grow upon germination (N = 15) grew only three cm in one year. Attempts at tissue culture were equally unsuccessful.

Bernhardt (1990) describes mistletoe as having an intimate connection with the host xylem; and he goes onto say that this connection may cut off water to the extremities of the host branches. This statement appears anecdotal with no evidence to support it. In contrast, all the host trees encountered during the course of this study did not have branches which ended abruptly at the site of the mistletoe attachment but appeared to end similarly to branches of the same size which did not support mistletoe (Figure 1.1). Also several attempts to grow *P. serotinum* in tissue culture over a five month period also supports this slow growth characteristic. However, a slow growth rate does not relegate this angiosperm to the state of a photosynthate parasite.

Besides this possible destructive role as a water and nutrient parasite, the role of *P. serotinum* in forest ecology is poorly understood. It has been suggested that mistletoe aids the survival of host species during defoliation, which may result from herbivore attack. Hull and Leonard (1964) hint that mistletoe is known to store nutrients. These stored nutrients might be shared with hosts and permit hosts to withstand defoliation longer than uninfected members of the same species. However, these authors are only speculating and present no experimental evidence to support their speculation.

Hemmerly (1989) quotes Gattinger in 1901 as saying that American mistletoe is parasitic on all deciduous trees. Many authors report over 100 potential hosts for *P. serotinum*. Table 3.1 is a list of hosts reported in the literature. These citations were found by searching several databases including: Agricola, Biosis, Chem. Abstracts, carl.org., and the United States Department of Agriculture Mistletoe Center. It should be noted that one master's thesis by McAlister was not available (McAlister, 1984) and another report by Karalamangala and Nickrent was only available in an abstract. The authors indicate that they collected mistletoe from 30 different host species but they do not mention the specific epithets in the abstract (Karalamangala and Nickrent, 1987).

Table 3.1. Species reported to serve as hosts for *P. serotinum*.

1. *Acacia roermeriana*
2. *Acer pseudoplatanus* (Steyermark 1962)
3. *Acer rubra* (Harris and Harris, 1976; Musselman et al., 1977)
4. *Acer saccharinum* (Mckinney and Hemmerly, 1977; McAlister, 1984)
5. *Acer saccharum* (Mckinney and Hemmerly, 1977; Homoya, 1986)
6. *Aesculus sylvatica* (Henderson and Pekala, 1980)
7. *Algorobia* (Engelmann, 1850)
8. *Betula nigra* L. (Steyermark, 1962)
9. *Carya cordiformis*
10. *Carya glabra* (Mckinney and Hemmerly, 1977)
11. *Carya illinoensis* (Eleuterius, 1976; Henderson and Pekala, 1980; Hemmerly, 1981)
12. *Carya ovata* (Rucker and Hemmerly, 1976; Mckinney and Hemmerly, 1977; Henderson and Pekala, 1980; Hemmerly, 1981; McAlister, 1984)
13. *Carya tomentosa* (Mckinney and Hemmerly, 1977)
14. *Celtis laevigata* (Rucker and Hemmerly, 1976; Mckinney and Hemmerly, 1977)
15. *Celtis mississippiensis*
16. *Celtis occidentalis* L. (Spooner, 1983)
17. *Cercis canadensis* (Mckinney and Hemmerly, 1977)
18. *Cornus florida* (Eleuterius, 1976)
19. *Diospyros virginiana* (Henderson and Pekala, 1980; Hemmerly, 1981)

20. *Fagus grandifolia* (Rucker and Hemmerly 1976)
21. *Fraxinus americana* (Rucker and Hemmerly, 1976; Mckinney and Hemmerly, 1977; Hemmerly, 1981)
22. *Fraxinus caroliniana*
23. *Gleditsia aquatica*
24. *Gleditsia triacanthos* L. (Ridgeway, 1872; Rucker and Hemmerly, 1976; Spooner, 1983)
25. *Juglans nigra* L. (Rucker and Hemmerly, 1976; Mckinney and Hemmerly, 1977; Henderson and Pekala, 1980; Hemmerly, 1981; Spooner, 1983)
26. *Liquidambar styraciflua* (Mckinney and Hemmerly, 1977)
27. *Maclura pomifera* (Rucker and Hemmerly, 1976; Mckinney and Hemmerly 1977; Hemmerly, 1981; McAlister, 1984)
28. *Nyssa aquatica* (Steyermark, 1962; Hemmerly, 1981)
29. *Nyssa sylvatica* (Steyermark, 1962; McAlister, 1984)
30. *Papyrus papyrifera*
31. *Plantanus occidentalis* (Eleuterius, 1976; Rucker and Hemmerly, 1976; Mckinney and Hemmerly, 1977)
32. *Prosopsis juliflora* var. *glandulosa*
33. *Prunus serotina* Ehrh. (Eleuterius, 1976; Hemmerly, 1981; Spooner, 1983)
34. *Quercus alba*
35. *Quercus falcata* (Mckinney and Hemmerly, 1977)
36. *Quercus kelloggi*
37. *Quercus macrocarpa* (Rucker and Hemmerly, 1976)

38. *Quercus marilandica*
39. *Quercus minor*
40. *Quercus muehlenbergii* (Rucker and Hemmerly, 1976; Mckinney and Hemmerly, 1977)
41. *Quercus nigra* (Eleuterius, 1976)
42. *Quercus phellos*
43. *Quercus rubra* (Eleuterius, 1976; Mckinney and Hemmerly, 1977)
44. *Quercus velutina* (Henderson and Pekala, 1980)
45. *Quercus virginiana* (Au, 1974)
46. *Robinia pseudoacacia* L. (Rucker and Hemmerly, 1976; Hemmerly, 1981; Spooner, 1983)
47. *Sapindus marginatus*
48. *Sassafras albidum* (Eleuterius, 1976)
49. *Sorbus aucuparia* (McAlister, 1984)
50. *Toxylon pomiferum*
51. *Tilia americana* (James, 1958)
52. *Ulmus alata* (Rucker and Hemmerly, 1976)
53. *Ulmus americana* (Rucker and Hemmerly, 1976; Mckinney and Hemmerly, 1977; Henderson and Pekala, 1980; Hemmerly, 1981)
54. *Ulmus crassifolia*
55. *Ulmus serotina* (Rucker and Hemmerly, 1976; Hemmerly, 1981)
56. *Ulnus rubra*
57. *Xanthoxylum clava-herculis*

58. *Myrica cerifera* (Stalter, 2003)

While a broad range of hosts is common for mistletoe species in general, it is also common for *P. serotinum* to show a preference for one species in a small geographic area (Stalter, 1971). Nickrent and his coworkers have found Karalamangala attempted to find a genetic explanation for this phenomenon (Karalamangala and Nickrent, 1987). Despite looking at many enzymatic alleles, these authors failed to identify a genetic cause.

The sites in this study support typical James River flora with many anthropomorphic additions, evident in the neatly, evenly spaced rows of some of the older ornamental trees. Ware describes the area around the James River as part of the former long-leaf pine forest, which has become a mixed hardwood forest. He reports that along the James River, the tributaries have extensive flood plains. These areas support oak-hickory associations (Ware et al., 1993).

Presque Isle, was part of the mainland until a channel was dug making it an island. It is now in the James River and is maintained as a National Wildlife Refuge. The flora listed by Parker (1977) on Presque Isle, includes each of the host species identified in this study. *Gleditsia triacanthos* is listed as a field-border tree and rare. *Carya cordiformis* is listed as both river escarpment and a field-border tree and is described as occasional. *Carya illinoensis* is a field border tree and rare. *Acer saccharinum* is a river escarpment tree and described as rare. *Diospyros virginiana* is also found on River escarpments and listed as rare.

### **Materials and Methods**

The two study sites, Carter's Grove and Capital Landing road are the same as those described in Chapter 2. Host taxa were identified using Gleason and Cronquist (1991) and Harlow (1957). Mistletoe concentrations and host taxa were mapped as

described above using the Geo Explorer II in the UTM coordinate system mode. Again, at least three satellites were used to triangulate all positions and the USGS Quad maps described above were also used.

In addition to measurements done on infected trees, measurements were also done on uninfected trees. In an area 30 meters in diameter immediately surrounding an infected host, potential hosts were identified at Carter's Grove. If the potential hosts had a DBH equal to or greater than the minimum DBH of infected hosts, then DBH, height, and UTM coordinates were recorded for each of these uninfected trees.

Core samples were taken from five infected trees. Several core samples were also taken from two uninfected trees. Only *Carya cordiformis* was used for core sampling. These samples were not extensive enough to determine the age of the tree but were used only for comparing annual growth in infected trees to that in uninfected trees. Mistletoe was only collected from two of the cored hosts.

Distances between neighbors were measured using the measuring tool in ARC View GIS, 3.2 (ESRI 1992-1997). The only field measurements of distance done were those employing the GPS, described above.

Statistical analysis was done using JMP (1989-2000) and Statview (1992-1998).

## **Results**

The predominant host species in the entire study (Route 60 in Williamsburg County, York County, James County, and Norfolk County) is *Carya cordiformis* (N = 35). This is followed by *Gleditsia triacanthos* (N=9), *Carya illinoensis* (N=7), and *Diospyros virginiana* (N = 1). However, *P. serotinum* has been known to grow on *Quercus alba* and *Carya ovata* at Carter's Grove. Both *Quercus alba* and *Carya ovata*

were recorded as host trees used for harvesting mistletoe by the chief horticulturist at Carter's Grove (Kelly 2002) and by members of the tree crew. No mistletoe was found infecting the latter two species (*Quercus alba* and *Carya ovata*) during the course of this study.

Both the Colonial Williamsburg Foundation and the City of Williamsburg harvest mistletoe for sale in December. At Carter's Grove the horticulturist has takes note of the host trees used for this purpose but the City of Williamsburg harvests street trees and even those on front lawns with the permission of homeowners, quite liberally and without note.

At the northwestern limit of our study site, mistletoe was growing primarily on *Gleditsia triacanthos* (Figure 3.1). The mean number of mistletoe bushes per *G. triacanthos* host was 6.9 bushes/host. At Carter's Grove the predominant host was *C. cordiformis* (Figure 3.2). The mean number of mistletoe bushes /host for *C. cordiformis* was 16.7 bushes /host. These two sites are less than 10 km apart but both again are on Route 60 (Figure 3.3).

There were a total of 587 mistletoe bushes on 35 *C. cordiformis* hosts. There were 62 bushes on nine *G. triacanthos* hosts. The six *C. illinoensis* hosts supported 127 bushes and had a mean of 21.2 bushes/host. The 10 *A. saccharinum* hosts supported 30 bushes, resulting in a mean of 10 bushes per host and the single *D. virginiana* host supported 22 bushes. The minimum DBH for *Carya cordiformis* hosts was 38.81 cm, for *Acer saccharinum* 48.51 cm, for *Carya illinoensis* 67.91, and for *Gleditsia triachanthos*, 22.0 cm.

**Table 3.2. Descriptive statistics for mistletoe concentration on trees of host species at Carter’s Grove and Capital**

**Landing Road.**

	<i>Acer saccharinum</i>	<i>Carya illinoensis</i>	<i>Gleditsia triacanthos</i>	<i>Carya cordiformis</i>	<i>Diospyros virginiana</i>
<b>100.00% maximum</b>	20	55	21	85	
<b>99.50%</b>	20	55	21	85	
<b>97.50%</b>	20	55	21	85	
<b>90.00%</b>	20	55	21	42.8	
<b>75.00% quartile</b>	20	32.5	13	24	
<b>50.00% median</b>	6	18	3	9	
<b>25.00% quartile</b>	4	6.8	2.5	4	
<b>0.00% minimum</b>	4	3	1	1	
<b>Mean</b>	10	21.2	6.9	16.7	22
<b>Std Dev</b>	8.7	18.5	7.0	18.8	.
<b>Std Err Mean</b>	5.0	7.6	2.3	3.2	.

<b>upper 95% Mean</b>	31.7	40.6	12.3	23.2	.
<b>lower 95% Mean</b>	-11.7	1.7	1.5	10.3	.
<b>N</b>	3	6	9	35	1
<b>Sum</b>	30	127	62	586	
<b>Skewness</b>	1.6	1.4	1.3	1.9	
<b>Kurtosis</b>	.	2.4	0.4	4.3	
<b>CV</b>	87.2	87.5	101.7	112.5	

**Table 3.3. Descriptive statistics for height in meters of mistletoe host trees at Carter’s Grove and Capital Landing Road.**

	<i>Acer saccharinum</i>	<i>Carya illinoensis</i>	<i>Carya cordiformis</i>	<i>Gleditsia triacanthos</i>
<b>100.00% maximum</b>	17.597	24.38	30.479	20
<b>90.00%</b>	17.597	24.38	28.48	20
<b>75.00% quartile</b>	17.597	24.38	24.68	19.5
<b>50.00% median</b>	17.246	24.38	18.679	16
<b>25.00% quartile</b>	16.895	16.079	16.738	12.659
<b>10.00%</b>	16.895	13.312	14.865	8.74
<b>0.00% minimum</b>	16.895	13.312	11.093	8.74
<b>Mean</b>	17.246	21.613	20.298676	15.895333
<b>Std Dev</b>	0.496389	5.534	5.1243565	4.2169024
<b>Std Err Mean</b>	0.351	2.767	0.8788199	1.4056341
<b>upper 95% Mean</b>	21.705806	30.418977	22.086637	19.136763
<b>lower 95% Mean</b>	12.786194	12.807023	18.510716	12.653904

	<i>Acer saccharinum</i>	<i>Carya illinoensis</i>	<i>Carya cordiformis</i>	<i>Gleditsia triacanthos</i>
<b>N</b>	2	4	34	9
<b>Sum</b>	34.492	86.452	690.155	143.058
<b>Skewness</b>	.	-2	0.4744831	-1.014452
<b>Kurtosis</b>	.	4	-0.802384	-0.189337
<b>CV</b>	2.8782846	25.60496	25.244781	26.529185

**Table 3.4 Descriptive statistics for basal area (cm<sup>2</sup>) of host trees at Capital Landing Road and at Carter's Grove.**

	<i>Acer saccharinum</i>	<i>Carya cordiformis</i>	<i>Carya illinoensis</i>	<i>Gleditsia triacanthos</i>
<b>100% maximum</b>	4349.9	12012.	14483.	754.4
<b>90.00%</b>	4349.9	8464.	14483.	754.4
<b>75% quartile</b>	4349.9	7386.	12993.	747.6
<b>50% median</b>	1847.3	4728.	4692.	572.3
<b>25.00%</b>	1847.3	2298.	3845.	433.7
<b>0% minimum</b>	1847.3	1182.	3620.	379.9
<b>Mean</b>	2681.496	4753.645	7317.132	576.287
<b>Std Dev</b>	1444.908	2726.518	4840.933	155.614
<b>Std Err Mean</b>	0834.218	460.866	1976.303	51.871
<b>upper 95% Mean</b>	6270.885	5690.231	2397.306	695.904
<b>lower 95% Mean</b>	-0907.89	3817.06	2236.958	456.67
<b>N</b>	3	35	6	9

	<i>Acer saccharinum</i>	<i>Carya cordiformis</i>	<i>Carya illinoensis</i>	<i>Gleditsia triacanthos</i>
<b>Sum Wgts</b>	3	35	6	9
<b>Sum</b>	8044.487	166377.59	43902.794	5186.582
<b>Variance</b>	208.776	743.39	2343.463	2.422
<b>Skewness</b>	17320.508	5711.17	1.0073.84	377.685
<b>Kurtosis</b>	.	-2547.19	-14426.11	-20861.67
<b>CV</b>	538843.93	573563.57	661588.81	270028.9

**Table 3.5. Descriptive statistics for DBH (cm) of host trees at Capital Landing Road and at Carter's Grove.**

	<i>Acer saccharinum</i>	<i>Carya cordiformis</i>	<i>Carya illinoensis</i>	<i>Gleditsia triacanthos</i>
<b>100% maximum</b>	<b>74.44</b>	<b>123.70</b>	<b>135.83.</b>	<b>31.00</b>
<b>90.00%</b>	<b>74.44</b>	<b>135.83</b>	<b>135.83</b>	<b>31.00</b>
<b>75% quartile</b>	<b>74.44</b>	<b>103.81</b>	<b>128.59</b>	<b>30.86</b>
<b>50% median</b>	<b>48.51</b>	<b>77.61</b>	<b>77.31</b>	<b>27.00</b>
<b>25.00%</b>	<b>48.51</b>	<b>54.11</b>	<b>69.91</b>	<b>23.50</b>
<b>0% minimum</b>	<b>48.51</b>	<b>38.81</b>	<b>69.91</b>	<b>22.00</b>
<b>Mean</b>	<b>57.15</b>	<b>74.51</b>	<b>92.53</b>	<b>26.877</b>
<b>Std Dev</b>	<b>14.97</b>	<b>22.74</b>	<b>30.18</b>	<b>3.694</b>
<b>Std Err Mean</b>	<b>8.648</b>	<b>3.84</b>	<b>12.32</b>	<b>1.23</b>
<b>upper 95% Mean</b>	<b>94.34</b>	<b>82.33</b>	<b>124.20</b>	<b>29.71</b>
<b>lower 95% Mean</b>	<b>-19.96</b>	<b>66.70</b>	<b>60.85</b>	<b>24.03</b>
<b>N</b>	<b>3</b>	<b>35</b>	<b>6</b>	<b>9</b>

	<i>Acer saccharinum</i>	<i>Carya cordiformis</i>	<i>Carya illinoensis</i>	<i>Gleditsia triacanthos</i>
<b>Sum Wgts</b>	3	35	6	9
<b>Sum</b>	8044.487	166377.59	43902.794	241.83
<b>Variance</b>	208.776	743.39	2343.463	13.64
<b>Skewness</b>	17320.508	5711.17	1.0073.84	-0.04
<b>Kurtosis</b>	.	-2547.19	-14426.11	-2.05
<b>CV</b>	538843.93	573563.57	661588.81	13.75

## Conclusion

Despite the many references arguing for regional host preference in the literature (Hemmerly, 1981; Anderson and Mundy, 1980; Henderson and Pekala, 1980; Kuijt, 1979), there is a lack of proof. Identifying UTM coordinates for each host tree with GPS and mapping these positions makes it possible to measure regional host preferences.

At the northwestern limits of the study site, *Gleditsia triacanthos* is the preferred host (Figure 3.1). Nine small *G. triacanthos* hosts support 62 mistletoe bushes. These hosts are in close proximity to one another. In the southeastern limits, *Carya cordiformis* is the preferred host (Figure 3.2). On the fringes of both sites, there is a mixture of all the species. The literature offers no clear explanation of this phenomenon. Thus far attempts to demonstrate genetic divergence have not been fruitful.

Mize (1985) observed that mistletoe often becomes established in areas where birds will find other berries such as holly. Migrating species can easily spot the mistletoe as they fly over the barren canopy in winter. The birds have established their food source along their flight path.

Many of the 58 species, which are listed in the literature are present along route 60. *Carya ovata* and *Quercus* are found at both Carter's Grove and the Capital Landing Road sites. Yet, *Carya cordiformis* was the most widely parasitized host. Not one host belonging to the genus *Quercus* was found and there was no evidence of *Carya ovata* serving as a host.

The absence of parasitized *Quercus* and *Carya ovata* is partially accounted for by over-harvesting. The *G. triacanthos* might have supported more mistletoe bushes but is probably also over-harvested. There is no way to correct for harvesting.

There was no overall effect of basal area or height on mistletoe concentration within species. However, there does seem to be a minimum size before a tree is infected and this diameter is dependent on the species. It is not clear whether this is due to genetics or to the vector again.

The literature emphasizes that the birds are actively seeking out the mistletoe and not hosts. While they might fly to a tree because it is heavily infected, they will not choose a larger oak tree over one that is slightly smaller. However, it has been well established that birds establish patterns in food seeking habits and it is safe to conjecture that they might seek a particular tree species in which to look for food. Further, once having established a food source, it is logical that they might choose other trees of the same species in their search for food.

Future efforts should be directed toward documenting the coordinates of potential hosts, which remain mistletoe free and identifying these species and learning more about the feeding habits of the birds which act as vectors.

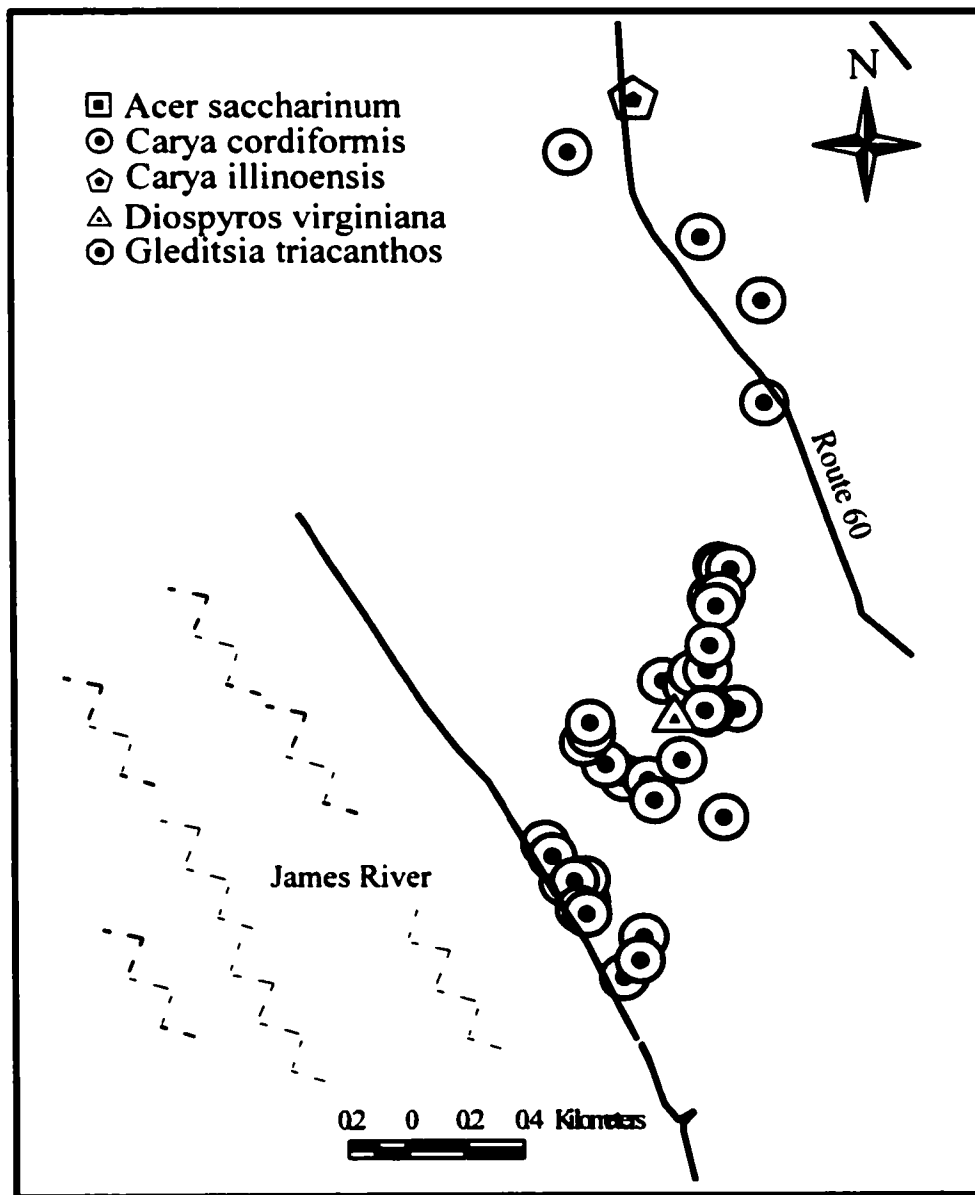


Figure 3.1. *Carya cordiformis* is the predominant host at the Carter's Grove site.

