THE EFFECTS OF PINE PHENOLICS ON GYPSY MOTH AND A PATHOGENIC NUCLEAR POLYHEDROSIS VIRUS

Clifford W. Beninger

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ABSTRACT

Over 400 tree species are primary or secondary hosts of gypsy moth. Interactions between host plant compounds and a viral pathogen such as nuclear polyhedrosis virus (NPV) in vivo may explain gypsy moth host preference in later instars. Phenolic extracts from pines negatively affected growth of 2nd and 3rd but not 4th instars when incorporated into artificial diet at low concentrations. The aglycone quercetin, and two of its glycosides found in pines, rutin and quercetin-3-O-glucoside, also reduced growth for 2nd and 3rd instars. When 3rd instars were dosed with NPV (60,000 PIBs/larva) and fed pine extract (0.1%) in diet, growth was reduced and mortality increased. Survivorship to adulthood was not significantly higher than control for 3rd instars fed pine extracts over a range of viral doses, nor was it higher for 4th instars dosed at 60,000 PIBs/ larvae. The three flavonoids also tended to have negative and synergistic effects on growth and survivorship of 3rd and 4th instars dosed with 60,000 PIBs/larva. These results suggest that early instars of gypsy moth are quite sensitive to pine phenolics in diet, which may explain why they do not feed on pines in those instars. Gypsy moth is more tolerant of pine phenolics in later instars but these results do not support the hypothesis that gypsy moth later instars which feed on plant hosts such as pine, gain some protection from infectivity of NPV due to the phenolics present in those hosts.

RÉSUMÉ

Plus de 400 espèces d'arbres sont les hôtes primaires et secondaires de la spongieuse (*Lymantria dispar* L.). Les interactions entre les composés chimiques de ces plantes et un agent pathogène viral comme le virus nucléopolyèdre (VNP) in vivo peuvent expliquer la préférence alimentaire des spongieuses âgées, c-à-d celles du quatrième stade et plus. Lorsque des extraits phénoliques provenant des pins sont inclus dans un régime artificiel à faible concentration, ceux-ci influencent d'une façon négative la croissance des larves des deuxième et troisième stades mais non celle du quatrième stade. Deux glycosides, la rutine et la quercetine-3-O-glucoside, ainsi que la quercetine sans glycoside, des flavonols retrouvés dans les pins, réduisent la croissance des larves aux deuxième et troisième stades. De plus, les larves du troisième stade dosées avec 60 000 corps poly-inclusionnaires (CPI/larve) du virus nucléopolyèdre et nourries avec ces extraits de pins à 0.1% présentent une réduction de croissance et une augmentation de la mortalité. Cependant, les études de survie jusqu'au stade adulte n'ont pas démontré des survies supérieures des larves de troisième stade infectées avec le VNP (60 000 CPI/larve) par rapport aux larves nourries avec des extraits des pins, elles aussi infectées avec une gamme variée de concentrations du virus nucléopolyèdre. Des résultats identiques ont été obtenus pour la survie des larves de quatrième stade infectées avec le VNP (60 000 CPI/larve). Ceci indique que les trois flavonols nommés ci-haut ont un effet négatif et synergique sur la croissance et la survie des spongieuses de troisième et de quatrième stades dosées avec 60 000 CPI/larve. Les résultats précédents suggèrent que les jeunes spongieuses sont sensibles aux composés phénoliques des pins. Ceci explique pourquoi ces jeunes larves ne s'alimentent pas des pins. Une fois rendue au quatrième ou au cinquième stade, la spongieuse est plus tolérante aux phénols de pins. Nos observations ne supportent pas l'hypothèse que ces spongieuses âgées s'alimentent des pins pour obtenir une protection ou une inhibition du virus nucléopolyèdre causée par l'ingestion des phénols retrouvés dans leurs hôtes, .

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CHAPTER ONE: General Introduction

It is well known that the introduction of exotic species, plant or animal, into areas where they are not endemic can severely disrupt native communities of plants and animals (Liebhold et al. 1995). In particular, accidental or intentional introduction of plant feeding insects can cause severe disruption and economic damage to commercial crops and forests. Once such an introduction occurs and the exotic insect becomes established it is essential that we understand the potential range of host species on which it feeds, and look for potential control agents, be they chemical or biological. In a practical effort to control introduced species we must: 1) identify plant hosts, 2) determine host species composition of areas in the path of spread, 3) examine the secondary chemistry of hosts, and 4) determine the effect that host chemistry has on feeding of the insect. In addition, knowledge of the impact of plant compounds on viral pathogens may be important for determining the utility of viruses as pest management tools.