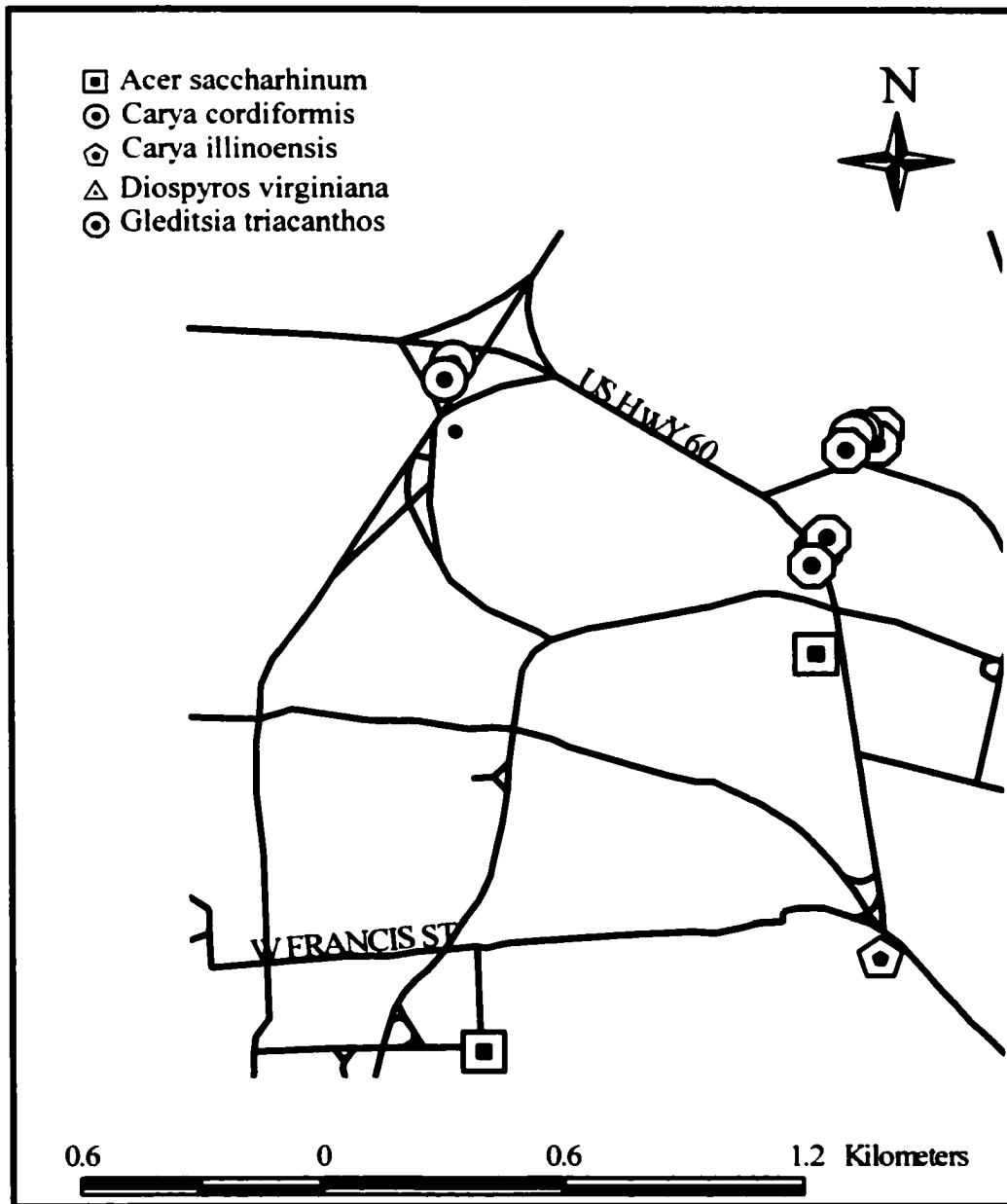


Figure 3.2. *Gleditsia triacanthos* is the predominant host species at the Capital Landing Road Site.



**Figure 3.3.** There is a distance of 9.59 km between the concentration of *Gleditsia triacanthos* hosts at the Capital Landing Road in the north and the concentration of *Carya cordiformis* hosts at Carter's Grove site in the south.

Figure 3.4a. Frequency of mistletoe bushes per *Carya cordiformis* host, N= 35 (log normal). KSL Goodness of Fit Test (D = 0.100043; P = 0.1500)

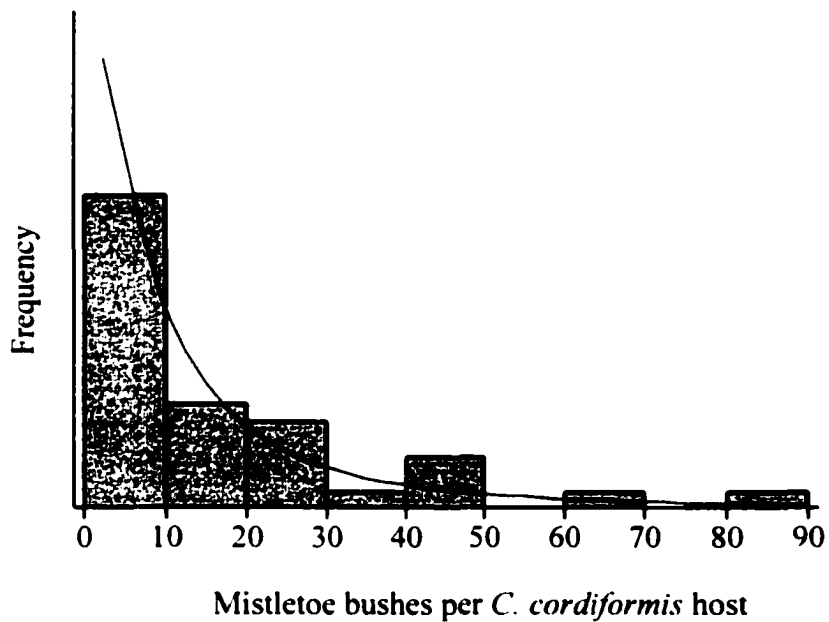


Figure 3.4b. Frequency of mistletoe bushes per *Gleditsia triacanthos* host, N=9 (log normal). KSL Goodness of Fit Test (D = 0.312956; P = 0.0140).

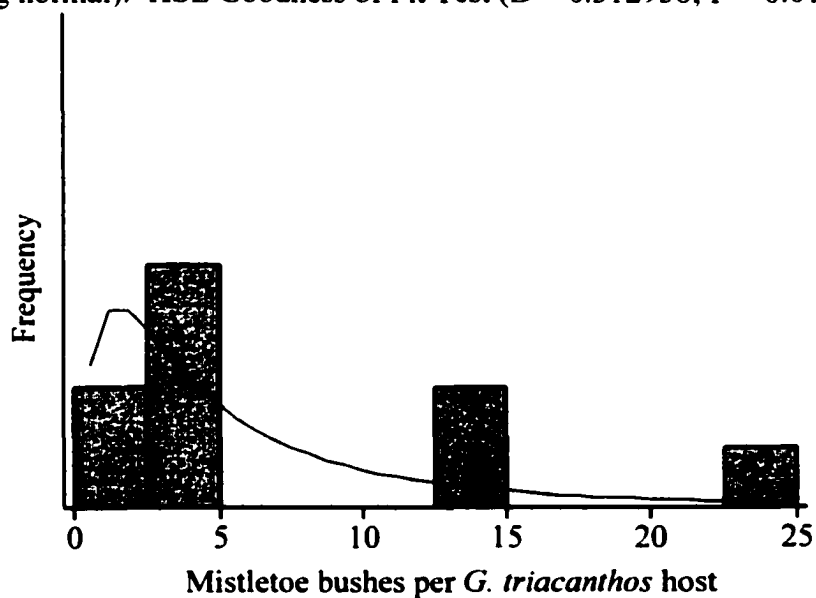


Figure 3.5. Frequency of mistletoe bushes per *Carya illinoensis* host (log normal). KSL Goodness of Fit Test ( $D = 0.154926$ ;  $P = 0.1500$ ).

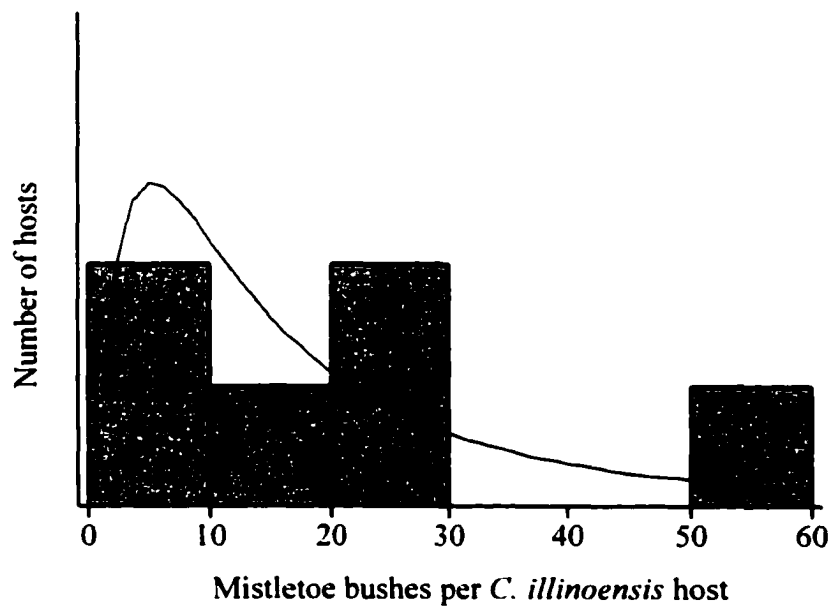


Figure 3.6a. The effect of basal area on the number of mistletoe bushes per *Carya cordiformis* host. Nominal Logistic Regression ( $P = 0.01$ ).

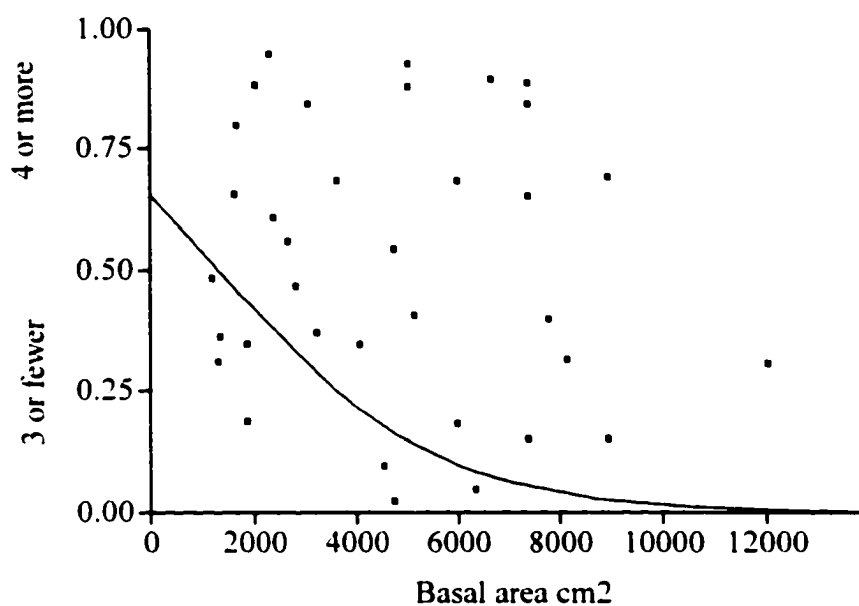


Figure 3.6a. The effect of height on the number of mistletoe bushes per *Carya cordiformis* host. Nominal Logistic Regression ( $P = 0.004$ ).

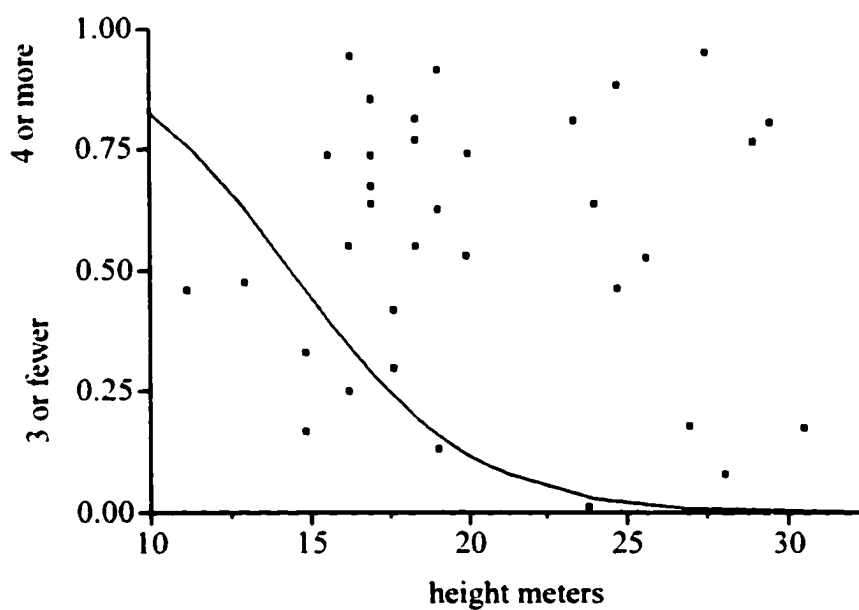


Figure 3.7. The effect of DBH on the number of mistletoe bushes per *Carya cordiformis* host. Nominal Logistic Regression ( $P = 0.01$ ).

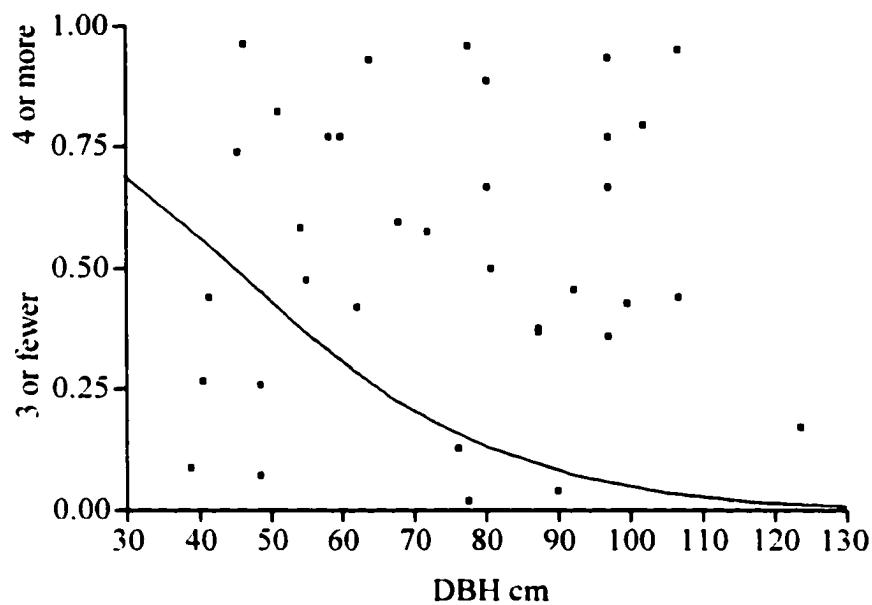


Figure 3.8a. The effect of basal area  $\text{cm}^2$  on the number of mistletoe bushes per *Gleditsia triacanthos* host. Nominal logistic Regression ( $P = 0.25$ ).

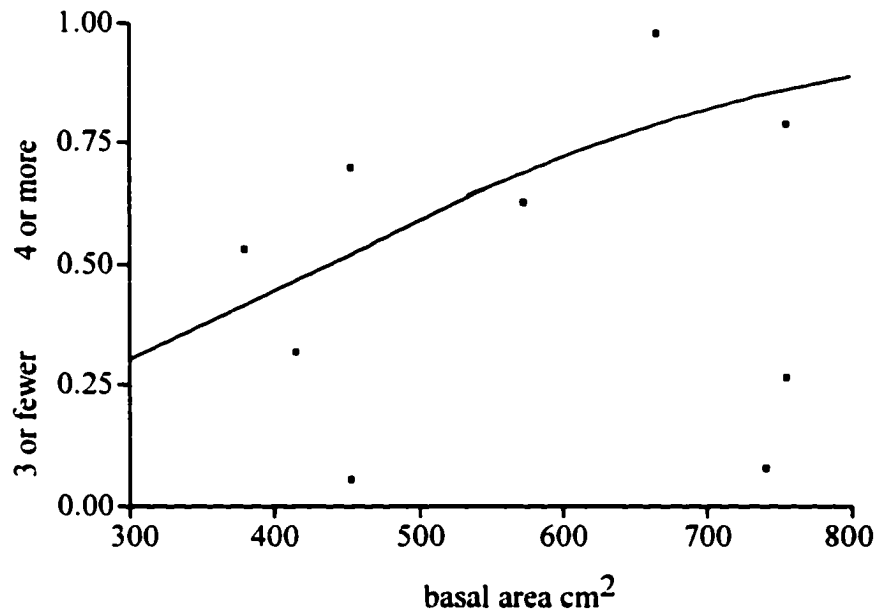


Figure 3.8b. The effect of height on the number of mistletoe bushes per *Gleditsia triacanthos* host. Nominal logistic Regression ( $P = 0.55$ ).

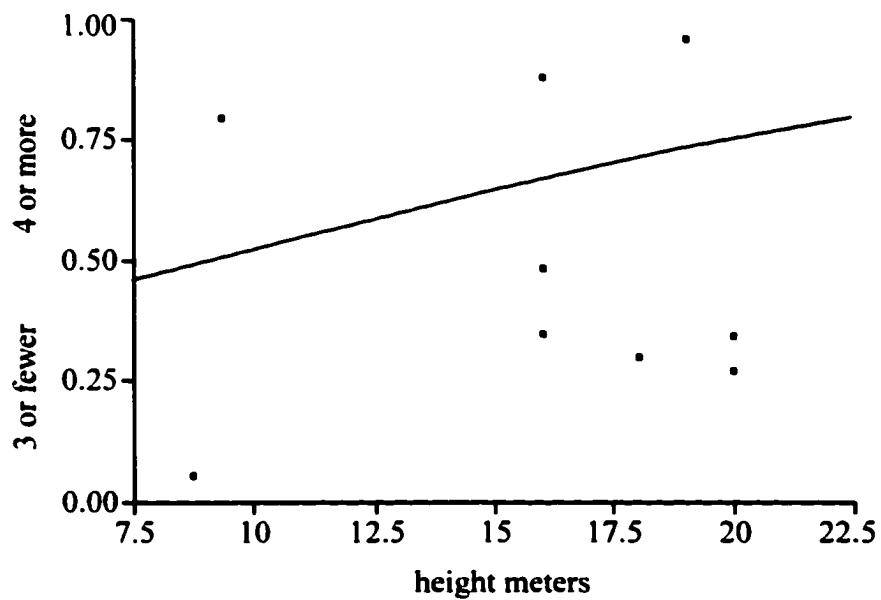


Figure 3.9. The effect of DBH on the number of mistletoe bushes per *Gleditsia triacanthos* host. Nominal Regression ( $P = 0.25$ ).

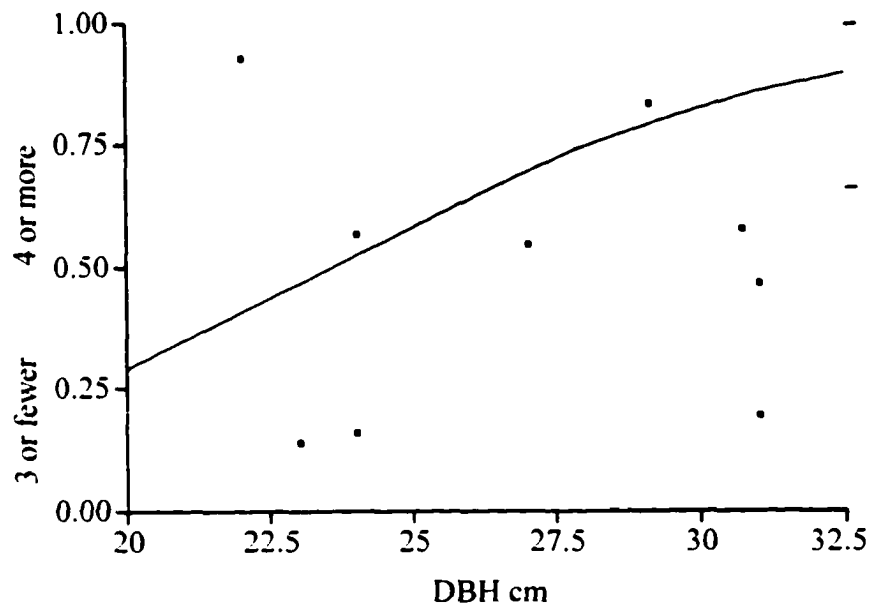


Figure 3.10a. The effect of basal area on the number of mistletoe bushes per *Carya illinoensis* host. Nominal Logistic Regression ( $P = 0.02$ ).

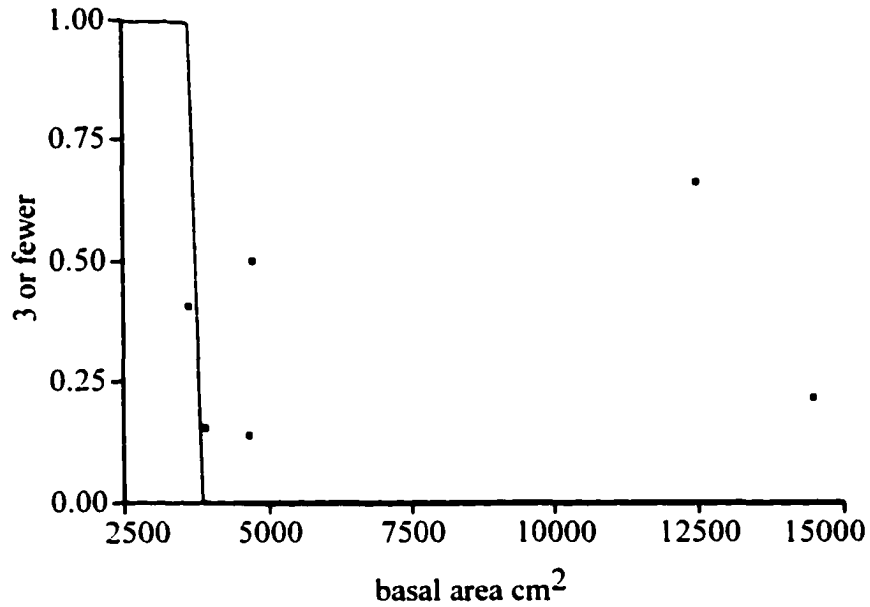


Figure 3.10a. The effect of height (m) on the number of mistletoe bushes per *Carya illinoensis* host. Nominal Logistic Regression ( $P = 0.02$ ).

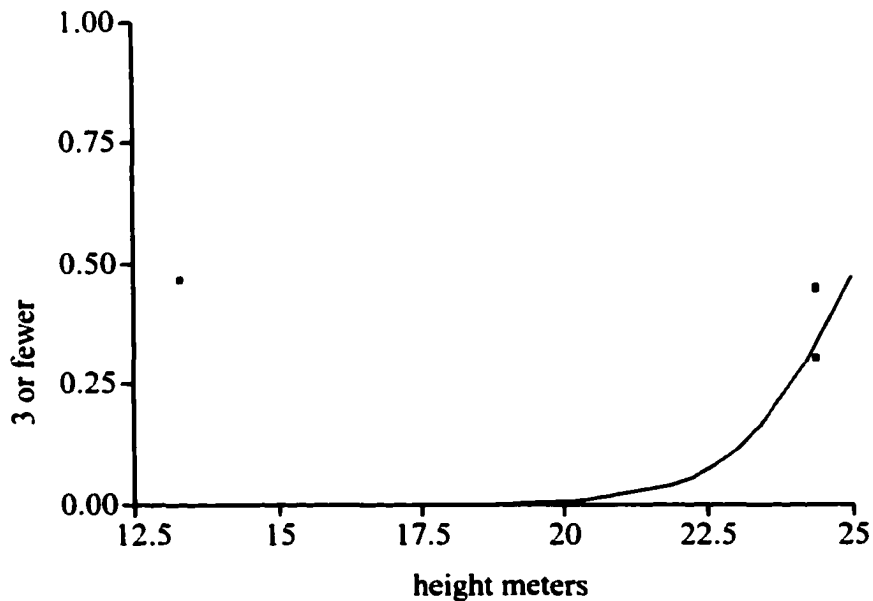
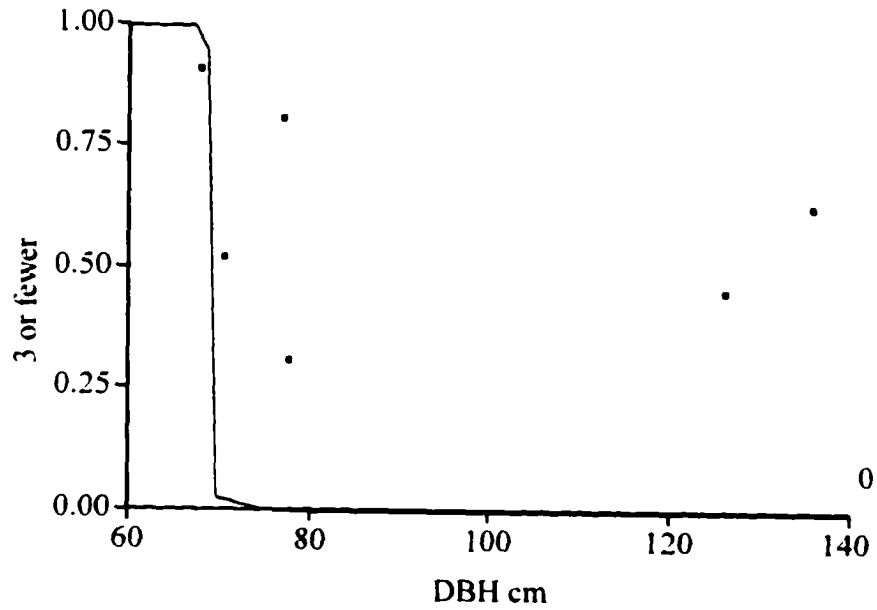


Figure 3.11. The effect of basal area on the number of mistletoe bushes per *Carya illinoensis* host. Nominal Logistic Regression ( $P = 0.02$ ).



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## **Chapter 4. Secondary Metabolites: protection and defense**

### **Introduction**

Not all the romantic myths about mistletoe depict it as evil. Keats refers to mistletoe in *Endymion* as being capable of warding off evil forest spirits. Although the “spiritual” potency Keats describes has not been conclusively quantified, it is possible that other protective relationships involving mistletoe may exist. Several areas have been investigated, among them allelopathy and anti-insect feedant activity.

Although potential destructive roles for mistletoe have been well characterized, other roles in forest ecology remain poorly understood. It has been suggested that mistletoe may assist the survival of host species during defoliation subsequent to insect attack. Hull and Leonard (1964) note that mistletoe is known to store nutrients and they intimate that the parasites may share these nutrients with hosts. This would enable the hosts to withstand defoliation longer than uninfected members of the same species. Their conjecture is not based on experimental evidence. Although intriguing, there is no data in the literature that supports this theory.

Field observations and samples collected at Carter’s Grove and Williamsburg, Virginia may support the anti-insect feedant properties of *Phoradendron seritonum*. In the process of harvesting mistletoe, host branches must be collected with the mistletoe. Harvesting methods are discussed below. Several hosts with insect damage have been observed, but amidst the 10 kg (wet mass) of mistletoe collected to date, from bushes growing on the herbivore damaged hosts, fewer than twenty mistletoe leaves have shown serious herbivore damage. The production of secondary metabolites by *P. seritonum* might be responsible. This observation and a reference in the literature by Hartzell (1947)

that acetone extracts of the leaves of American Christmas mistletoe were toxic to mosquito larvae, (*Culex quinquefasciatus*) led to the study described below of the production of secondary metabolites, which may act as deterrents to herbivores.

Humans have long recognized the capacity for plants to interfere with the growth and development of other plants. Theophrastus and Pliny noted that certain plants “exhausted” the ground. Although, the phenomena was recognized by Candolle, it was not until the early 1900s that research on allelopathy began. Schreiner showed that monocropping without crop rotation is detrimental to the growth of crops not because the soil is exhausted but because allelochemicals are exuded which result in a poisoning of the soil (Rice, 1974).

A field observation of *P. serotinum* suggested that allelopathy might occur. Specifically, the lack of new growth under host trees with heavy mistletoe infestation was observed. In one instance, a tree heavily laden with *P. serotinum* that had fallen during the ice storm of 1998 at the Carter’s Grove Site was observed to have a distinct lack of spring growth beneath the fallen crown in subsequent months. This storm sheared off a great deal of mistletoe along with host branches (Figure 4.1). The storm brought down many trees and did severe damage to the forests of the southeastern Coastal Plain (Elstner and Ware, 2001).

Although field observations suggested that allelopathy may have been responsible, unless quantified they can only result in anecdotal evidence. This led to a study of allelopathy under controlled conditions.

Inderjit and Weston (2000) list several areas of investigation required to characterize the ecological significance of allelopathy:

1. Aqueous media should be used to extract the secondary metabolites.
2. Soil from the geographical area of the aggressive plant should be used.
3. Species representative of natural conditions should be used.
4. Dose dependent response must be demonstrated.

The authors identify other criteria which should be met for an understanding of the mechanisms involved in allelopathic activity:

1. An ecological proof that the effect exists in the field.
2. Chemical characterization of the active compound/s.
3. Physiological identification of the mode of activity.
4. The response of the whole homogenate should be compared to that of any isolated compounds known to cause an allelopathic effect. This will help to establish the presence of synergistic effects.

The investigations described below meet three of Inderjit and Weston's (2000) requirements. Aqueous extracts are compared to methanolic extracts, during studies of dose dependency. Soil, collected from the study sites was used in experiments. However, seeds that are representative of the species which grow in Williamsburg were not used, nor have any of the active compounds been isolated.

The term, allelopathy is often defined in its broadest sense, i.e. the ability of plants to communicate with other organisms in the environment through the release of secondary metabolites. In the experiments described below, a very narrow working definition, the ability of a plant to produce and release compounds which interfere with the growth and development of other plants, was employed. This definition was adapted from Einhellig (1995). This same author takes care to point out that this definition does

not rule out synergy, stating that “almost all cases of allelopathic inhibition in a plant community result from the combined effects of several compounds” (Einhellig, 1995).

Allelopathic effects may be direct or indirect. They are direct when a plant competitor itself is affected, but considered indirect when the effect is on microorganisms that the competitor needs to complete their growth cycles. We therefore did an axenic study, and also looked at the effect of extract on four bacterial cultures, *Escherichia coli*, *Candida albicans*, *Staphalococcus aureus*, and *Enterococcus faecalis*<sup>1</sup>.

In the rich ethnobotanical literature available on the use of mistletoe, we know that different hosts appear to affect the production of a different complement of secondary metabolites. Pliny (Pliny ) refers to differences in poison found in mistletoe depending upon the host species. He goes onto say that the bitter, sticky leaves and berries give off different odors based on host species. Although Pliny was referring to *Viscum album*, a common European species, the American species is also bitter and sticky. Reports in the ethnobotanical literature indicate that production of secondary metabolites differs by host. Therefore, we compared the allelopathic effects of mistletoe from three species of hosts, *Gleditisa triacanthos*, *Acer saccharinum*, and *Carya cordiformis*.

## **Materials and Methods**

### **Harvesting**

A total of six collections of mistletoe from three species of hosts were harvested. Four collections were completed with the aid of the Colonial Williamsburg tree crew, their cherry picker, and their chain saw (Figure 4.2). These four collections were from

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<sup>1</sup>American Type Culture Collection 25922, 60193, 25923, 29212, respectively.

*Acer saccharinum* and *Carya cordiformis* hosts. Two collections were completed with the aid of a ladder and a tree pruner. These two collections were from *Gleditsia triacanthos* hosts, which were considerably shorter than the *A. saccharinum* and *C. cordiformis*.

The host branch was cut at least 300 cm proximal to the haustoria. The branch with attached mistletoe bush was submerged in water and remained in water until all mistletoe was removed. The mistletoe was frozen at -20° C within 12 hours of having been cut.

### **Allelopathy**

#### **Experiment 1. Comparison of several plant species to mistletoe.**

To compare allelopathy of *P. serotinum* to other species, homogenates of the known allelopaths, Pokeweed, *Phytolacca americana*, marigold *Tagetes erecta* L., and kale, *Brassica rapa* L. were prepared and used to test the effect on the germination of millet, *Sorghum vulgare* Pers. and alfafa, *Medicago sativa* L. *P. serotinum* was collected from *G. triacanthos* host at the Capital Landing Rd. site in Williamsburg, Virginia.

Ninety grams of leaves from each species were homogenized in a blender in 150 mL of MeOH. For *P. americana*, the leaves and the berries (10 g) were used separately. Two grams of *P. serotinum* berries were used because they are rare and difficult to find. Birds, which eat the berries rich in protein, had a faster and more efficient harvesting technique. Only leaves were used from all other plants. The homogenate was filtered through Whatman #1 Paper. Ten mL of the filtrate was put into each of four petri dishes containing Whatman #1 filter paper inserts. The paper was dried at 40° C. Two days later, 1.5 mL of deionized water was added to each of the dishes. 100 seeds of sorghum

were added to triplicate dishes for each of the potential allelopaths. The same was done for the alfafa. Equal aliquots of water were added to each dish, daily, to prevent the seeds from drying out. The seeds were kept at room temperature for three days. Germinated and ungerminated seeds were then counted.

### **Experiment 2. Comparison of mistletoe from three different host species.**

The host species, *Carya cordiformis* (hickory), *Gleditsia triacanthos* (honey locust), and *Acer saccharinum* (silver maple) were used. One-gram aliquots of mistletoe leaves were removed from samples stored at -20° C. The leaves were dipped into 90% methanol, 15% hypochlorite and then washed with water. They were then divided into 0.5g aliquots frozen in liquid nitrogen, and ground using a mortar and a pestle. Six homogenates were created, by resuspending two ground mistletoe aliquots from each of the three hosts in H<sub>2</sub>O and methanol (0.5g/10mL of solvent). The samples were centrifuged for 15 minutes and the supernatants were filtered on Whatman #1. The filtrates were dried and stored at -20° C until needed. Samples were never stored for more than 12 hours.

Under axenic conditions petri dishes (60x15 mm) were lined with Whatman Filter Paper (55mm). The previously centrifuged homogenates were removed from the freezer and resuspended in sterile distilled water or MeOH and sterilized by filtration. Aliquots of each extract, water and MeOH (*C. cordiformis*, *G. triacanthos*, *A. saccharinum*) 0.25 mL, 0.5 mL, 1.0 mL, 2.0 mL were sterilized by filtration and dispensed onto the filter paper. Juglone, (from Sigma) an identified allelopathic secondary metabolite of *Juglans nigra* was also sterilized by filtration and used as a positive standard. It was used at concentrations of 5 mg/mL, 2.5 mg/mL, 1 mg/mL, 0.75 mL of 1mg/mL, 0.5 mL of

1mg/mL, and 0.25 mL of 1mg /mL. Water was used as a negative standard. Warming the petri dishes to 40° C dried the filter paper.

Lettuce seeds (Plant Hart's Seeds) were used for the experimental model. To prevent the growth of microbes and avoid the confounding that such infection might cause, we sterilized the lettuce seeds. Seeds were immersed in 90% ethanol for one minute, then transferred to 15% hypochlorite (Clorox), for 5 minutes. This was followed by two rinses in distilled sterile H<sub>2</sub>O.

1.5 mL of H<sub>2</sub>O was added to each dish and 13 lettuce seeds were placed on each dish. A template was used to ensure that the seeds remained equidistant on the petri dishes. The controls were well developed on Day 3 and measurements were taken. Statistical analysis was performed using the JMP statistical program (1989-2000).

### **Experiment 3. Allelopathy using soil from Williamsburg as the growth media.**

Soil was collected from the Carter's Grove site in the spring of 1998 from under the crowns of infected and uninfected trees. The soil from under uninfected trees was used as a negative control. About 200 mL of soil was placed in a collection box. In the laboratory the soil was sieved to remove rocks larger than 1 cm in diameter and dried at 40° C to remove excess moisture and maintain uniform water content during the germination period.

Petri dishes (100 mm x 15 mm) were filled with the dried soil and weighed. Twenty-five lettuce seeds (Plant Hart's seeds) were evenly distributed on top of each dish and lightly covered with soil. Each dish was then weighed. Five grams of distilled water was added to each petri dish. The dishes were weighed on day 2, day 4, and day 6 and brought back up to the weight recorded after 5 g of distilled water had been added on day

0. On Day 7 the number of seeds, which had germinated were counted and the length of each seedling was measured from hypocotyl to epicotyl. Results were analyzed using StatView (1992-1998)

### **Antimicrobial Experiments**

#### **Experiment 4. A bioassay to determine antimicrobial activity against *Candida*, *Staphaloccus*, *Enterococcus*, and *Escherichia coli*.**

One hundred grams of mistletoe leaves were extracted in MeOH. The extract was dried on a flash evaporator and resuspended in 10 mL of MeOH. Three petri dishes containing the appropriate medium in agar were inoculated with cultures of *Candida*, *Staphaloccus*, *Enterococcus*, and *Escherichia coli*. Discs containing sterilized extracts of mistletoe or the positive control, Gramicidin (an antibiotic obtained from Sigma) were applied to the plates.

### **Herbivore Deterrents.**

#### **Experiment 5. Insect anti-feedant activity.**

A bioassay to determine insect deterrent efficacy was developed. When possible, second and third instar larvae of *Spodoptera eridania*, Southern army worm, were obtained from Dr. Mark Kao of American Cyanamid, Princeton, New Jersey. These larvae were grown in cages in the laboratory (Figures 4.3-4.6).

The larvae lived on *Brassica rapa*, bok-choy and the adult moths were fed sugar water. Southern army worms are commonly used in anti-feedant bioassays (Kao et al., 1995). The larvae were maintained on a diet of *Brassica rapa*, until they reached the fourth instar stage (Figures 4.3-4.6), at which time they were used in the experiments described below.

Several experiments were done to develop the assay. In the first experiment the larvae were fed only whole leaves after a three-hour period of food deprivation. Control larvae were similarly fed. A variety of feeding media and schedules were tried to maximize weight gain of the larvae. During the course of these experiments the homogenate was tested with and without cutin removal (Martin and Juniper, 1970; Kao 1997) to determine if the wax content was a confounding variable. The results showed no significant differences. However, as a precaution the cutin was always removed.

Two trips were necessary to collect the mistletoe used in these studies because the first samples collected were covered with an epiphytic fungus (Figure 1.9). The second host, from which mistletoe was harvested, however, was free from these epiphytic hyphae. This second host, which supported the fungally infected *P. serotinum* was *Carya cordiformis* and the host, which bore mistletoe free of fungus was *Gleditsia triacanthos* L., a honey locust tree. Both trees were heavily infected with mistletoe. Interestingly, the mistletoe extract from the plant infected with fungus was more potent than the mistletoe that was free of the fungus.

Different plant insults are known to stimulate the production of different sets of protective secondary metabolites. However, there was no way to know if the fungus was producing the substances that prevented the larvae from feeding, so the infected mistletoe was not used in the anti-insect feedant assays.

Mistletoe collected in the spring of 1997 from *Gleditsia triacanthos* was homogenized in MeOH and flash evaporated. The homogenate was applied to a polyamide column and eluted with a discontinuous gradient described in Table 4.1 below.

Table 4.1. Column Chromatography Gradient.

Fraction #	Eluent
1-3	H <sub>2</sub> O
4-5	H <sub>2</sub> O: MeOH 60:40
6-7	H <sub>2</sub> O: MeOH 80:20
8-9	100% MeOH
10-13	100% MeOH and 0.05% NH <sub>4</sub> OH
14-15	100% MeOH and 0.10 % NH <sub>4</sub> OH

Fractions from the gradient were collected, flash evaporated and resuspended in MeOH to a concentration of 10 mg/mL. The bok choy leaves were painted with the extract. Two mL of extract were used for every 5 grams of bok choy. An effort was made to coat the leaves evenly. Plain methanol served as a control. No positive controls were used.

#### **Experiment 6. Insecticidal activity.**

A bioassay to test insecticidal activity against mosquito larvae (*Aesculus aegypti*) was attempted using whole homogenate. A standard methanolic extract was used. The homogenate proved entirely ineffective in killing the larvae. Hartzell does not described a hexane extraction to remove cutin in his study (Hartzell 1947). However, to prevent the cutin from interfering with the activity of any secondary metabolites, the hexane extraction was done as described above.

## **Results**

### **Allelopathy**

#### **Experiment 1. Comparison of several plant species.**

In preliminary experiments we found crude aqueous and MeOH extracts of mistletoe leaves to have as much allelopathic effect on lettuce seed germination trials as pokeweed (*Phytolacca americana*). Pokeweed is a weedy species of the eastern United States that is in clinical trials for its strong antiviral (including HIV) and anti-cancer properties. All of the plants used were more allelopathic than the controls.

*Phoradendron serotinum* proved to be as allelopathic as *Phytolacca americana*. The berry had the same effect as the leaves (Figure 4.7 and Table 4.2).

Table 4.2. Percent ungerminated seeds after 72 hours of incubation.

Seed	Control	<i>B. rapa</i>	<i>T. erecta</i>	<i>P. americana</i>	<i>P. americana</i>	<i>P. americana</i>	<i>P. seritonum</i>	<i>P. seritonum</i>
				berries	leaves	berries	leaves	leaves
<i>S. vulgare</i>	17.0	46.0	33.5	92.5	92.5	91.1	90.0	90.0
	N=100	N=100	N=100	N=99	N=98	N=20	N=100	N=100
<i>M. sativa</i>	26.5	16.5	32.0	93.5	90.0	88.3	94.0	94.0
	N=96	N=100	N=99	N=100	N=100	N=20	N=97	N=97

**Experiment 2. Comparison of allelopathy in mistletoe from three different host species.**

Analysis of variance showed significant differences between controls and the mistletoe for both water and methanol extraction,  $P = 0.0001$  for each method. Pairwise comparison (Tukey-Kramer HSD,  $\alpha = 0.05$ ) showed no significant differences between hosts or extraction techniques, i.e.  $H_2O$  and methanol (Figures 4.8-4,11 and Table 4.3).

**Experiment 3. Allelopathy using soil samples**

Although our data do not exhibit dramatic effects when soil from the study site is introduced as a growing media, allelopathy is still demonstrated. There are significant differences as shown by ANOVA ( $P = 0.0001$ ) demonstrating allelopathy when soil from under a tree with heavy mistletoe infestation (90 plants) is used as the growth media for lettuce seeds. Regression analysis shows a downward trend of the mean even with a 95% confidence interval. Pairwise tests (Tukey Kramer) show significant differences between all classes of soil with mistletoe bushes overhead and soil taken from under a tree with no mistletoe bushes (Figure 4.12 and Table 4.4). There is no difference noted between the tree with 23 bushes and the tree with 29 bushes. There is also no difference between the tree with 47 bushes and the tree with 90 bushes.

**Table 4.3. Analysis of variance for the effects of mistletoe from three different host species on seedling growth.**

**R square 0.06261**

<b>Source</b>	<b>DF</b>	<b>Sum of Squares</b>	<b>Mean Square</b>	<b>F Ratio</b>
<b>mistletoe concentration</b>	<b>4</b>	<b>15772.68</b>	<b>3943.17</b>	<b>21.7737</b>
<b>Error</b>	<b>1304</b>	<b>236151.67</b>	<b>181.1</b>	
<b>Total</b>	<b>1308</b>	<b>251924.36</b>		

**Table 4.4. Comparisons of soil samples effect on seedling growth for all pairs using Tukey Kramer HSD,  $\alpha = 0.05$   $q^* = 2.73163$ . Positive values indicate a significant difference.**

	0	29	23	47	90
0	-8.2199	4.556	5.4085	9.2204	10.5977
29	4.556	-2.7063	-1.8458	1.5639	3.3292
23	5.4085	-1.8458	-2.6775	0.7289	2.4974
47	9.2204	1.5639	0.7289	-4.1099	-2.4852
90	10.5977	3.3292	2.4974	-2.4852	-2.7286

## **Anti-Microbial Experiment**

### **Experiment 4. A bioassay to determine antimicrobial activity against *Candida*, *Staphaloccus*, *Enterococcus*, and *Escherichia coli***

We found only a weak anti-bacterial effect against *E. coli*. The effect in the crude extracts was only 25% of the effect found with the positive control. Further attempts to enhance the effect through purification failed. (Refer to description of column chromatography in the anti-insect feedant study below.) Two of the fifteen fractions showed positive but weak anti-microbial activity. The effect was only about 5 % reduced from that of the control in one fraction and 5% less in the other.

### **Experiment 5. Insect anti-feedant activity**

In the whole leaf experiment, there was negligible herbivory on the mistletoe leaves by *Spdoptera eridania* larvae, while the mulberry was consumed completely. Changes in feeding schedule and media quickly led us to the conditions described above. We found bok choy to be preferred food, as the fourth instars consumed it at a faster rate (when measured in grams eaten) than any other media including artificial media provided by Dr. Kao. After attempting several different feeding schedules, we found a two hour period of starvation followed by a 5 hour feeding frenzy, gave us measurable results.

All fractions from the polyamide column exhibited activity, resulting in a slower feeding rate of the fourth instar larvae as compared to the controls. Fraction 15 in particular was more active than the others (Figure 4.13). Preparative HPLC failed to isolate enough of any one compound to identify a specific active compound.

### **Experiment 6. Mosquito larvae lethality assay.**

Despite several attempts to kill mosquito larvae with mistletoe, they all appeared to be undergoing normal development. There were no differences between the controls and the larvae exposed to mistletoe extract at 10,000 ppm.

### **Conclusions**

While the data support the possibility that allelopathy is responsible for effects observed in the field, other explanations are possible. Although falling boughs are credited with preventing new growth, and allelopathy effects are only one of several possible mechanisms. As previously noted, observations and sample collections were taken after the ice storm of 1998 (Elstner and Ware, 2001). This storm was devastating and resulted in the destruction of many trees, including those with mistletoe at both the Carter's Grove site and at the Capital Landing Road site. The tree referred to with 47 mistletoe bushes growing in the crown was a much older tree with a DBH of 80 cm and a height of 27 m. This age difference may explain why soil from under this tree demonstrated an allelopathic effect that was so close to that of the soil taken from under the tree with 90 genets.

To further verify the results, the experiments described above will have to be repeated with seeds gathered from the field. Soil pH should be monitored. It is known that *P. serotinum* homogenates in water may have a pH as low as 3.8 and as high as 4.6. While under field conditions the acid could be expected to wash away quickly, in a petri dish it could be responsible for allelopathic effects, even when a methanol extraction is performed. When the extract is resuspended the pH must be checked to account for this potential confounder.

The anti-insect feedant activity appeared to be synergistic and no further attempts were made to isolate the compounds responsible. Activity was exhibited by all fractions in the column and when these activities were added together, they could not account for the activity found in the whole homogenate.

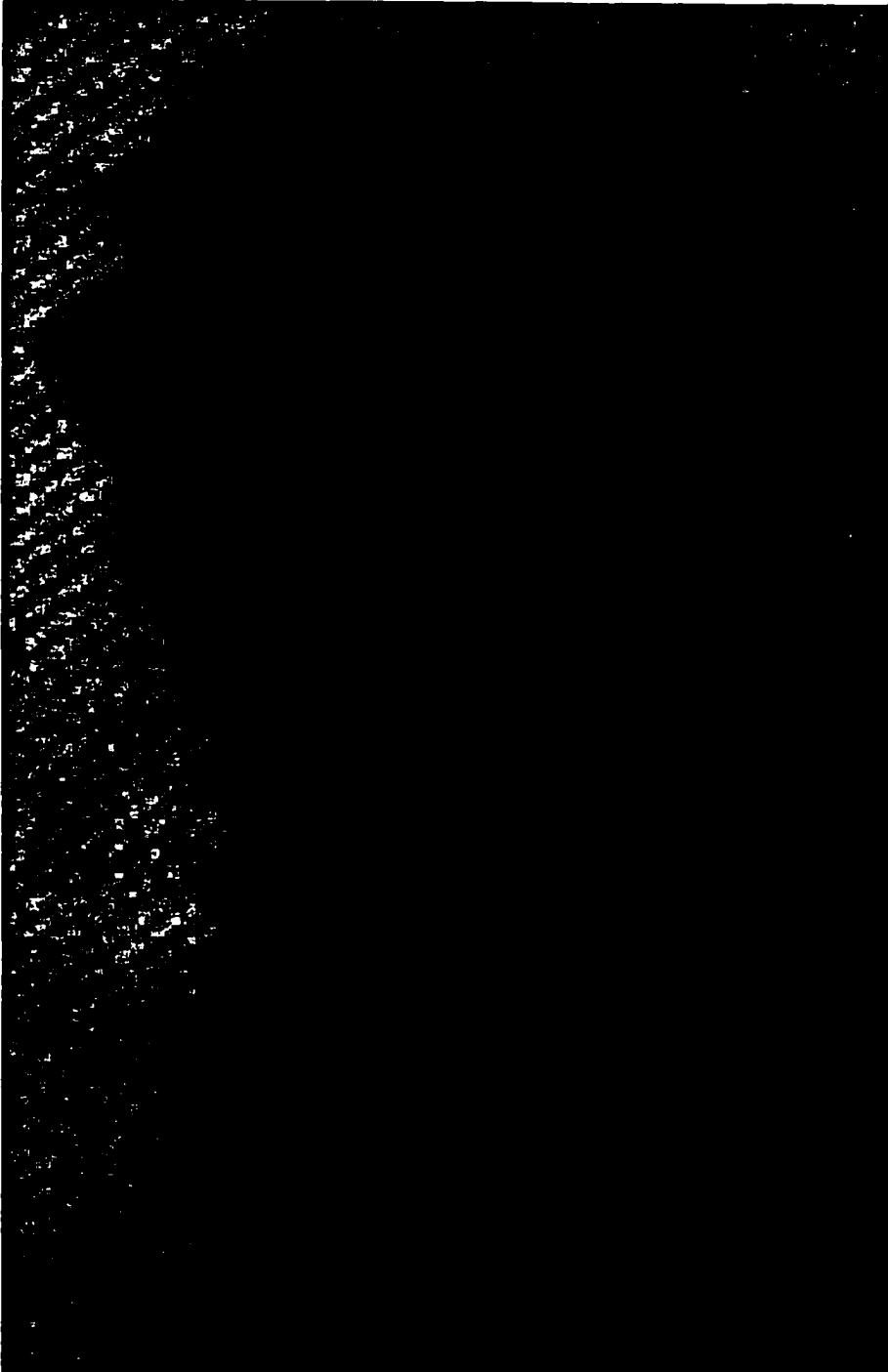
We could not verify the insecticidal activity reported by Hartzell using *A. aegypti*. However, we were using whole homogenate as opposed to an extract as described by Hartzell. Further the acetone extraction done by the author could have accounted for his results (Hartzell, 1947). An acetone extraction probably resulted in the presence of a great deal of cutin in the suspension, which would have precipitated out at later points in the experiment. This might explain Hartzell's positive reaction.



**Figure 4.1. Many trees and their mistletoe inhabitants suffered serious damage during a severe ice storm in 1998.**



**Figure 4.2.. Mistletoe distribution in a tree exposed on all sides on the front lawn of a house in Colonial Williamsburg.**



**Figure 4.3.** *Spodoptera eridania* eggs laid on a piece of nylon in the moth cage.



Figure 4.4. Seventh instar larvae, *S. eridania*, obtained from M. Kao, American Cyanamid Corporation, Princeton, New Jersey.



Figure 4.5. Pupal stage of *S. eridania* on the floor of the moth cage.

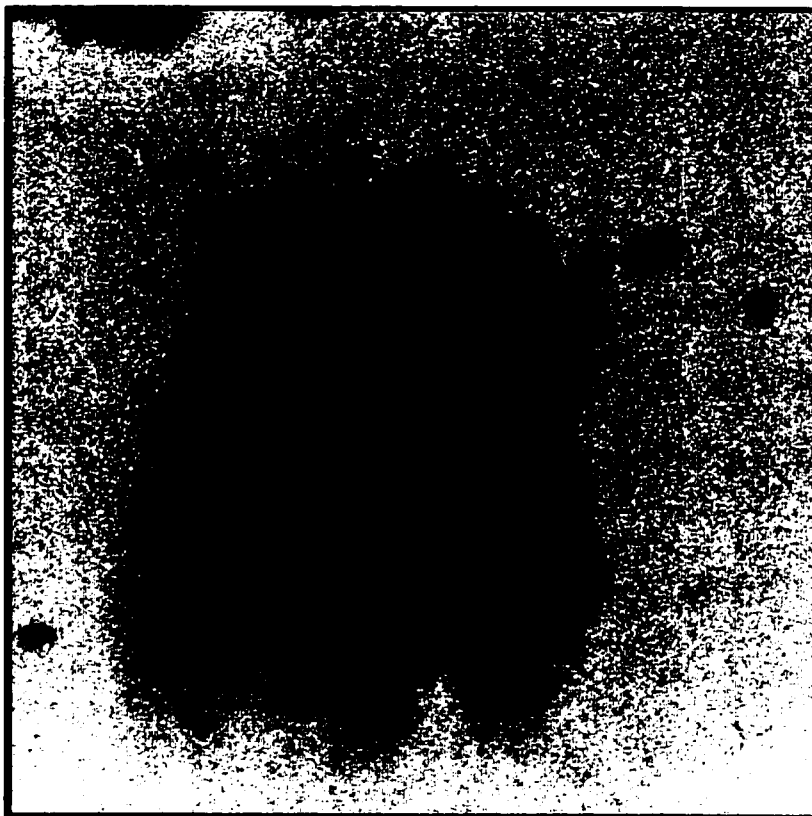


Figure 4.6. Adult *Spodoptera eridania*.

Figure 4.7. Allelopathic activity found in mistletoe extracts compared to allelopathic activity found in several other plant species.

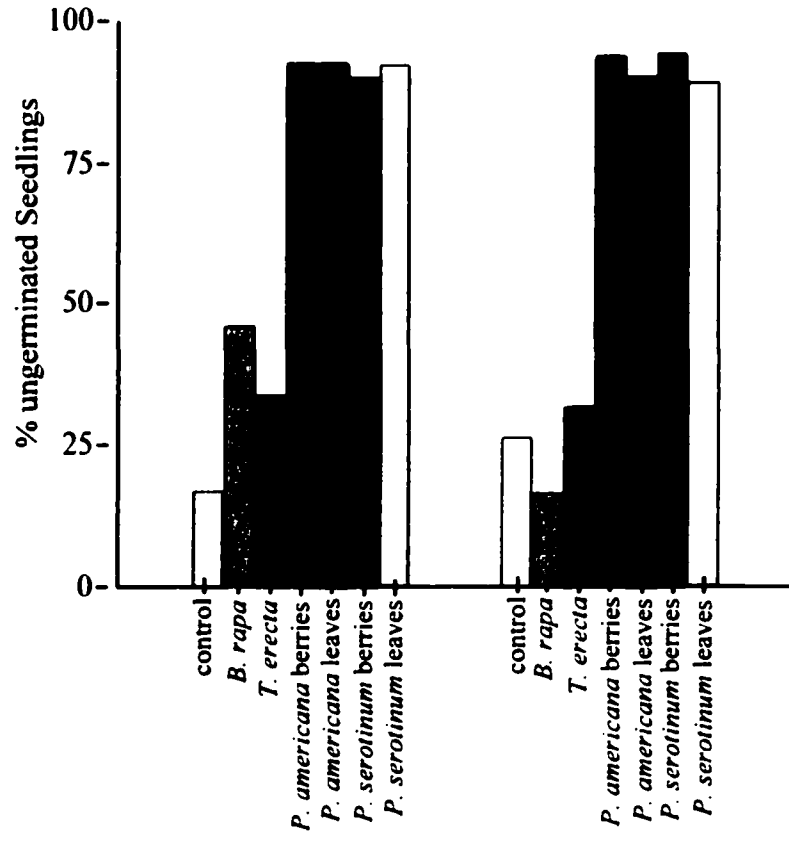


Figure 4.8. Dose dependent effects of mistletoe extracts from three different host species (water and methanol extracts) on seedling length. Both methanol and water extracts were compared

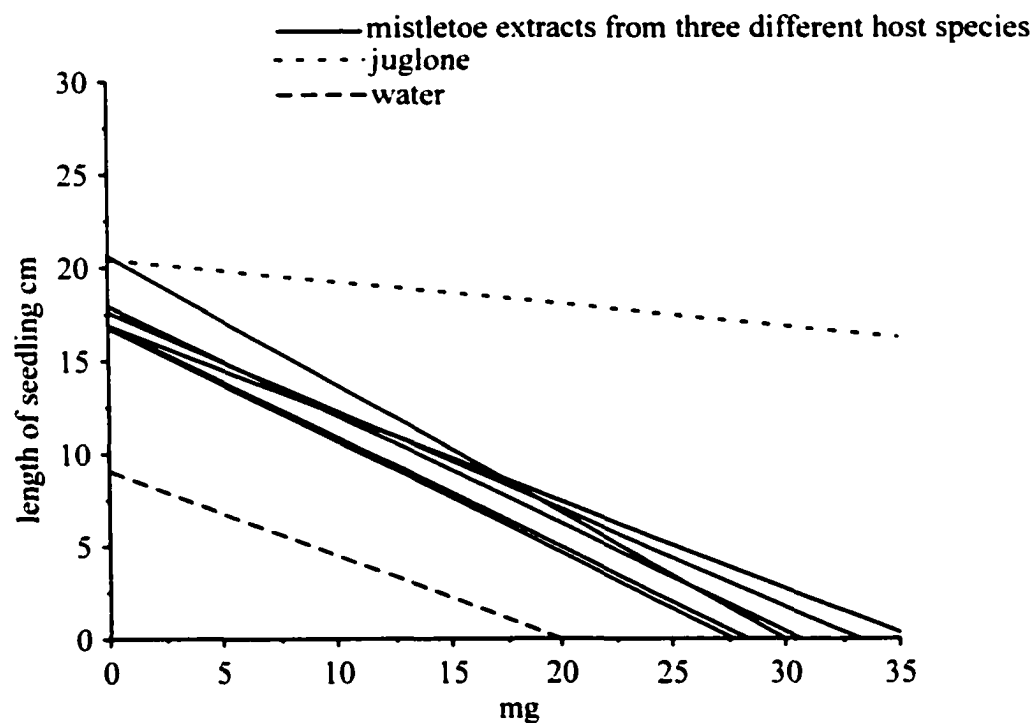


Figure 4.9a. Dose dependent linear regression using a water extract of mistletoe taken *A. saccharinum* host (R square = 0.313).

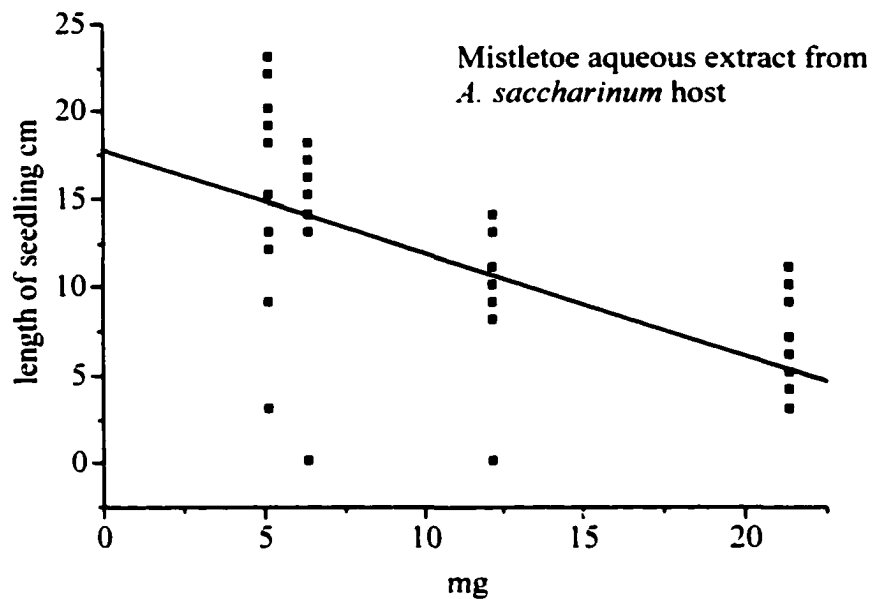


Figure 4.9b. Dose dependent linear regression using a water extract of mistletoe taken *A. saccharinum* host (R square = 0.447)

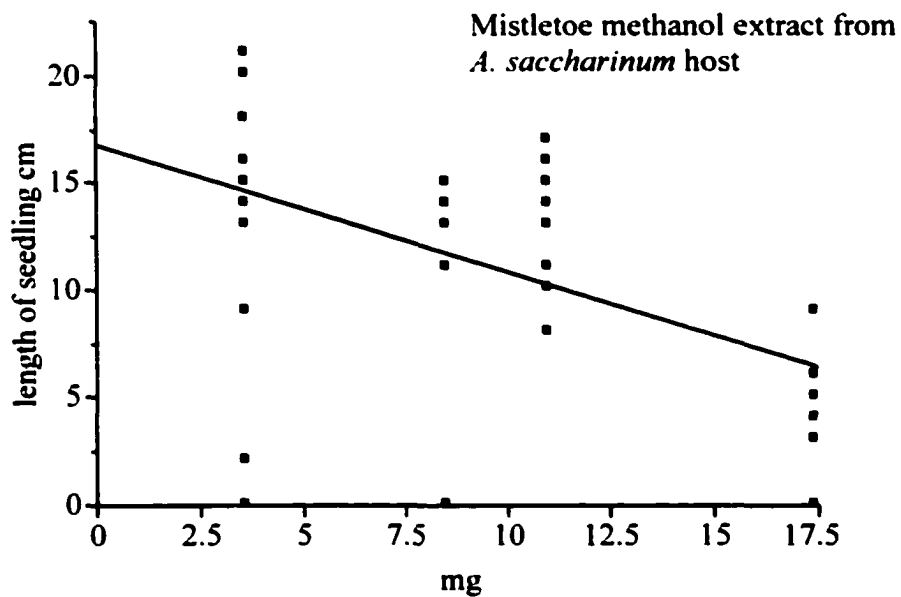


Figure 4.10a. Dose dependent linear regression using a methanol extract of mistletoe taken *C. cordiformis* host (R square = 0.250)

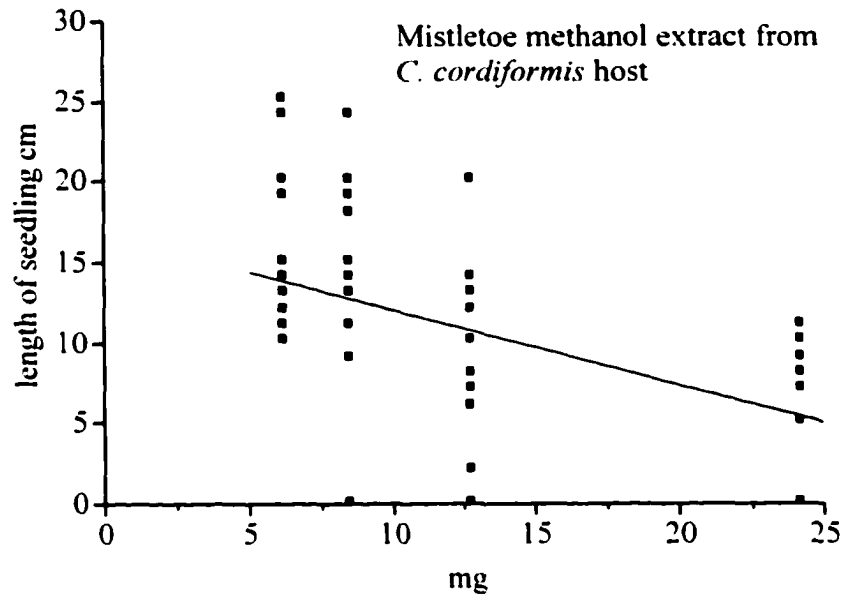


Figure 4.10b. Dose dependent linear regression using a water extract of mistletoe taken *C. cordiformis* host (R square = 0.532)

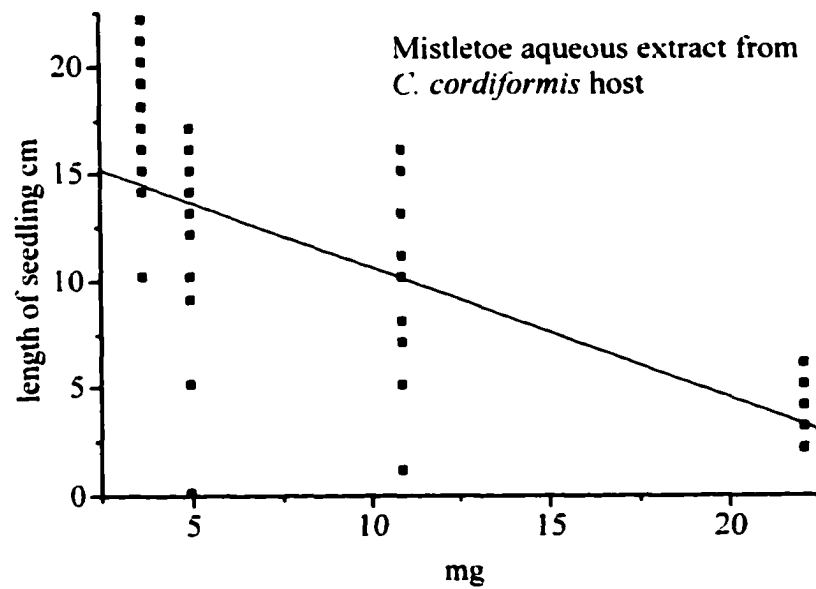


Figure 4.11a. Dose dependent linear regression using a water extract of mistletoe taken *G. triacanthos* host (R square = 0.511)

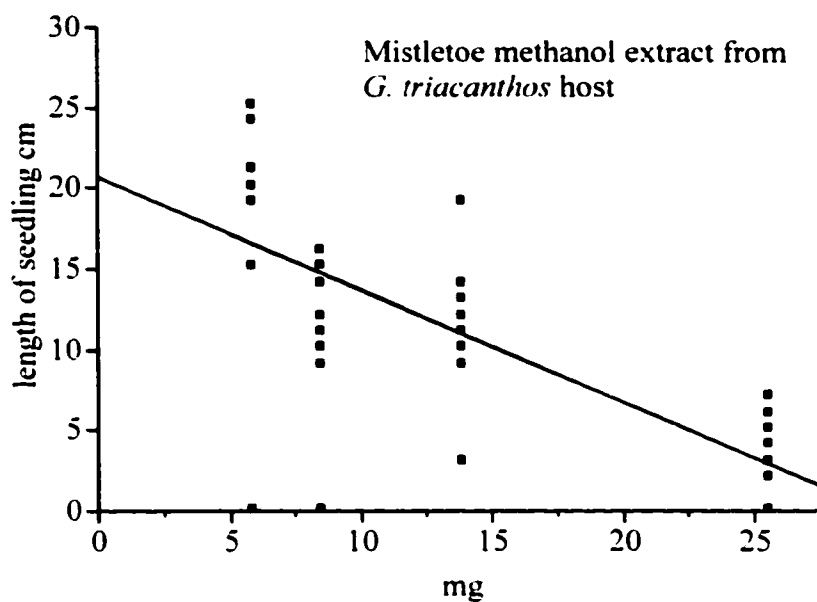


Figure 4.11b. Dose dependent linear regression using a water extract of mistletoe taken *G. triacanthos* host (R square = 0.596)

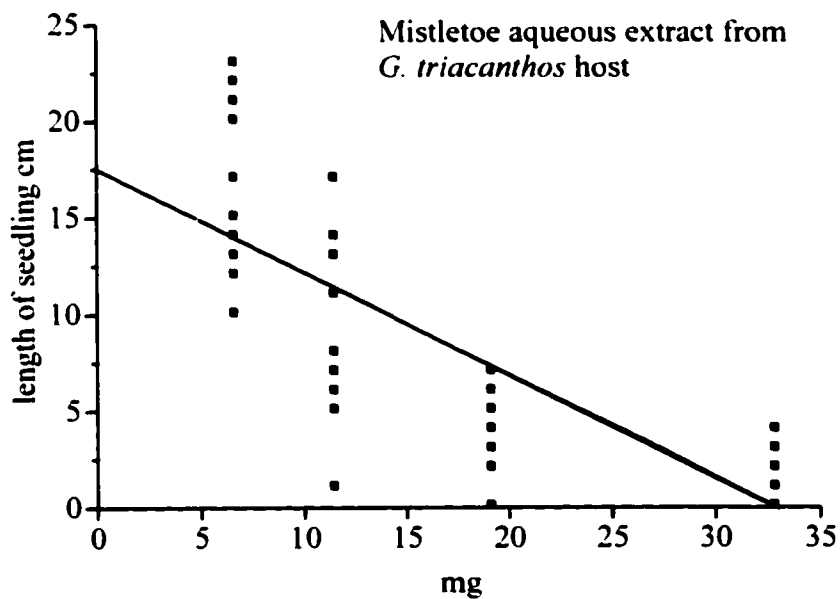


Figure 4.12. One-way ANOVA comparing allelopathy in soil taken from beneath the crowns of host trees at Carter's Grove.

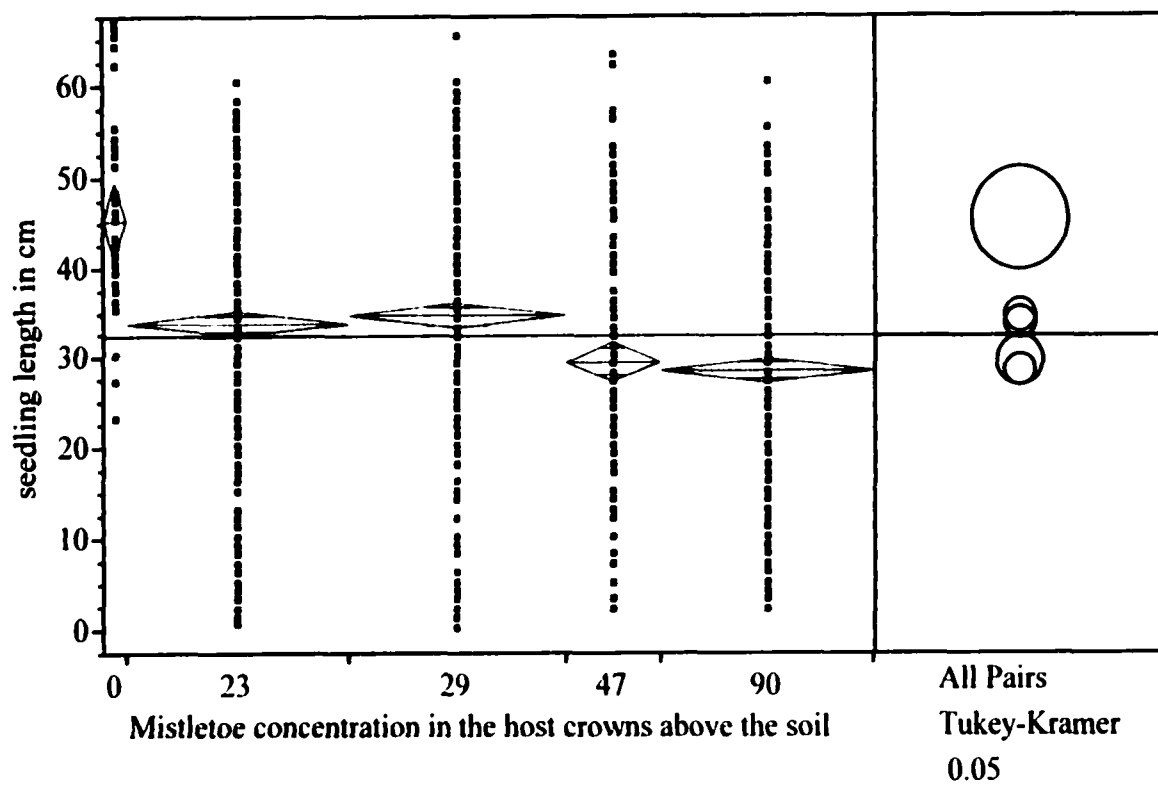
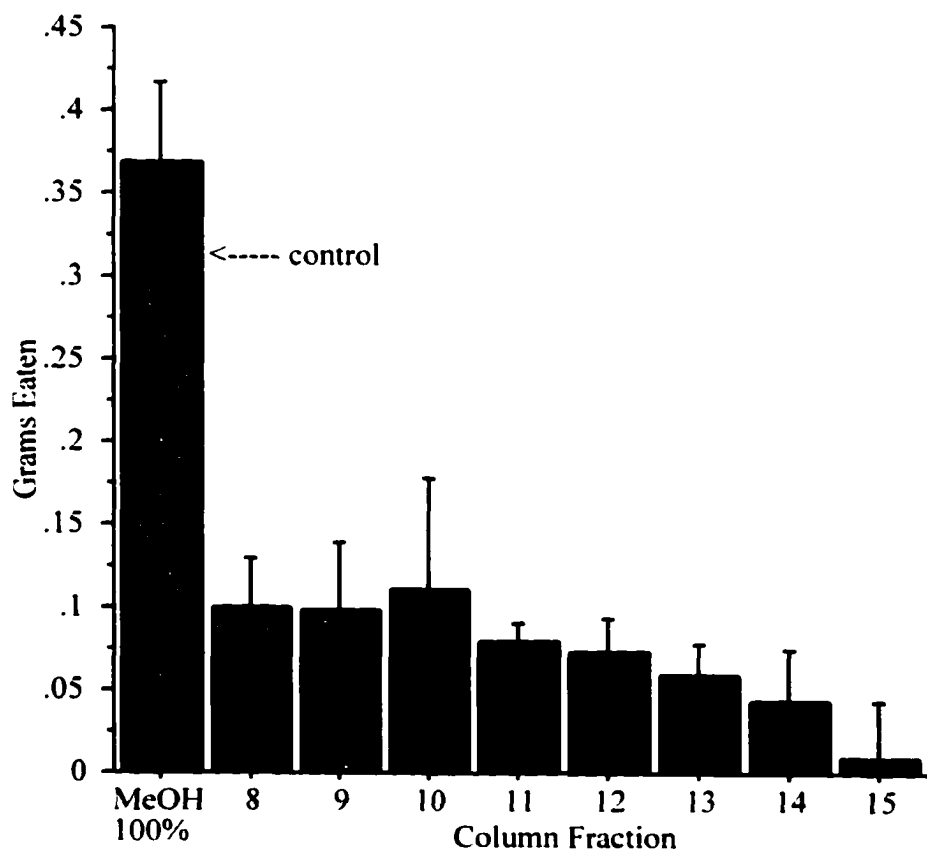


Figure 4.13. Grams of bok-choy eaten by larvae of *Spodoptera eridania* after three hours of starvation (95% CI).



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## **Chapter 5. Secondary metabolites with anti-oxidant activity and anti-cancer potential.**

### **Introduction**

The production of secondary metabolites by plants serves a variety of functions. Some are effective in attracting pollinators and seed dispersers, while others are effective in repelling herbivores. The array of compounds produced as secondary metabolites includes alkaloids, carbohydrates, water soluble organic acids, aromatic compounds, saponifiable lipids, terpenes, flavonoids, amino acids, peptides, alkaloids, and porphyrins (Robinson, 1991). A better understanding of these secondary metabolites will lead to a better understanding of the role played by *P. serotinum* in its niche.

*Homo sapiens* has used these compounds to draw pictures on the walls of caves, decorate pottery, and as medicinals from prehistoric times. Even animals have been credited with using plants rich in secondary metabolites for medicinal purposes. Native mistletoe species have a long and rich history of medicinal uses (Table 5.1) among the many nations of Native Americans who were superb phytopharmacologists (Moerman, 1998).

*Viscum album* is a common European species, which parasitizes a wide variety of hosts. Many of these host genera overlap with the genera parasitized by the American *P. serotinum*. *Viscum album* has been extensively investigated because of its widespread use in Europe in adjuvant cancer treatment. In 1916 Rudolf Steiner first advocated the use of the European species to treat cancer (Kleijnen and Knipschild, 1994). Today, *V. album* extracts are among cancer treatments of last resort in Europe and are used by about

4% of all cancer patients in the Netherlands and Switzerland (Bussing, 1997; Fischer et al., 1997).

Table 5.1. Native American medicinal uses of mistletoe.

Species	Use	Nation reported to have used the plant	Reference
<i>Arceuthobium americanum</i> Nutt. Ex. Engelm..	1. Antihemorrhagic: used to stop lung hemorrhages	Bella Coola	(Smith, 1929)
	2. Antihemorrhagic: Used to stop mouth hemorrhages	Southern Carrier	
	3. Dietary aid		
<i>Arceuthobium occidentale</i> Engelm.	4. Gastrointestinal aid	Mendocino	(Chestnut, 1902)
<i>Phoradendron californicum</i> Nutt.	5. Cathartic (berries only)	Pima	(Curtin, 1949)
	6. Dermatological aid (leaves)		
	7. Gastrointestinal aid		

Species	Use	Nation reported to have used the plant	Reference
<i>Phoradendron junipericum</i> Nutt.	8. Gastrointestinal aid	Hopi	(Whiting, 1939)
	9. Antidiarrheal	Keres	
	10. Antirheumatic: used as a rub		
	11. Dermatological: wart treatment	Navaho	(Hocking, 1956)
	12. Gynecological aid: promote muscular relaxation and stop excessive bleeding at childbirth	Zuni	(Steggerda, 1941)

Species	Use	Nation reported to have used the plant	Reference
<i>P. serotinum</i>	13. Analgesic	Cherokee	(Hamel and
	14. Treatment of hypotension		Chiltoskey, 1975)
	15. Anticonvulsive (from oak host)		
	16. Paralytic weakness and debility	Huoma	(Speck and Dexter, 1951)
	17. Abortifacient	Mendocino	(Chestnut, 1902)
	18. Toothache remedy		
	19. Antirheumatic	Seminole	(Sturtevant, 1955)
20. Tuberculosis Remedy	Creek	(Swanton, 1928)	

Species	Use	Nation reported to have used the plant	Reference
<i>Phoradendron macrophyllum</i> (Engelm) Cockerell	21. Dermatological aid: to treat dandruff	Diegueno	(Hedges, 1986)
	22. Disinfectant and sore eye remedy	Cahuilla	(Bean and Saubel, 1972)
	23. Analgesic for cramps	Papago	(Castetter and Underhill, 1935)

Iscador is a commercially available extract of mistletoe, which has been bacterially fermented. In one preclinical trial it was used to treat sarcomas on the dorsal surface of mice, which had received a single injection of 20 mg of 20-methylcholantrecene (Kuttan et al., 1997). Iscador treatments were administered twice weekly for fifteen weeks to the experimental group. Only 1 (N=45) of the mice treated with Iscador developed tumors. All of the untreated mice developed metastatic cancer.

The inhibition of metastasis in the mouse model is thought by some to be linked to the immunomodulatory effect of *V. album* due to its rich lectin content. However, besides lectins, this mistletoe is rich in other secondary metabolites. It is known to contain free amino acids, vitamin C, triterpenes, and flavonoids. It is possible that one or more of these secondary metabolites contributes to the anti-cancer effect. Kuttan and his coworkers offer three possible mechanisms by which the active component might be functioning. These mechanisms are apoptosis, induction of differentiation of tumor cells, or interference with the promotion of oncogenesis (Kuttan et al., 1997).

The brine shrimp assay is an excellent tool with which to begin investigations of an herb's biological activity. McLaughlin and his associates (Meyer et al., 1982; McLaughlin and Rogers, 1998) developed this assay in the early 1980s to identify anti-cancer compounds. This in-vivo assay is a reliable prescreen for the biological activity of plant secondary metabolites and it has been used successfully for almost twenty years to investigate biologically active botanicals (Alali et al., 1999). Although the Cox assay is a more definitive screen, it is costly and time consuming and beyond the limits of current funding.

Much of the use of *V. album* in adjuvant cancer treatment can be credited to its ability to produce lectins, which stimulate the immune system. Viscotoxin is one of these active agents in the European species. Phoratoxin is a lectin, which has been isolated from *P. serotinum* and it is currently being studied in several laboratories.

An assay, which was serendipitously carried out on *P. serotinum* homogenate during the course of these studies, was an anti-oxidant assay using 1,1-diphenyl, 2-picrylhydrazine as a free radical. Plants are a rich source of anti-oxidants because, as electrons are passed around the plant cell during cyclic and non-cyclic photophosphorylation, it is logical that some would leak and create free radicals. In addition to the production of free radicals related to photophosphorylation, plants also produce free radicals when under extreme environmental conditions. Conditions such as heat, high light, drought, paraquat, and chilling can trigger the release of oxidants (Becana, 1998; Bartoli et al., 1999). It should be remembered here that drought stressed plants lack CO<sub>2</sub> because the stomates close under these conditions (Tausz et al., 2001).

Mistletoe, as a resident of the canopy, is subject to drought, heat, and UV light. Its need for free access to the host's xylem establishes its constant need for water. There is disagreement in the literature as to whether free radical production within a plant leads to an increase in anti-oxidant levels or whether free radical production leads to lower levels of antioxidants. To clarify this issue, in a recent publication, Hansen and coworkers (2003) found tocopherol levels to exhibit a concentration gradient consistent with position in the canopy. It is clear that a plant subject to high levels of oxidants should have evolved to produce high levels of free radical scavengers for protection.

Plant derived antioxidants in the diet are highly correlated with reduced risk of several chronic diseases, including arthritis, cancer, and atherosclerosis. There is some indication that high concentrations of antioxidants are associated with longevity (Halvorsen et al., 2002).

As useful as these compounds might be, if they cannot be isolated in the laboratory and identified, then their utility is greatly diminished. The concentration and the nature of secondary metabolites can diminish depending on the season and the environmental conditions (Ratnayaka et al., 2002). Specific predators can initiate the production of specific secondary metabolites. These may be tied to compounds in the saliva of the predators. Little or nothing is known about these mechanisms.

Only one reference is listed in Table 5.1 (Use 15) above, which cites a specific host, an oak as important to medicinal value, but the literature on cancer efficacy of *Viscum album* also indicates that host specificity can play an important role. During the studies described below, mistletoe homogenates from three host species were compared to determine if there were significant differences in the production of the biologically active compounds in the brine shrimp lethality assay.

## **Materials and Methods**

### **Brine Shrimp Lethality Assays**

#### **Comparison of crude homogenates from three different host species.**

Mistletoe was collected as described above in Chapter 4. Two 1 g aliquots of leaves from each of three host species, *Gleditsia triacanthos*, *Carya cordiformis*, and *Acer sacharrinum* were frozen in liquid nitrogen and then ground with mortars and pestles. The samples were washed into test tubes with 5 mL aliquots of water or

methanol, and then vortexed. This was followed with quantitative transfers to centrifuge tubes.

The vortexed samples were centrifuged at 3000 rpm for 5 minutes and the supernatant was decanted. Aliquots (0.1mL, 0.2mL, 0.3mL, 0.4mL, and 0.5mL) of each of the six resulting homogenates were pipetted into pre-weighed 5 ml vials and dried. Each vial was weighed and the Brine Shrimp Lethality Assay was performed.

*Artemia salina* eggs were obtained from a pet shop. They were incubated in a hatchery prepared from a simple soap dish. The modified soapdish kept the eggs separated from the nauplii. This made it easier to harvest the *A. salina* (Opler et al, 2002).

Artificial seawater (phosphate free) was prepared by dissolving 32 g of synthetic sea salt in 500 mL of distilled water to a specific gravity of 1.02 at 24° C. The nauplii hatched after 24 hours and were incubated for another 24 hours. The nauplii were always used within 36 hours of hatching.

To prepare the extract or fraction samples for incubation, small aliquots were removed from the freezer, then resuspended in MeOH and distilled H<sub>2</sub>O in a 3:1 ratio. All aliquots were under 20 mg. They were placed in pre-weighed vials and dried at 40° C. A drop of DMSO was added to each vial to solubilize the sample and dilutions were made with artificial seawater. DMSO was added as needed; never exceeding 10 µL. Juglone was used as a positive control and water as a negative control.

For *A. salina* lethality assay, the 95% confidence interval for the Ld50 was calculated by bootstrap sampling using the computer program Toxicity (Kincaid, 2002). This is the same program written in True Basic, November 2002 Version described

above. It fits the dependent variable; in this case the proportion dead versus the independent variable, log dose in logistic regression.

### **Homogenization and extraction for all column chromatography separations.**

Mistletoe was collected from Williamsburg, Virginia as described in Chapter 3. Between 190 and 200 g of leaves were homogenized in 500 mL of methanol in a Waring Blender. The homogenate was flash evaporated on a Buchi Flash Evaporator to a volume of 1000 mL and extracted with hexane in a 3:1 ratio. The methanol fraction was also frozen and flash evaporated. It should be noted that it was impossible to completely dry the methanolic extract when large aliquots were involved. Even if butanol was added to the extract, a little water always persisted. Lyophilization also failed to yield dry samples. By the time the samples were removed from the lyophilizer and capped they were wet again. The samples were frozen in this semi-dry state. The hexane fraction was flash evaporated and frozen for possible future use. Both the methanol extract and the hexane extract were stored in the freezer at  $-20^{\circ}\text{C}$ .

### **Polyamide column chromatography**

Polyamide was obtained from Altech Associates. Ten grams of crude homogenate was always layered on top. This separation resulted in the production of between 17 and 30 fractions, which were subjected to the brine shrimp assay. Two active peaks always emerged. All fractions were tested for purity on TLC. If fractions appeared pure on TLC, HPLC was then run.

Thin layer chromatography was done on every column fraction using Silica Gel plates and chloroform:methanol:water, in an 8:1:0.05 ratio. If any fractions appeared pure, HPLC was done on those fractions to verify purity. A Waters System consisting of

717 Autosampler, 600 Controller, and a 996 Photodiode Array Detector with Millennium Software was used for this purpose. Wavelengths used were from 250 nm to 350 nm.

On one of these columns the two active peaks were subjected to preparative HPLC. Only two major peaks were obtained from preparative HPLC. However, it was not possible to test these peaks (from preparative HPLC) for activity because the yields were too low for the brine shrimp assay. The entire sample was used for NMR analysis.

#### **Vacuum liquid chromatography**

Vacuum liquid column chromatography was done using silica gel. Fifteen grams of crude homogenate were resuspended in 90% methanol and bound to 45 grams of silica gel. A Buchner Funnel was filled with silica gel and the bound crude extract was layered on top. The column was then extracted with chloroform and methanol in a discontinuous gradient from 100%  $\text{CHCl}_3$  to 30%  $\text{CHCl}_3$  (Table 5.2). Each fraction was eluted with 500 mL of solvent. Each fraction was flash evaporated on a Buchner Flash evaporator at 40° C and weighed. Dried fractions were stored at -20° C.

All fractions from VLC were tested in the Brine Shrimp Lethality Assay and if a fraction exhibited activity it was tested on TLC for purity. If the fraction appeared pure on TLC, it was subjected to HPLC for further verification.

**Table 5.2. Solvent concentration for the VLC column**

<b>Fraction</b>	<b>chloroform: methanol</b>
1.	100:0
2.	90:10
3.	80:20
4.	70:30
5.	60:40
6.	50:50
7.	50:50
8.	40:60
9.	30:70

### **Lipophilic Sephadex LH-20**

Sephadex LH 20 was obtained from Sigma Corporation. In an attempt to further purify the active components, several consecutive fractions from VLC columns, which proved to have the same R<sub>f</sub> values on TLC were pooled. The pooled fractions were concentrated on a flash evaporator and resuspended in methanol and water, 80:20. The pooled fractions were layered on a lipophilic sephadex LH-20 column.

All fractions from the LH 20 column were tested in the Brine Shrimp Lethality Assay and if a fraction exhibited activity it was tested on TLC for purity. If the fraction appeared pure on TLC, it was subjected to HPLC, as described above, for further verification.

### **Anti-oxidant assay**

The stable free radical, 1,1-diphenyl-2-picrylhydrazine (DPPH), obtained from Sigma Corporation, was used to identify anti-oxidant activity in *P. serotinum*. DPPH in MeOH absorbs at 517 nm even at a concentration of 500 μM in ethanol, DPPH is a deep violet color and is readily measured stoichiometrically. Ascorbic acid was used as positive control and ethanol as a negative control. Each molecule of ascorbic acid will reduce two molecules of DPPH (Blois, 1958).

Only fractions from one VLC column were used in the anti-oxidant assay. For this assay, small aliquots, under 500 mg of crude homogenate and 10 to 20 mg aliquots of column fractions were resuspended in 3:1 MeOH and H<sub>2</sub>O, dried and weighed. This step was necessitated by the hydrophilic nature of mistletoe extracts, upon removal from the freezer. The samples were again resuspended in 100 % ethanol.

A 400  $\mu\text{M}$  DPPH solution was prepared in 95% ethanol.

For the anti-oxidant assay, the 95% confidence interval for the  $\text{ED}_{50}$  was calculated by bootstrap sampling using the computer program Toxicity (Kincaid, 2002) written in True Basic, November 2002 Version. It fits the dependent variable, decrease in Absorbance at 515 m, versus the independent variable, log dose in logistic regression.

## **Results**

### **Brine Shrimp Lethality Assay**

No significant differences were observed among the water extracts of all three of hosts (Table 5.3). The 95% confidence intervals for all three overlap. The results were the same for the methanol extracts. There is no difference between the methanolic and water extracts within a species. Again the 95% confidence intervals overlap.

**Table 5.3. Comparison of Brine Shrimp Lethality Activity in extracts from three different host species.**

Host species	Extraction medium	Lethal Dose		Regression Analysis		
		LD <sub>50</sub> in raw dose units	95% CI	R square	F	P
<i>Acer saccharinum</i>	MeOH	39.2	3.4, 75.0	0.7351	11.10	<0.030
	H <sub>2</sub> O	8.1	3.5, 12.7	0.9814	210.79	<0.001
<i>Gleditsia</i>	MeOH	12.4	0, 37.6	0.9370	59.46	<0.005
<i>triacanthos</i>	H <sub>2</sub> O	9.0	3.9, 14.2	0.9185	45.09	<0.005
<i>Carya cordiformis</i>	MeOH	58.8	39.1, 78.6	0.9412	32.03	<0.025
	H <sub>2</sub> O	10.1	0, 45.4	0.9726	141.9	<0.001

All fractions from VLC columns showed some activity (Figures 5.1a-5.10b). However, on separation by TLC, all fractions had at least three distinct spots. This was further confirmed by HPLC. The LH 20 column was much worse than the VLC silica gel. The only type of column chromatography, which appeared to achieve any significant separation was the polyamide column. However, the yields were so poor that biological activity in the brine shrimp lethality assay could not be confirmed with this column. Even here the purity is questionable (Figure 5.1a- 5.11b).

#### **Anti-oxidant Assay**

Fractions from only one VLC column were tested on the anti-oxidant assay. These same fractions were subjected to the Brine Shrimp Lethality Assay (Figure 5.12). There was overlap. No attempt has been made at purifying anti-oxidants.

#### **Conclusion**

The polyamide column would yield high enough concentrations to make it possible to assay for anti-oxidant activity. However, it is questionable, given the inability to separate the active compounds whether it is prudent to continue using the brine shrimp assay as a prescreen for anti-cancer drugs. A large polyamide column without further purification on HPLC would yield enough of the active substances to be tested directly on cancer cells. If future funding permits, this will be done.

The antioxidant merits further investigation. The presence of high levels of antioxidants is consistent with the work done by (Hansen et al. (2003). The work by these authors leads to the expectation of high levels of antioxidants, which are positively correlated to light intensity among *Quercus robur* L.

Figure 5.1. Logistic regression for brine shrimp lethality using extracts from *Acer saccharinum* host. R square = 0.9814, LD<sub>50</sub> = 8.096 ppm, LD<sub>50</sub>: 95% CI for LD<sub>50</sub>: 3.494, 12.699 ppm

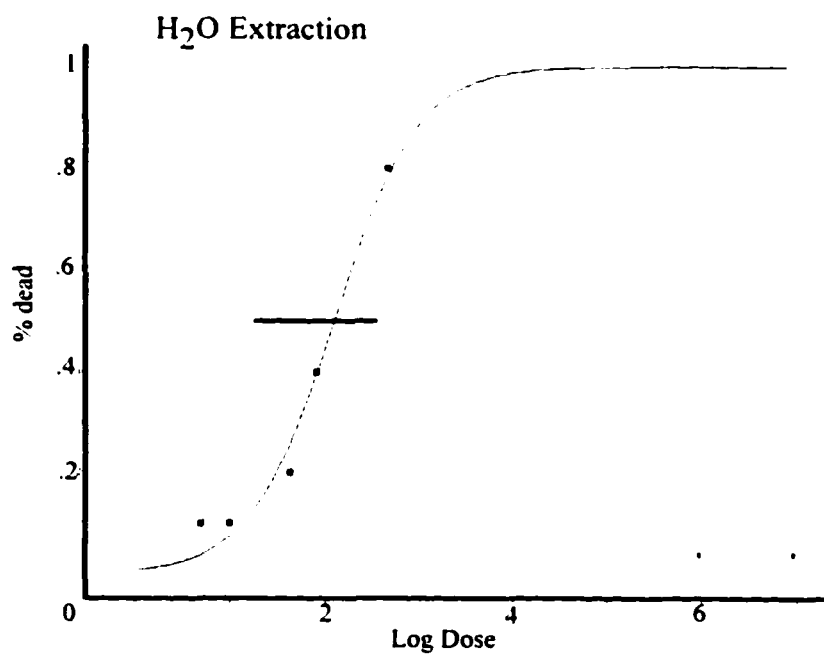


Figure 5.2. Logistic regression for brine shrimp lethality using extracts from *Acer saccharinum* host. R square = 0.7351, LD<sub>50</sub> = 39.661 ppm (using 100 bootstrap samples), LD<sub>50</sub>: 95% CI for LD<sub>50</sub>: 3.489, 75.834 ppm.

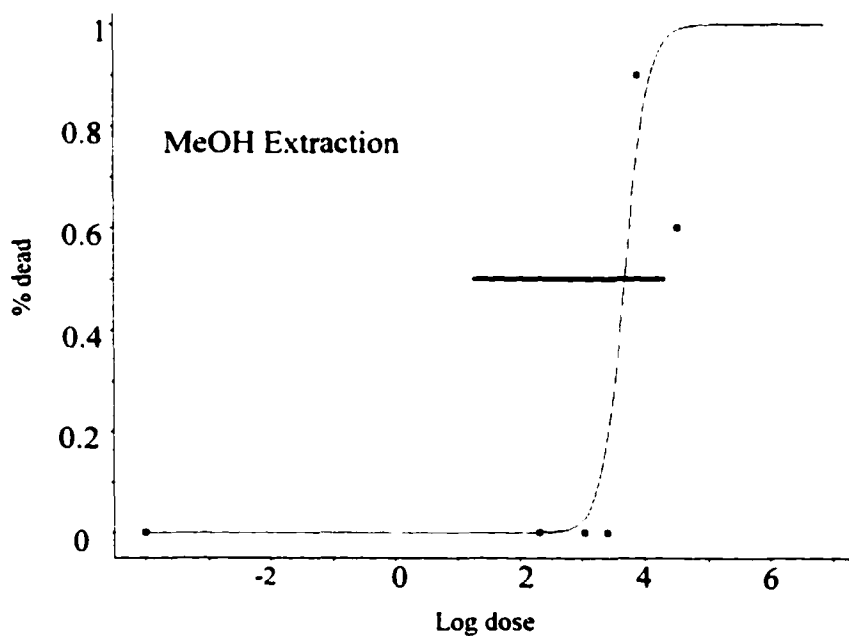


Figure 5.3a. Logistic regression for brine shrimp lethality using extracts from *Gleditsia triacanthos* host. R square = 0.9185, LD<sub>50</sub> = 0.032 ppm, LD<sub>50</sub>: 95% CI 3.845, 14.219 using 100 bootstrap samples.

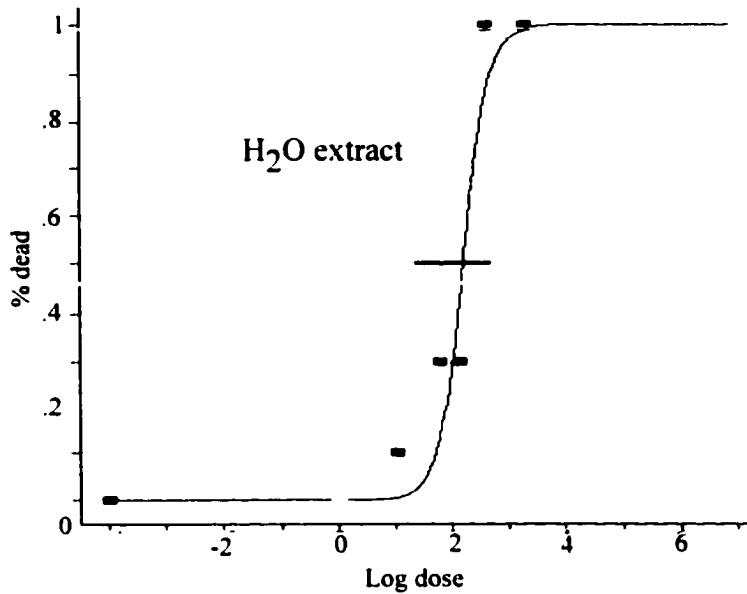


Figure 5.3b. Logistic regression for brine shrimp lethality using extracts from *Gleditsia triacanthos* host. R square = 0.9185, LD<sub>50</sub> = 12.376 ppm, LD<sub>50</sub>: 95% CI -12.793, 37.545 using 1000 bootstrap samples.

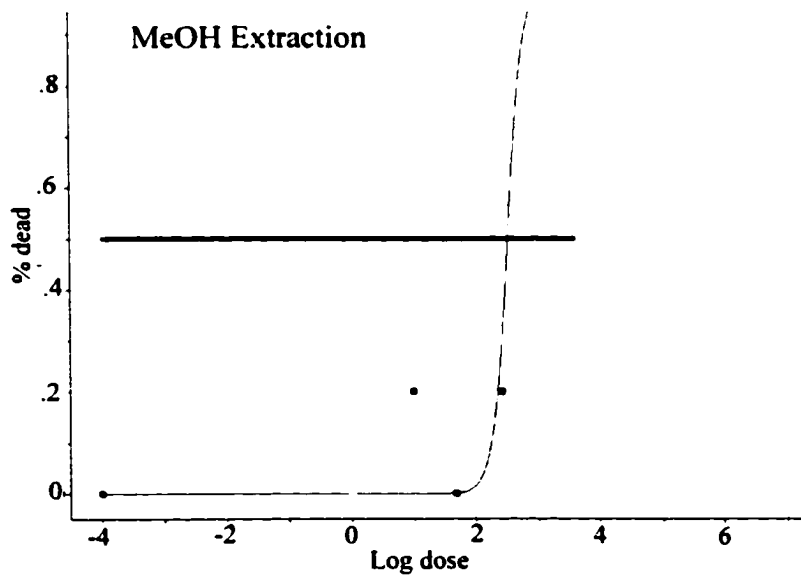


Figure 5.4. Logistic regression for brine shrimp lethality using extracts from *Carya cordiformis* host. R square = 0.377, LD<sub>50</sub> = 531.221 ppm, 95% CI for LD<sub>50</sub>: 527.3, 535.1 ppm.

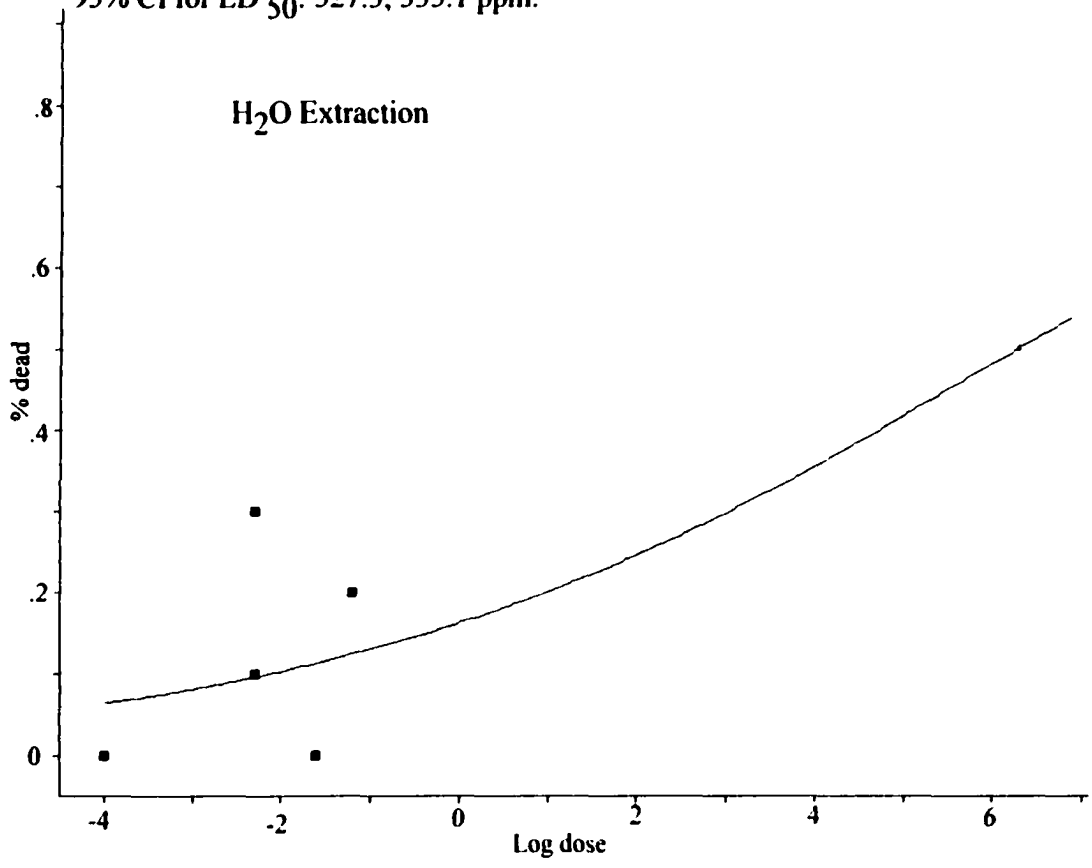


Figure 5.5a. Logistic regression for brine shrimp lethality using extract from *Carya cordiformis* host, R square = 0.791, LD<sub>50</sub> = 91.462, 95% CI for LD<sub>50</sub>: 12.308, 170.616 using 100 bootstrap samples.

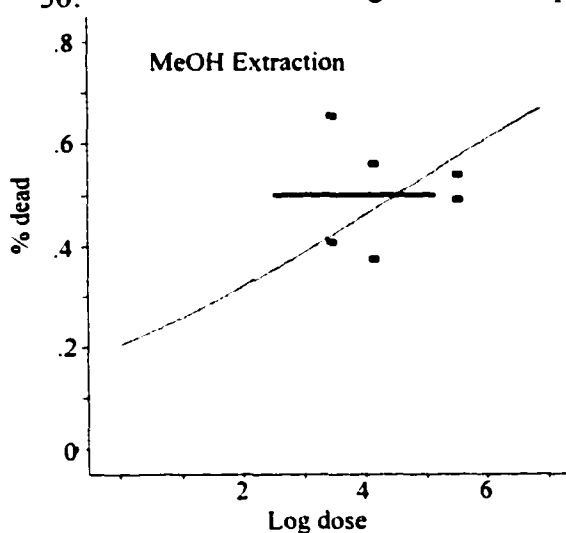


Figure 5.5b. Logistic regression for brine shrimp lethality assay using juglone standard. R square = 0.4445, LD 50 = 1.014 ppm, and 95% CI for LD 50: 0.718, 1.31 using 100 bootstrap samples.

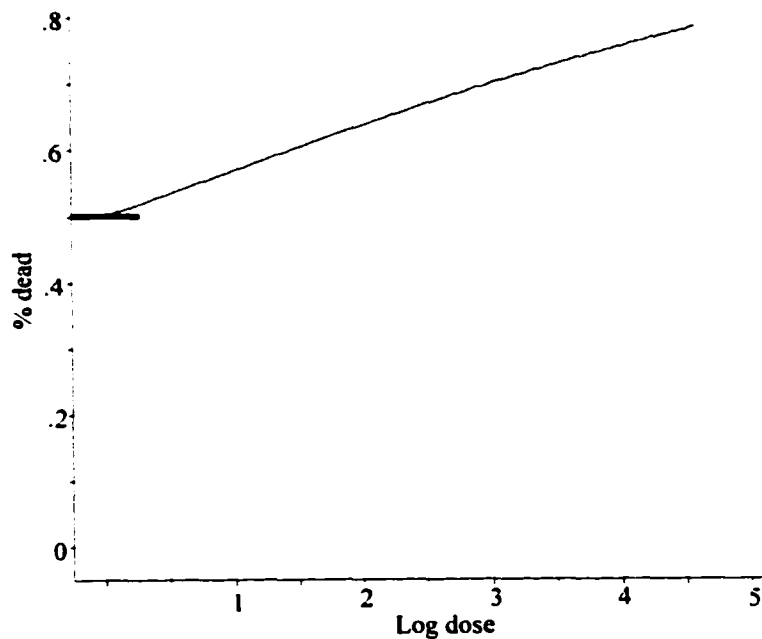


Figure 5.6. Logistic regression for Fraction 6 eluted with 20% methanol. F stat:13.98, R square = .6997,  $ED_{50} = 1.02$ ,95% Confidence Interval for  $ED_{50}$ : 1.007 to 1.033 using 100 bootstrap samples.

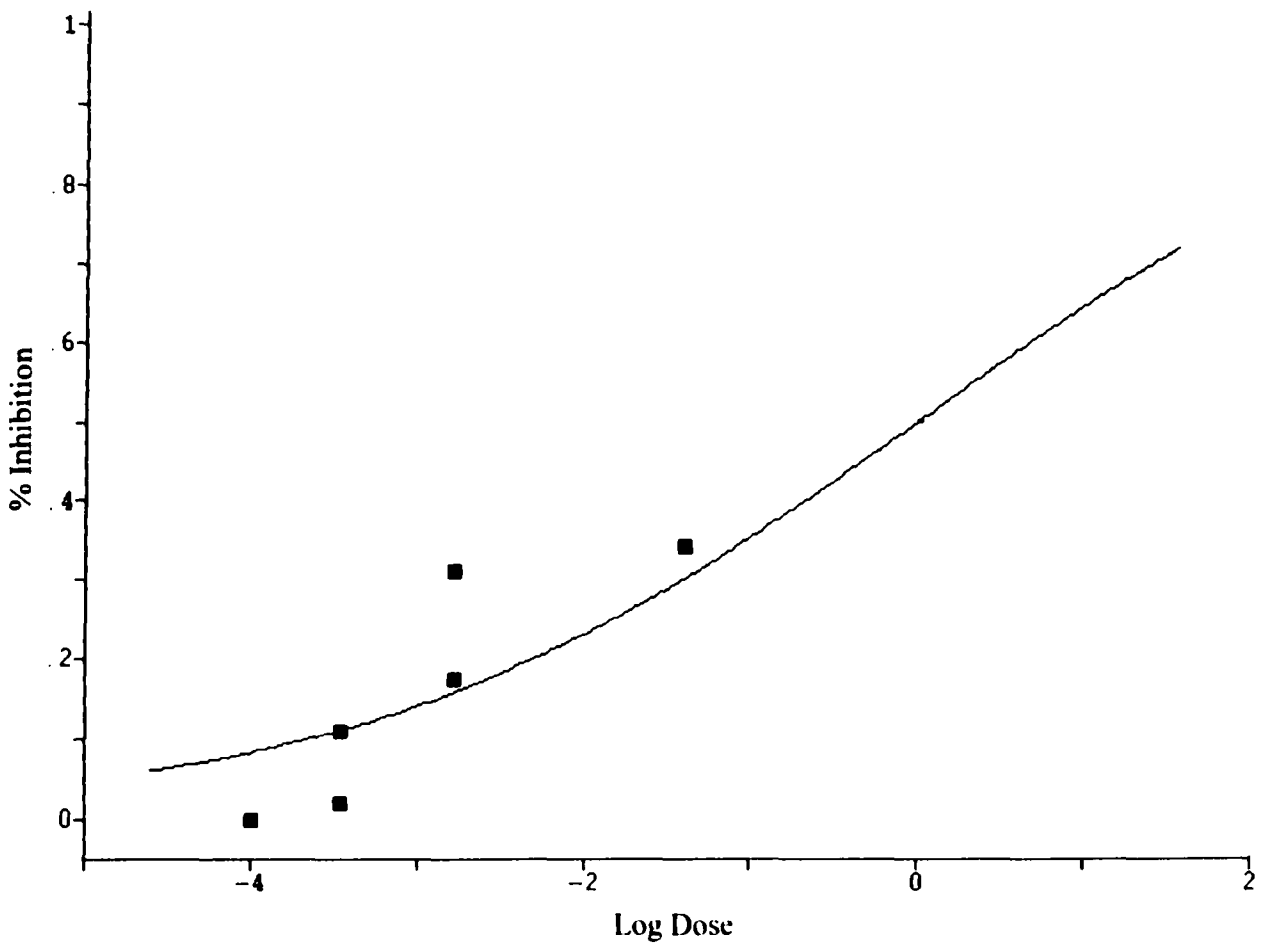


Figure 5.7. Logistic regression for Fraction 7 eluted with 20% methanol. F stat:8.43, R square = .5843, ED<sub>50</sub> = 1.241, 95% Confidence Interval for ED<sub>50</sub>: 1.108 to 1.375 using 100 bootstrap samples.

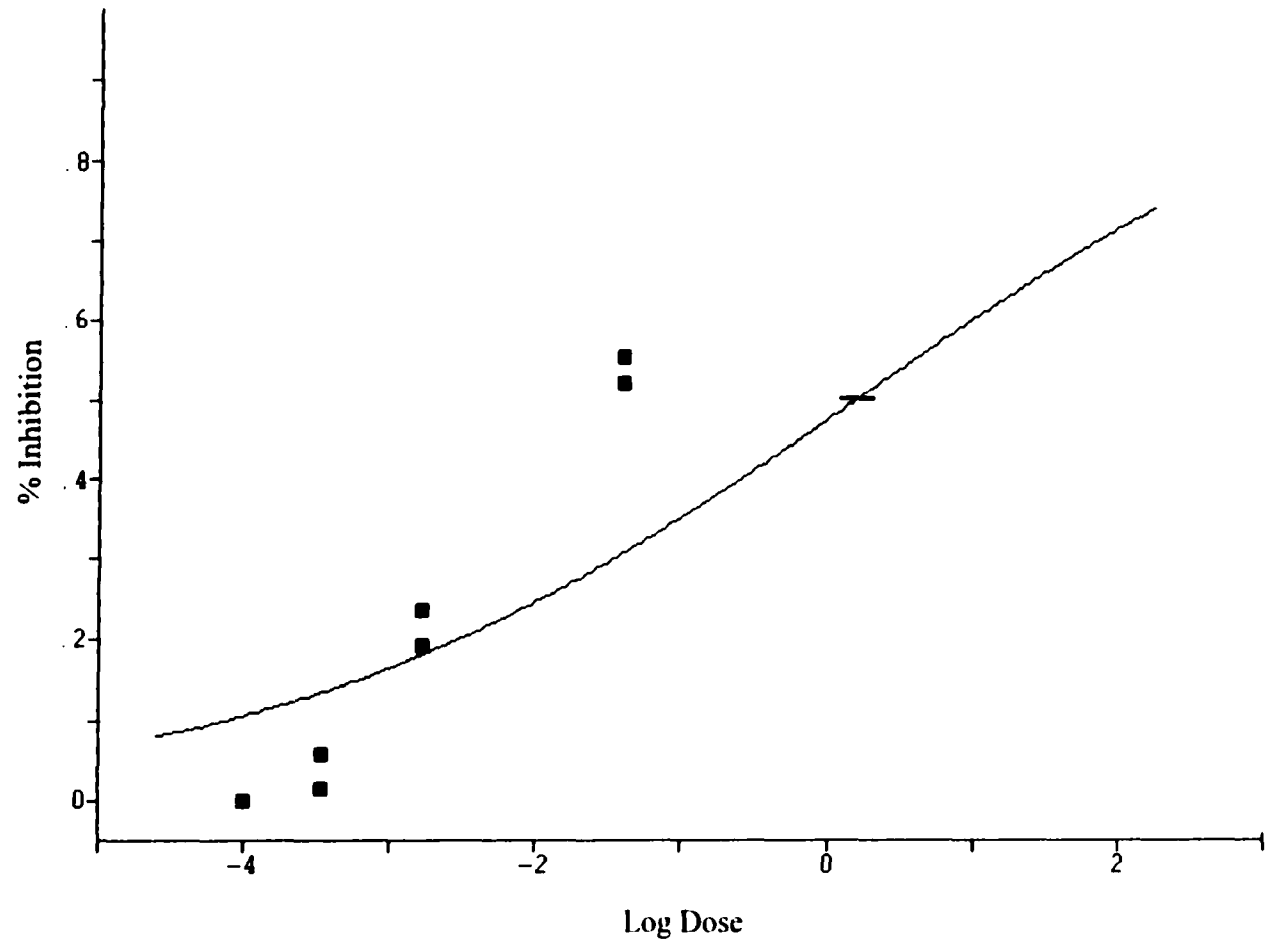


Figure 5.8. Logistic regression for Fraction 8 eluted with 20% methanol. F stat:35.14, R square = .8541  
ED<sub>50</sub> = 1.157, 95% Confidence Interval for ED<sub>50</sub>: 0.58 to 1.735 using 100 bootstrap samples.

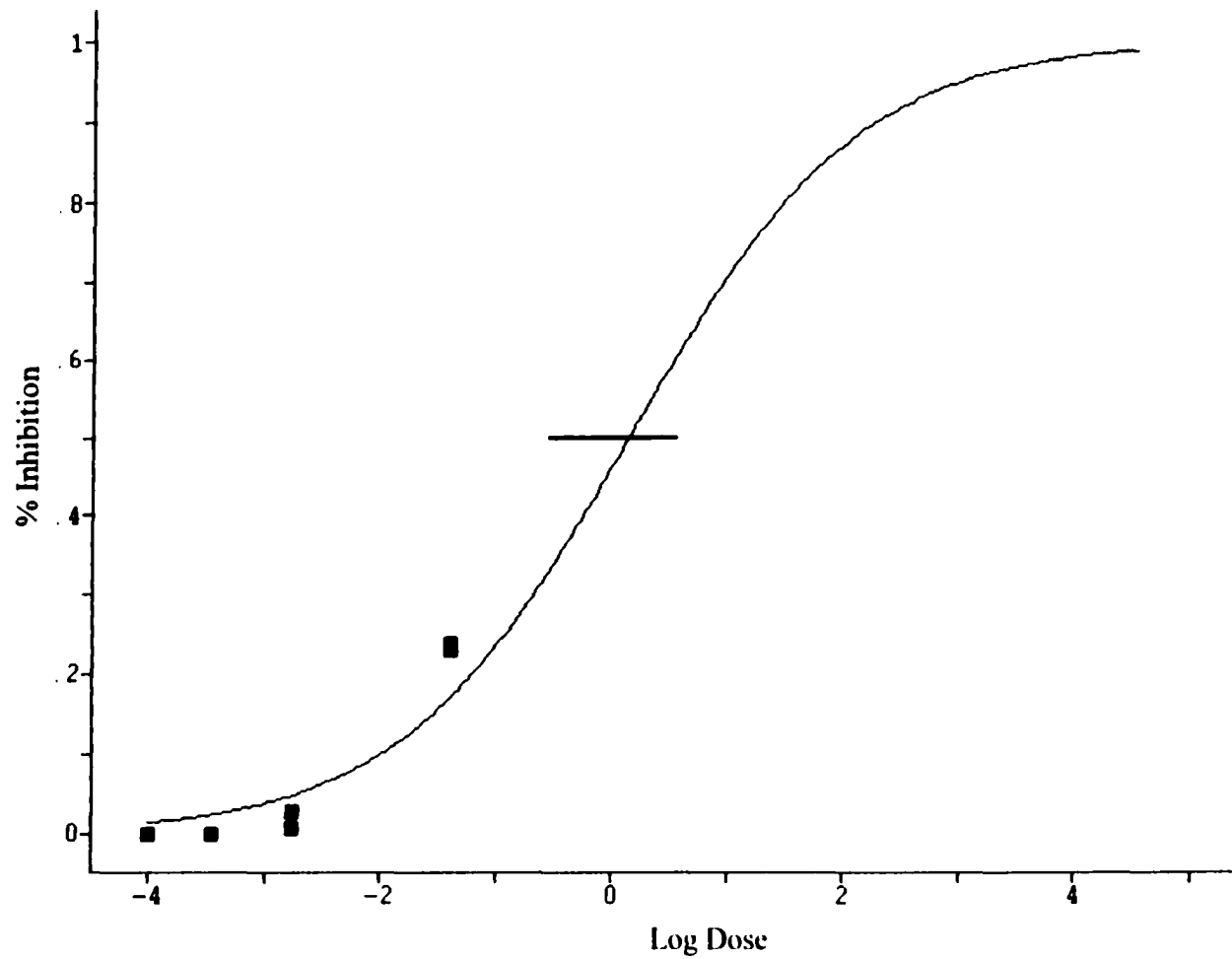


Figure 5.9. Logistic regression for Fraction 9 eluted with 20% methanol. F stat: 35.14, R square = 0.85413, ED<sub>50</sub> = 1.241, 95% Confidence Interval for ED<sub>50</sub>: 1.157 mg/ml to 1.735 using 100 bootstrap samples.

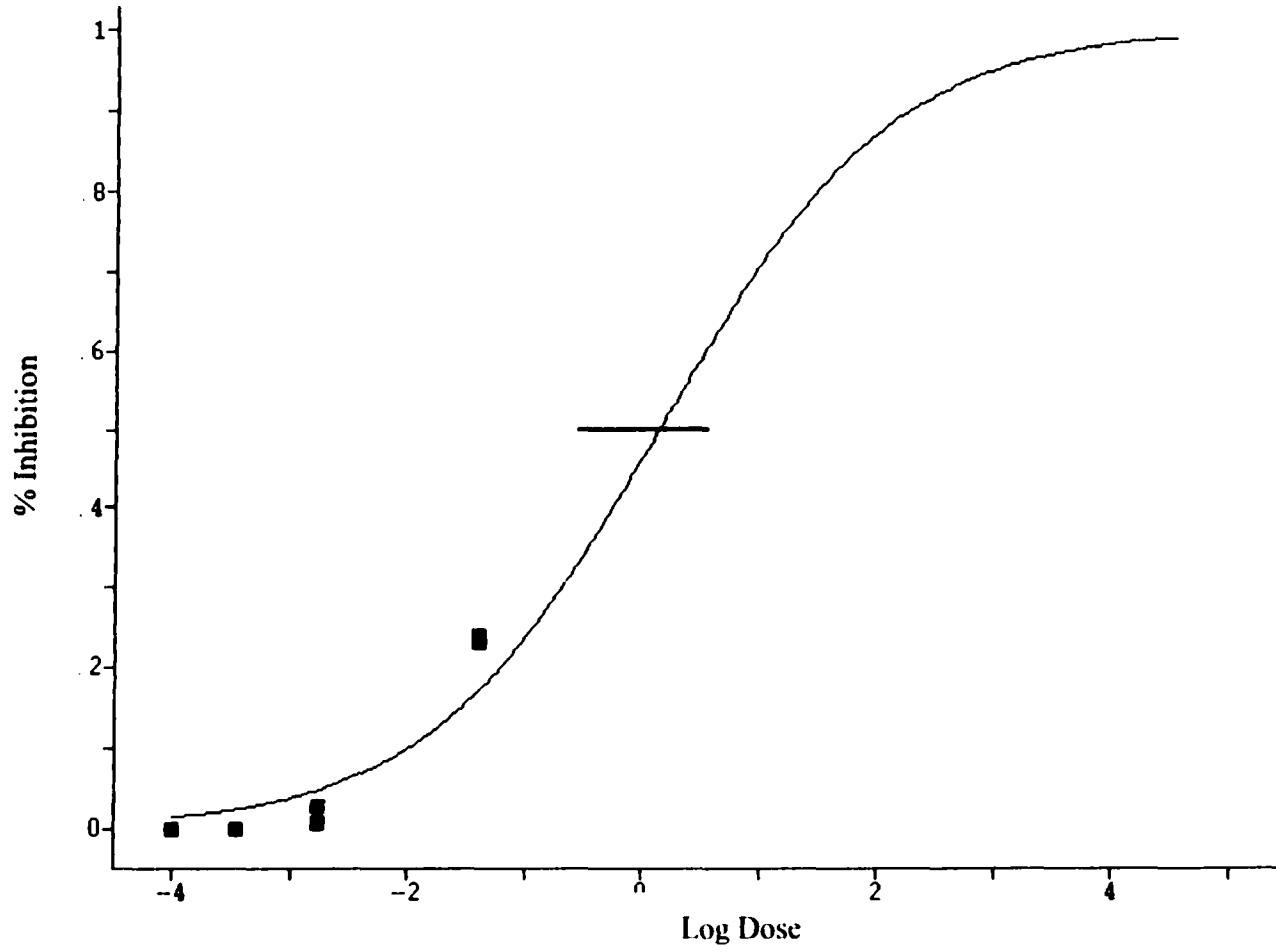


Figure 5.10. Logistic regression for Fraction 10 eluted with 30% methanol. F stat: 8.66, R square = 0.5906,  $ED_{50} = 1.211$ , 95% Confidence Interval for  $ED_{50}$ : 1.165 mg/ml to 1.257 using 100 bootstrap samples.

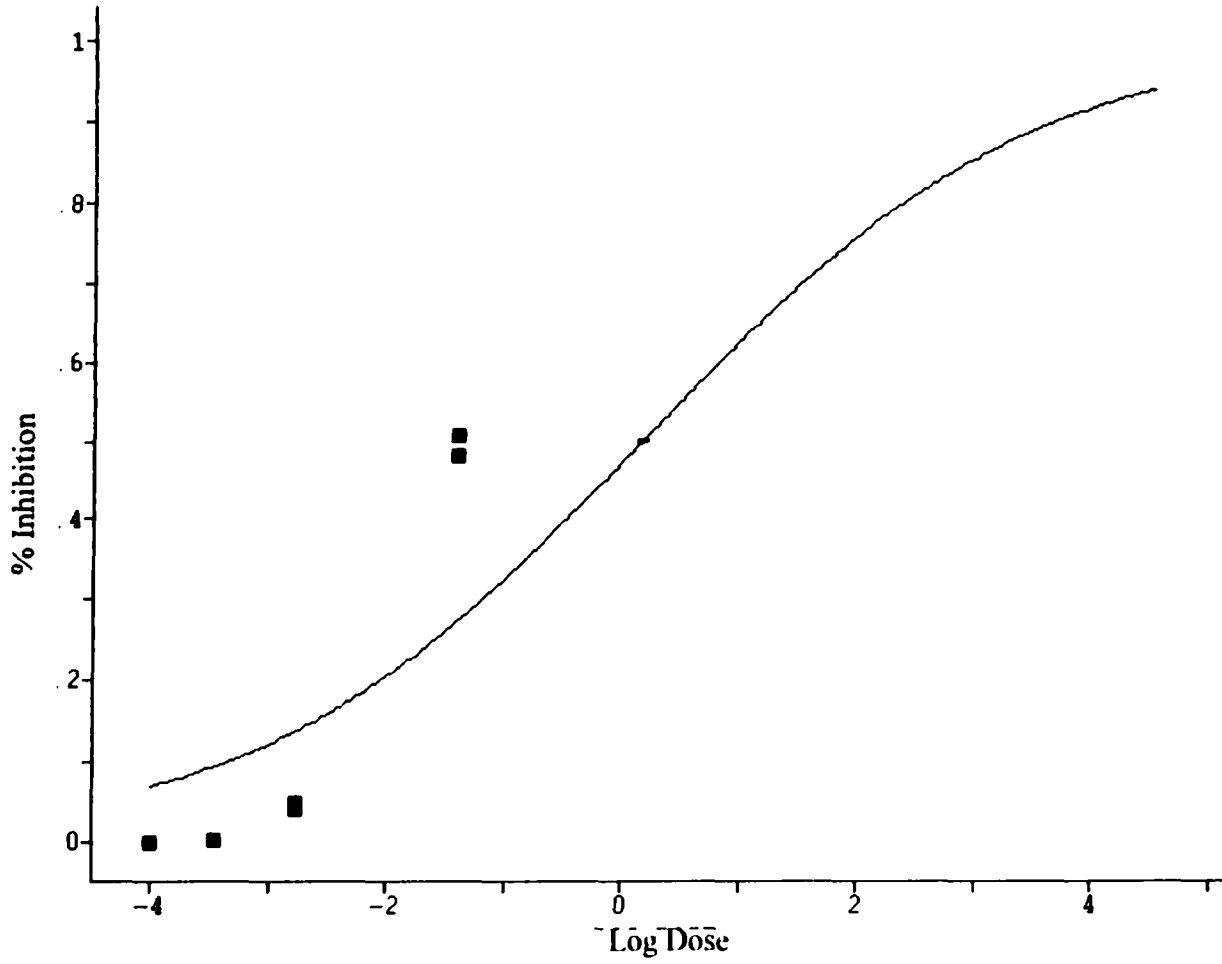


Figure 5.11. Logistic regression for Fraction 11 eluted with 30% methanol. F stat: 4.3, R square = 0.4173, ED<sub>50</sub> = 1.093, 95% Confidence Interval for ED<sub>50</sub>: .95 mg/ml to 1.235 using 100 bootstrap samples.

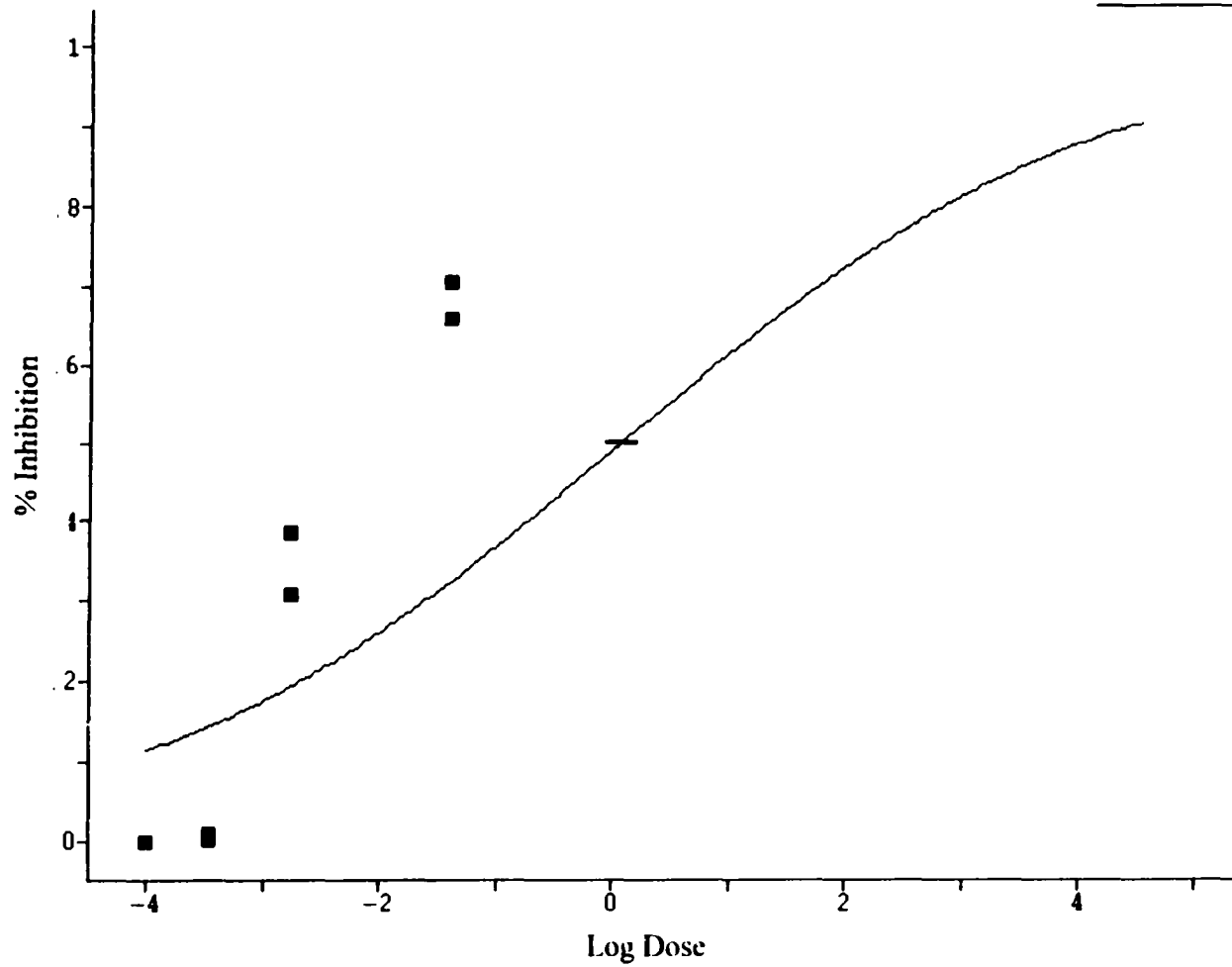


Figure 5.12. Logistic regression for Fraction 12 eluted with 30% methanol. F stat: 10.37, R square = 0.6335, ED<sub>50</sub> = 1.03, 95% Confidence Interval for ED<sub>50</sub>: .7 mg/ml to 1.36 using 100 bootstrap samples.

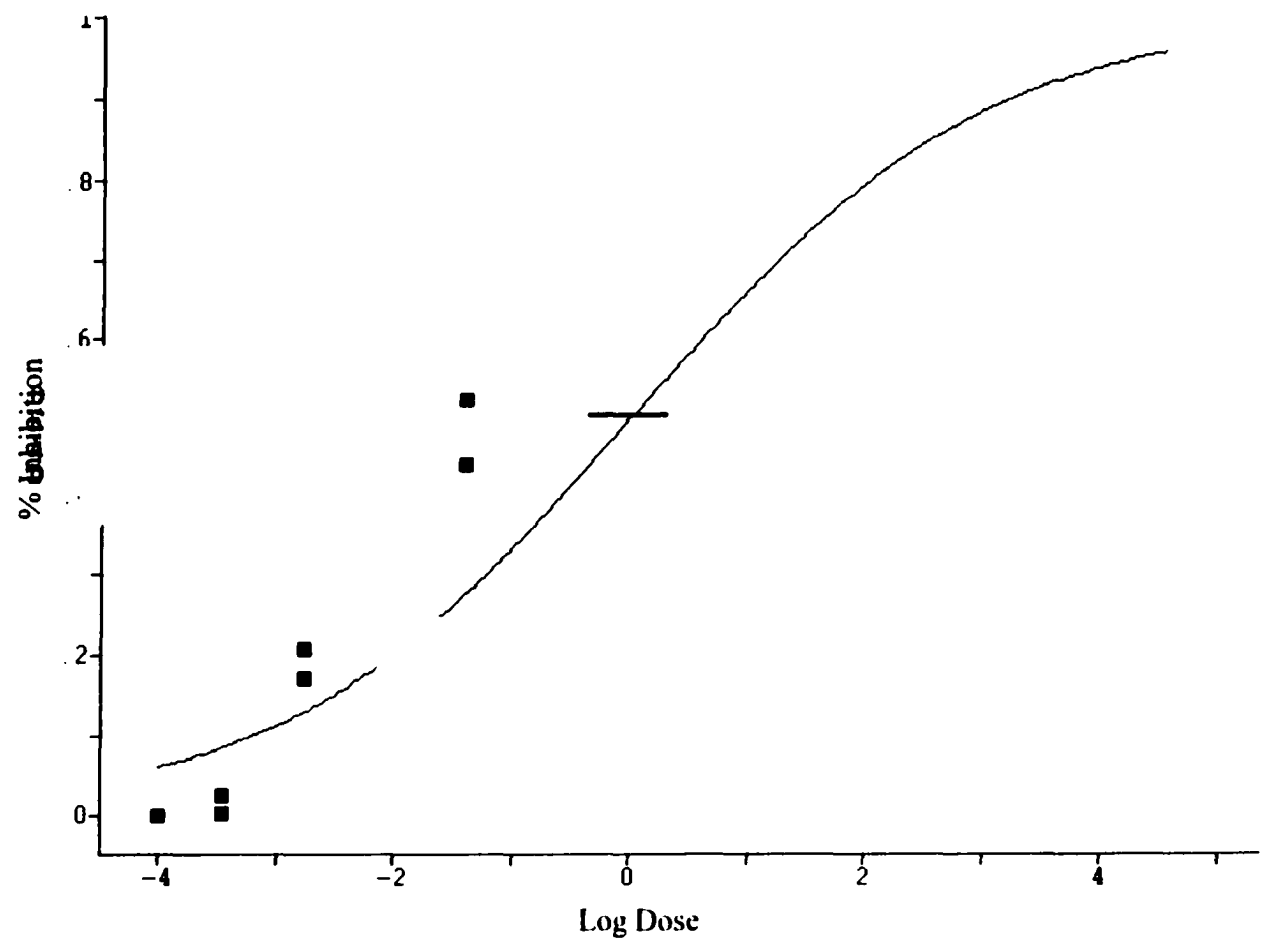


Figure 5.13. Logistic regression for Fraction 13 eluted with 40% methanol. F stat: 4.48, R square = 0.4275, ED<sub>50</sub> = 1.249, 95% Confidence Interval for ED<sub>50</sub>: 1.11 mg/ml to 1.39 using 100 bootstrap samples.

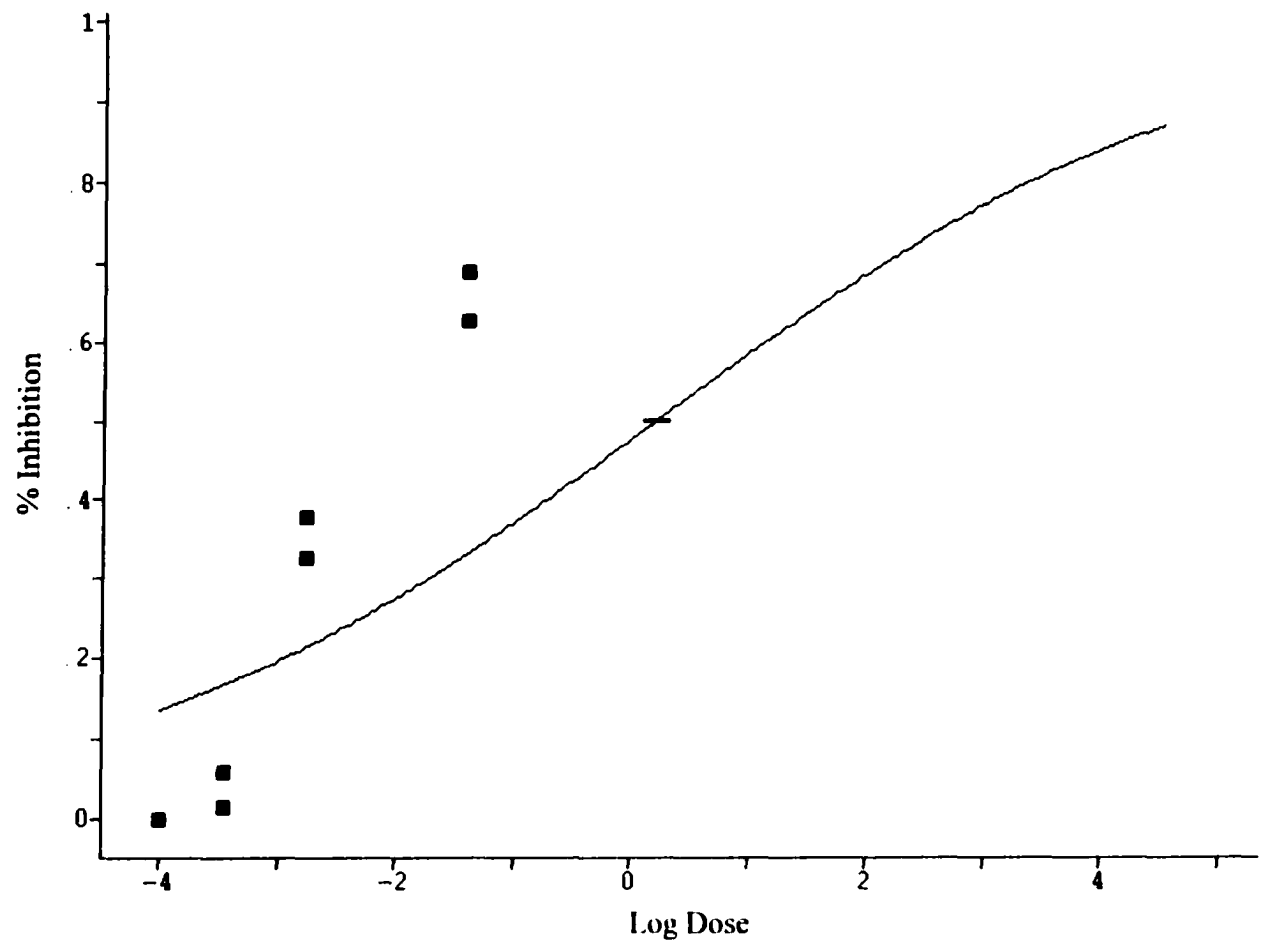


Figure 5.14. Logistic regression for Fraction 14 eluted with 40% methanol. F stat: 1.93, R square = 0.2437, ED<sub>50</sub> = 2.448, 95% Confidence Interval for ED<sub>50</sub>: 2.195 mg/ml to 2.701 using 100 bootstrap samples.

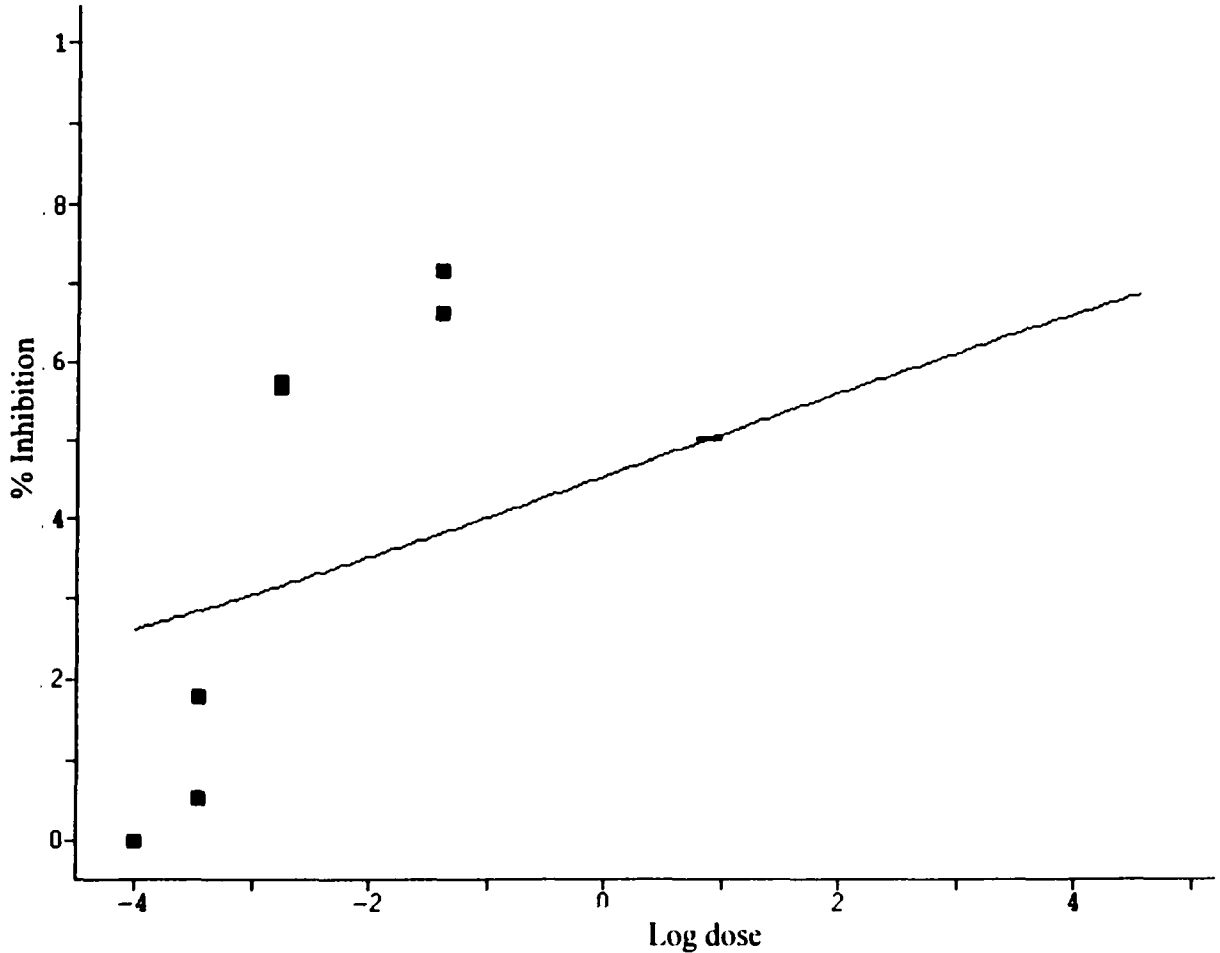


Figure 5.15. Logistic regression for Fraction 15 eluted with 40% methanol. F stat: 5.63, R square = 0.4842,  $ED_{50} = 1.012$ , 95% Confidence Interval for  $ED_{50}$ : 0.959 mg/ml to 1.066 using 100 bootstrap samples.

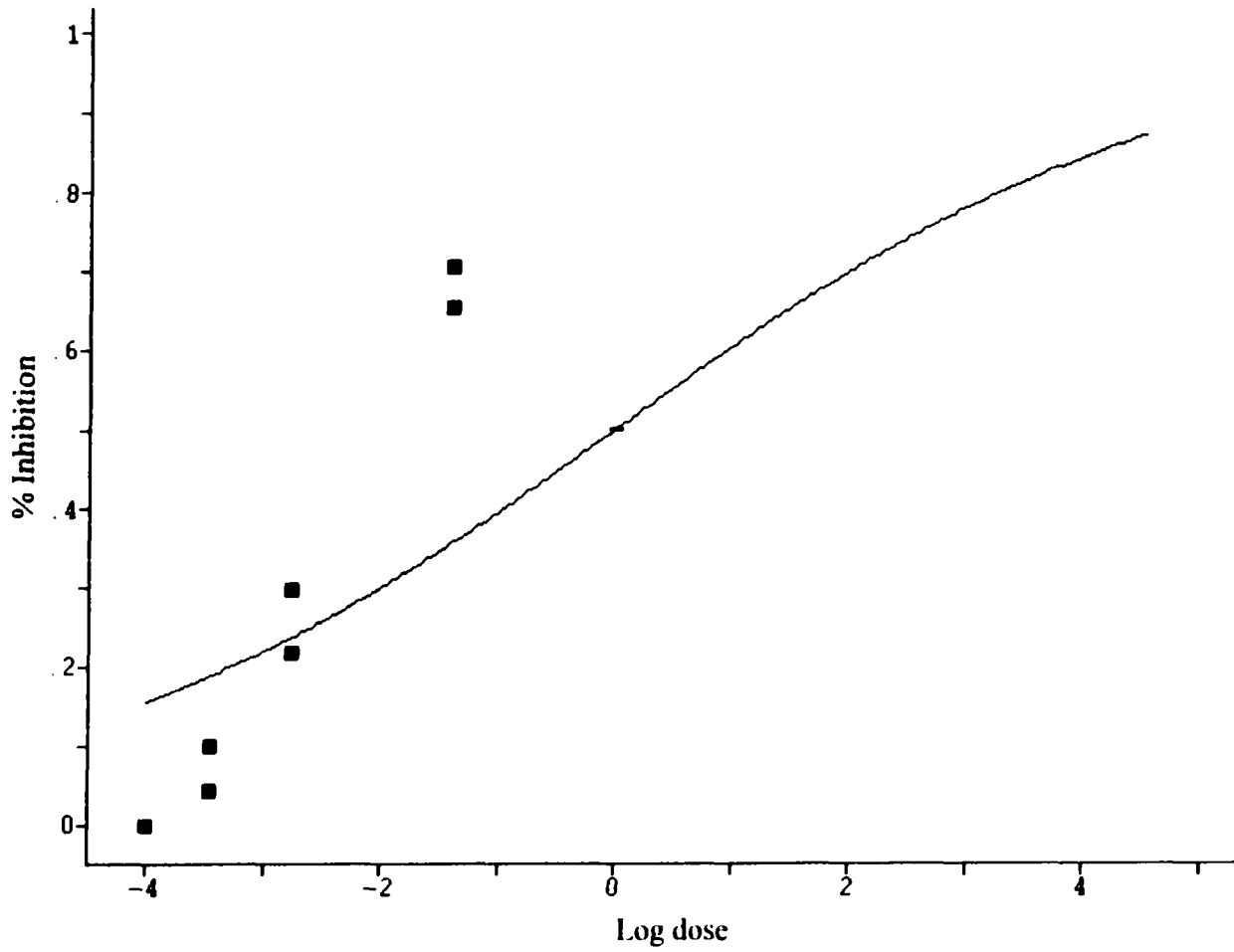


Figure 5.16. Logistic regression for Fraction 16 eluted with 50% methanol. F stat: 5.63, R square = 0.4842, ED<sub>50</sub> = 1.012mg/ml, 95% Confidence Interval for ED<sub>50</sub>: 0.959 mg/ml to 1.066mg/ml using 100 bootstrap samples.

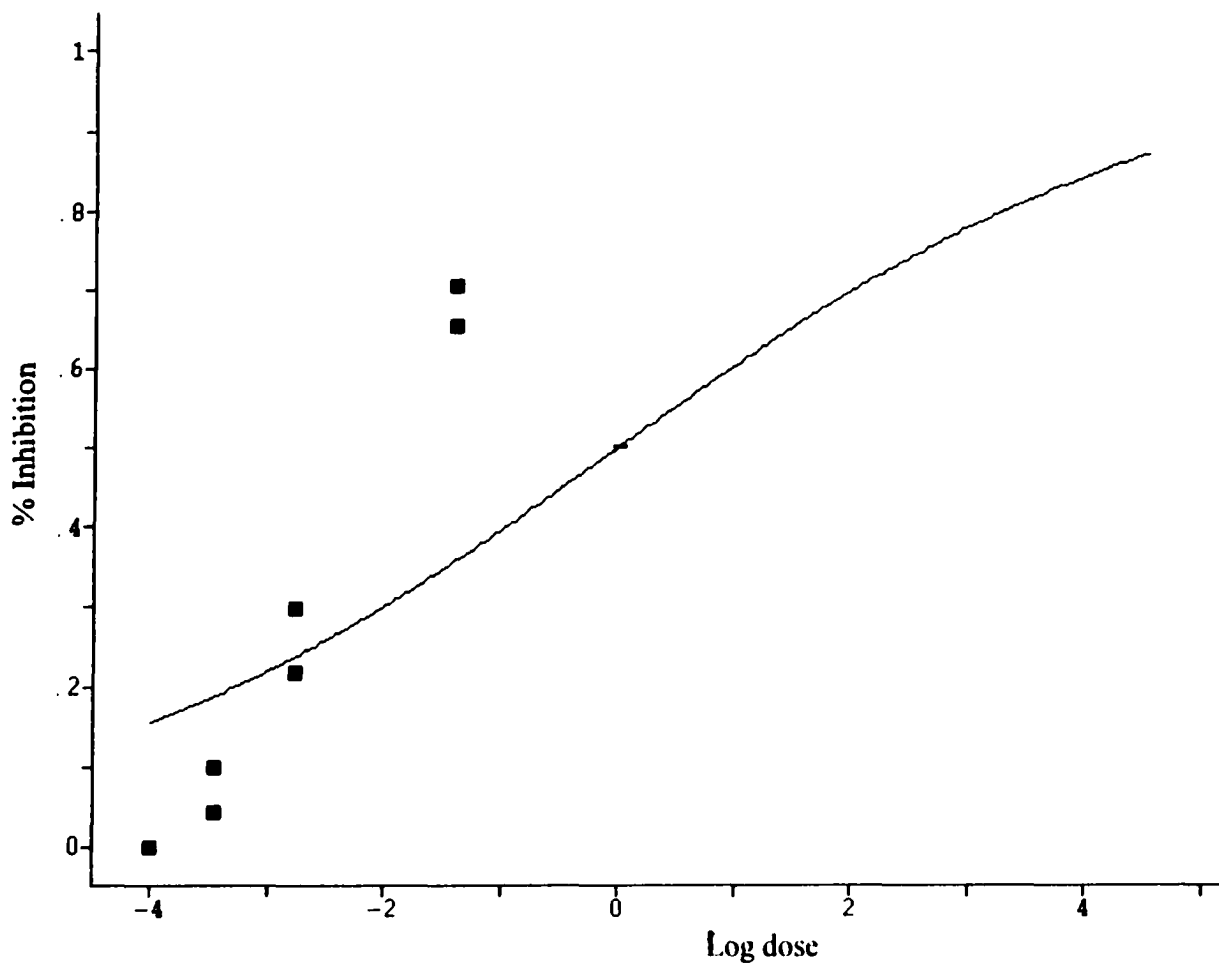


Figure 5.17. Logistic regression for Fraction 17 eluted with 50% methanol. F stat: 1.97, R square = 0.2473  
ED<sub>50</sub> = 1.0 mg/ml, 95% Confidence Interval for ED<sub>50</sub>: 0.678 mg/ml to 1.322 mg/ml using 100 bootstrap samples.

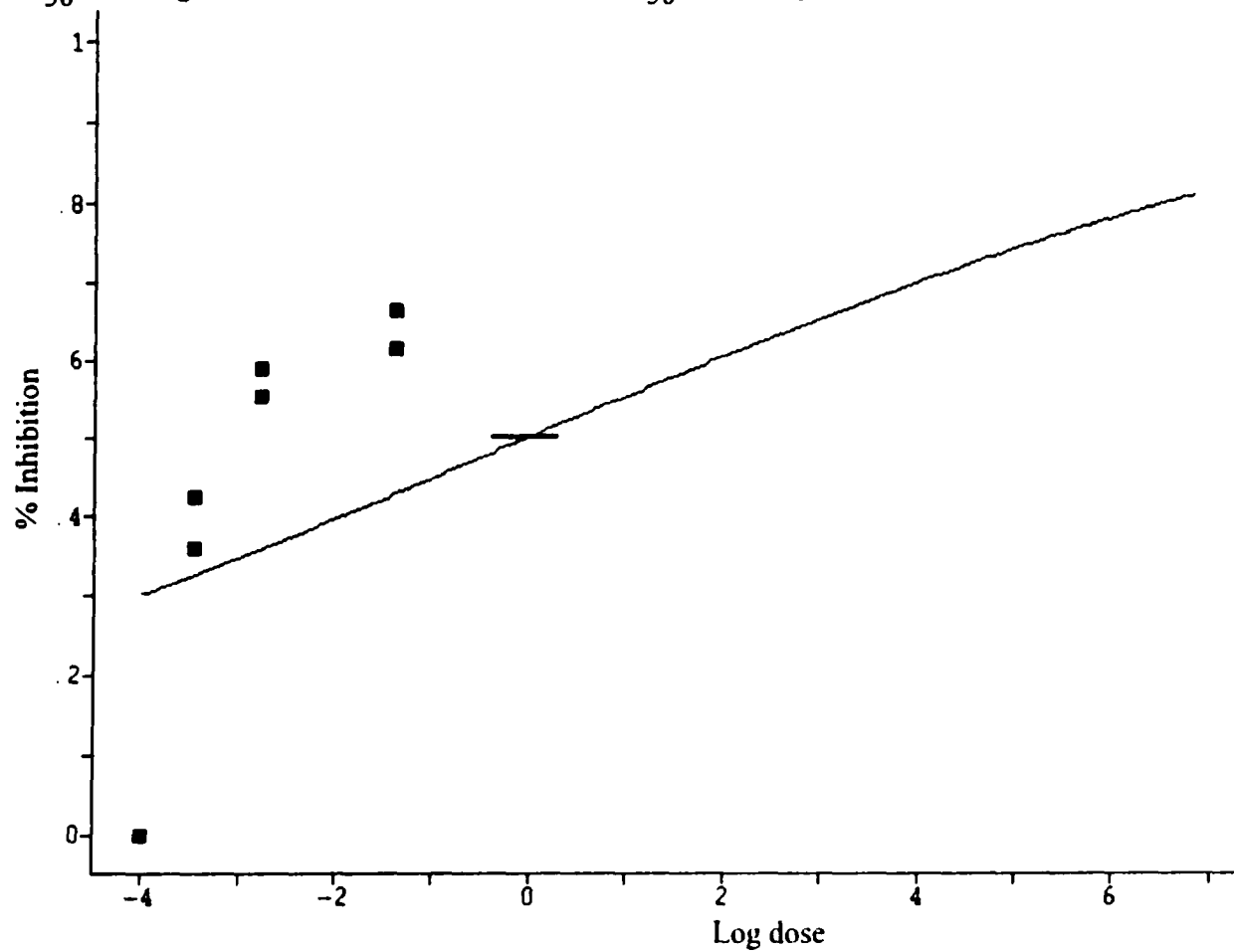


Figure 5.18. Logistic regression for Fraction 18 eluted with 50% methanol. F stat: 1.97, R square = 0.4391  
ED<sub>50</sub> = 1.047 mg/ml, 95% Confidence Interval for ED<sub>50</sub>: 0.873 mg/ml to 1.22 mg/ml using 100 bootstrap samples.

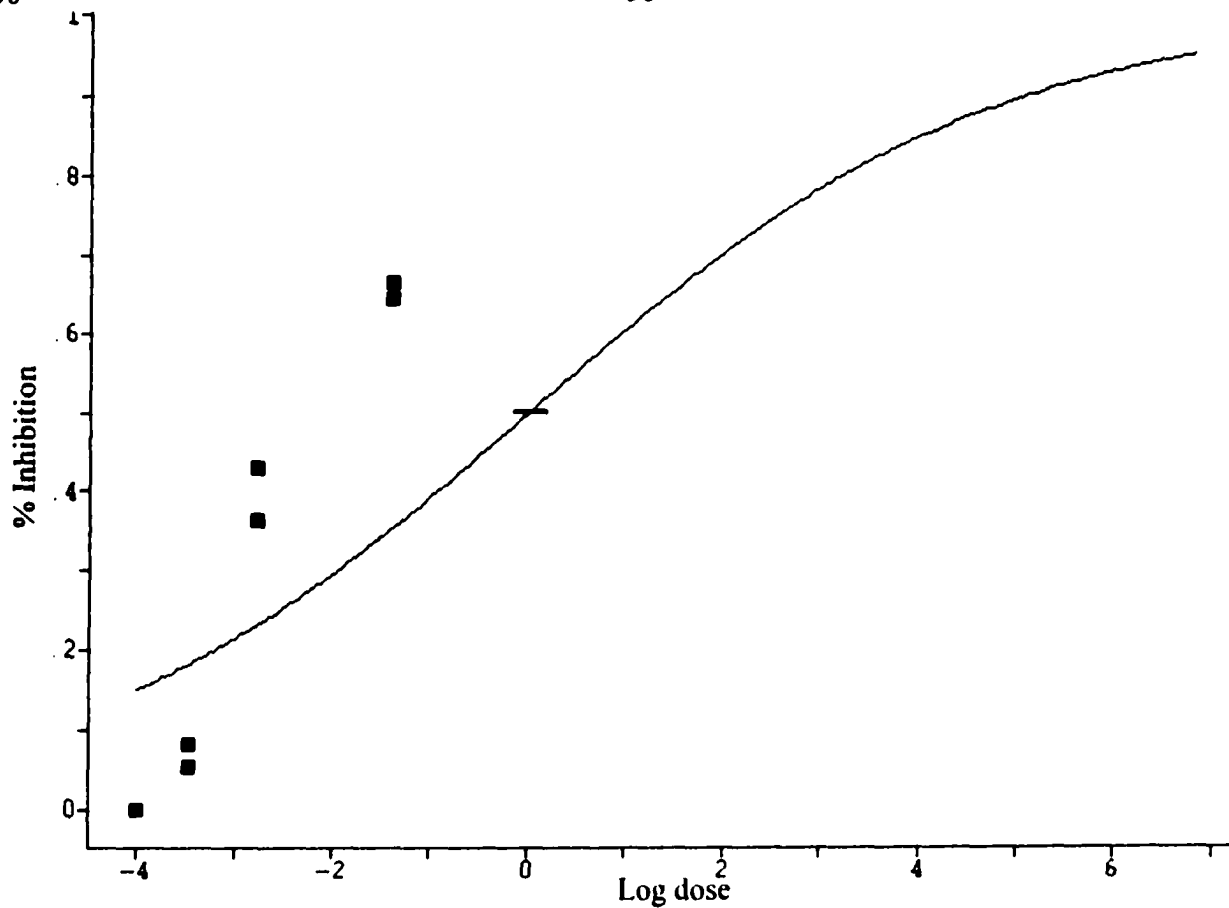


Figure 5.19. Logistic regression for Fraction 19 eluted with 50% methanol. F stat: 7.47, R square = 0.5545  
ED<sub>50</sub> = 1.001 mg/ml, 95% Confidence Interval for ED<sub>50</sub>: 0.999 mg/ml to 1.003 mg/ml using 100 bootstrap samples.

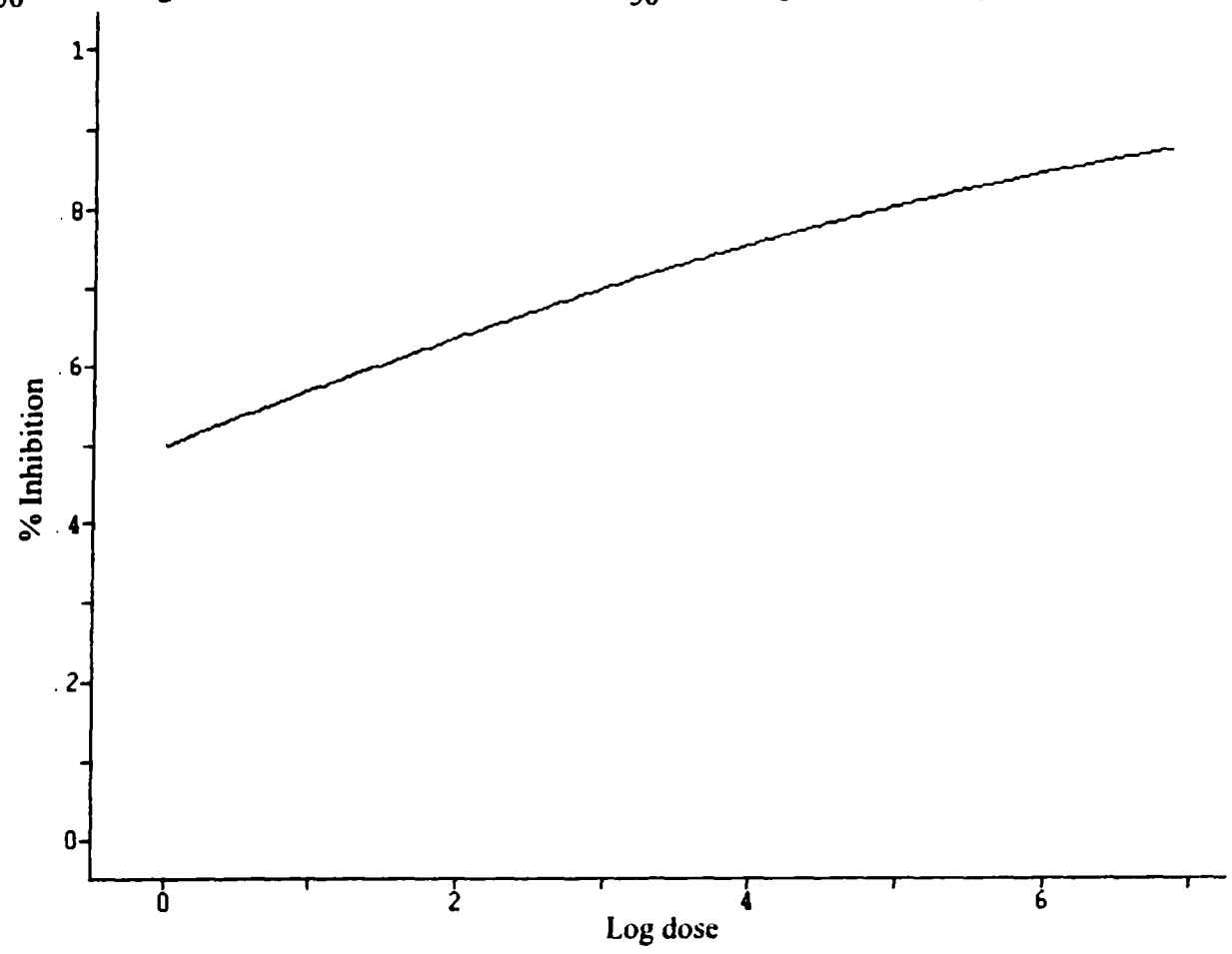


Figure 5.20. Logistic regression for Fraction 20 eluted with 60% methanol. F stat: 0.04, R square = 0.6012  
ED<sub>50</sub> = 1.001 mg/ml, 95% Confidence Interval for ED<sub>50</sub>: 0.900 mg/ml to 1.116 mg/ml using 100 bootstrap samples.

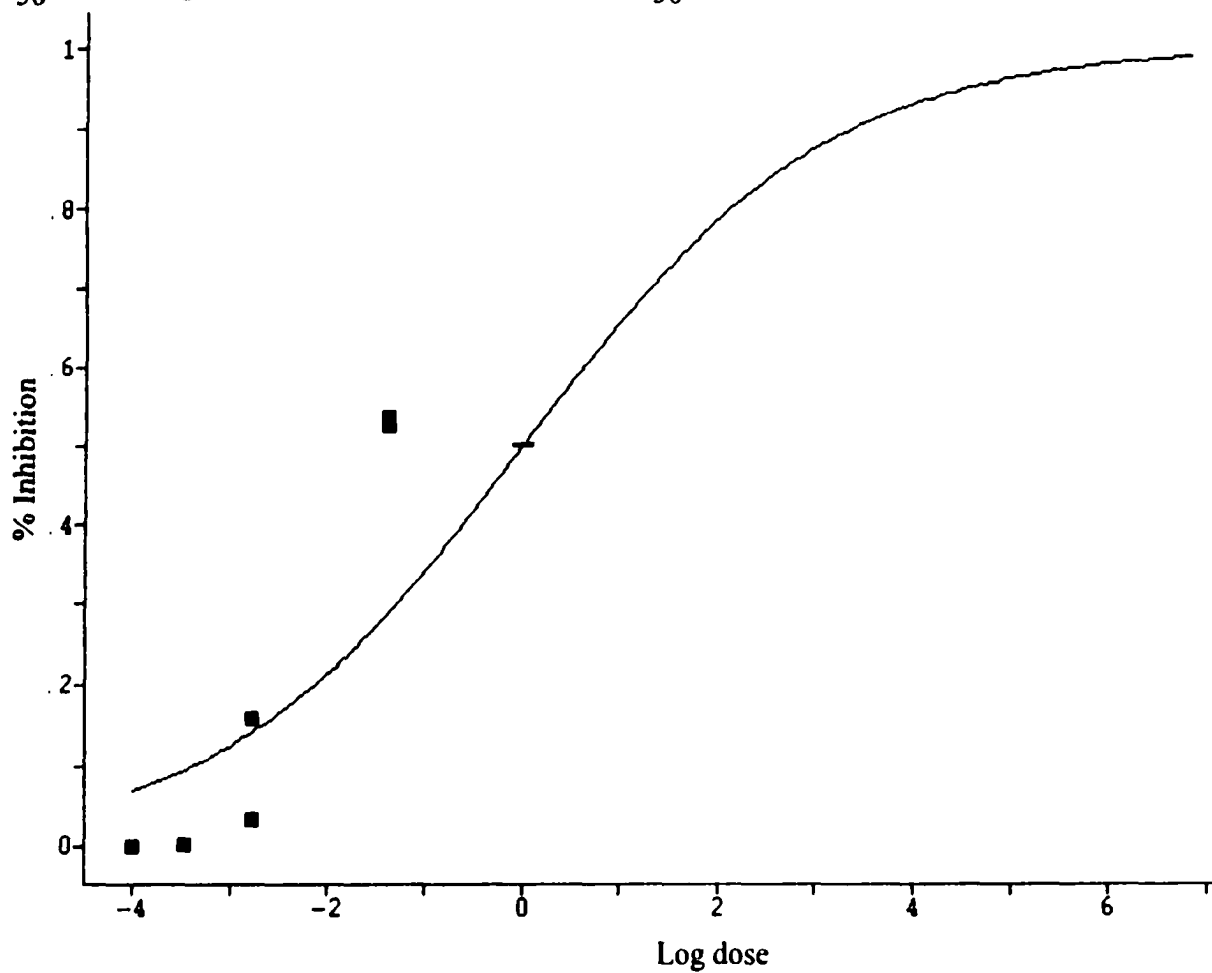
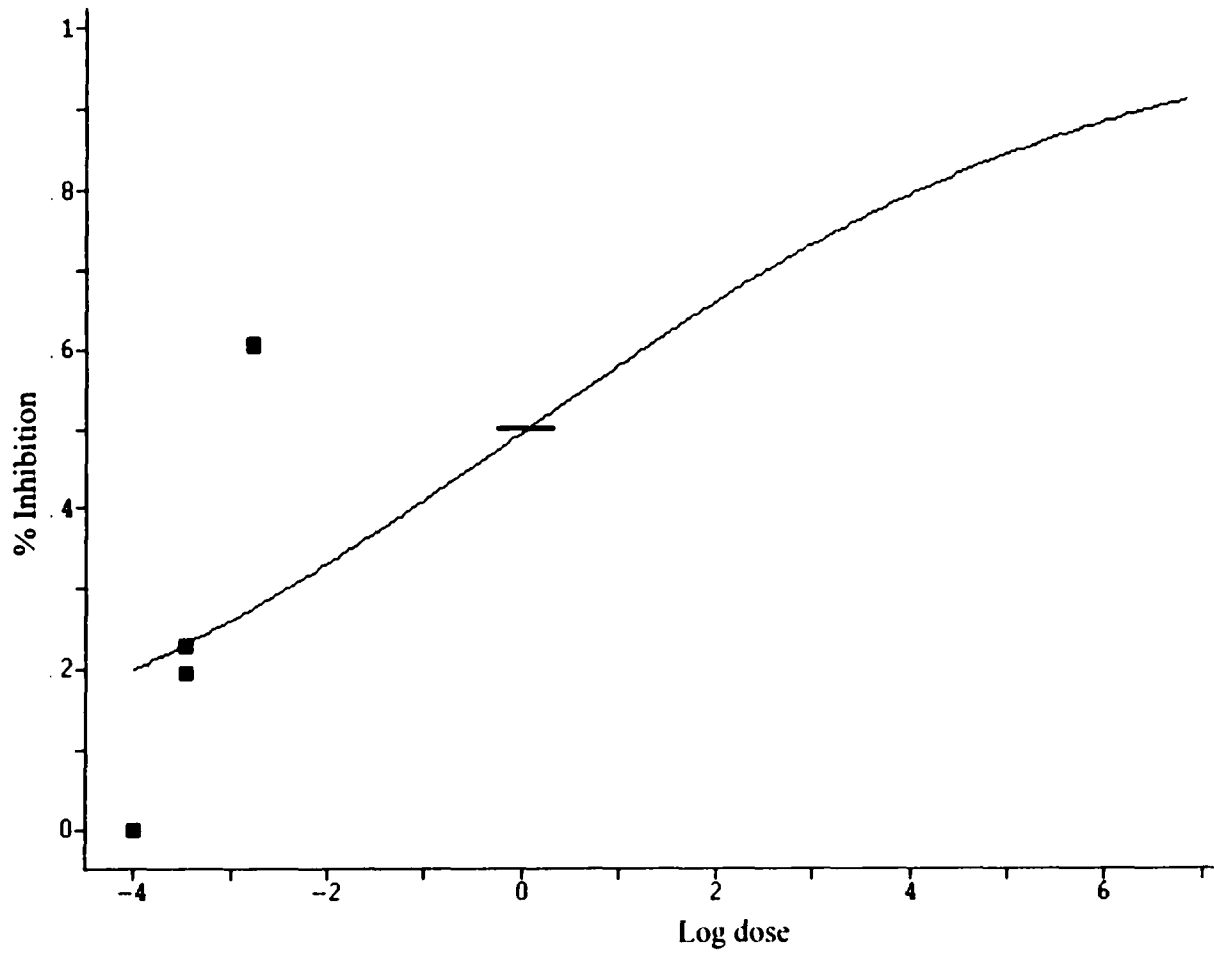


Figure 5.21. Logistic regression for Fraction 21 eluted with 70% methanol. F stat: 3.79, R square = 0.3871  
ED<sub>50</sub> = 1.075 mg/ml, 95% Confidence Interval for ED<sub>50</sub>: 0.769 mg/ml to 1.382 mg/ml using 100 bootstrap samples.



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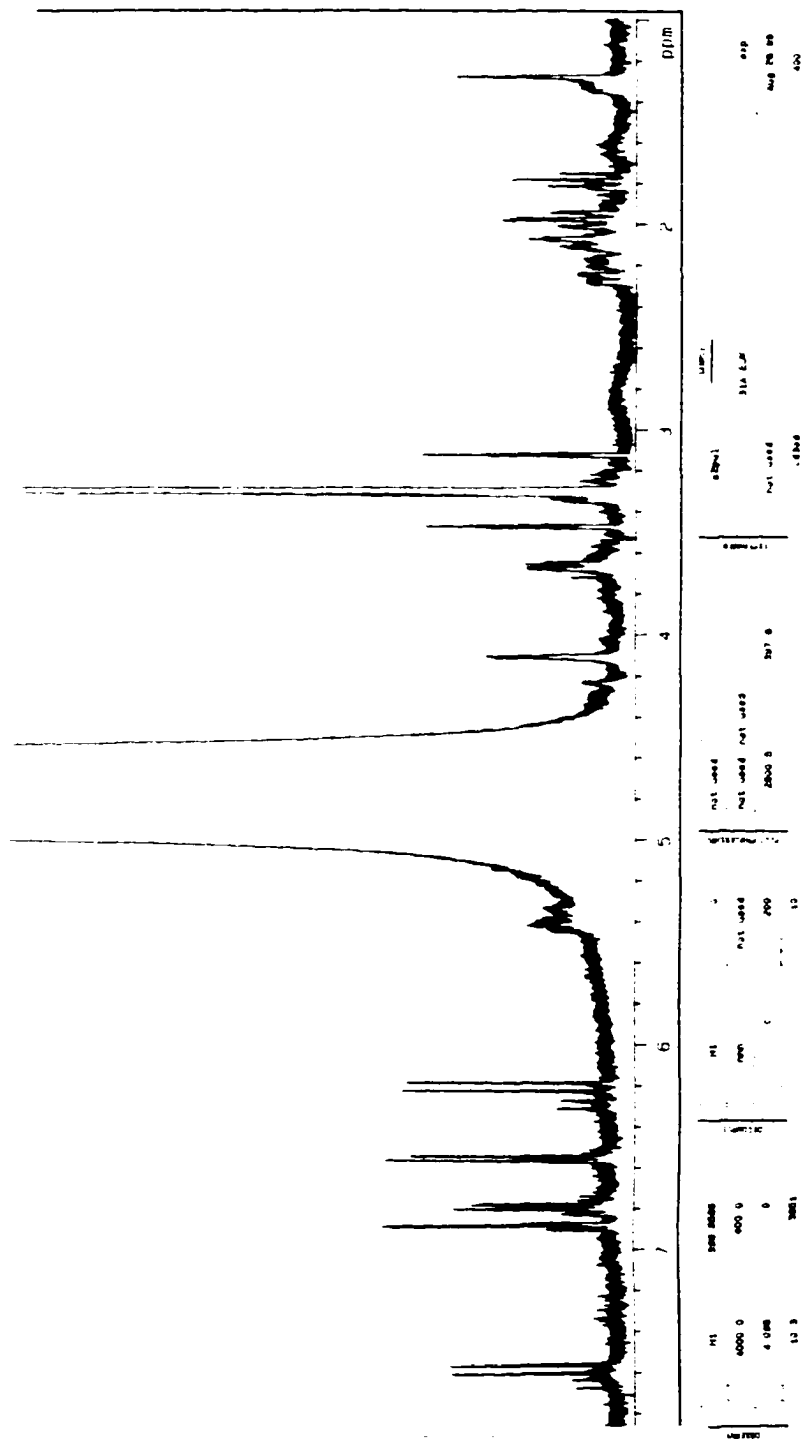
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## **Appendix**



Figure A.2 NMR of a pure fraction from preparative HPLC.



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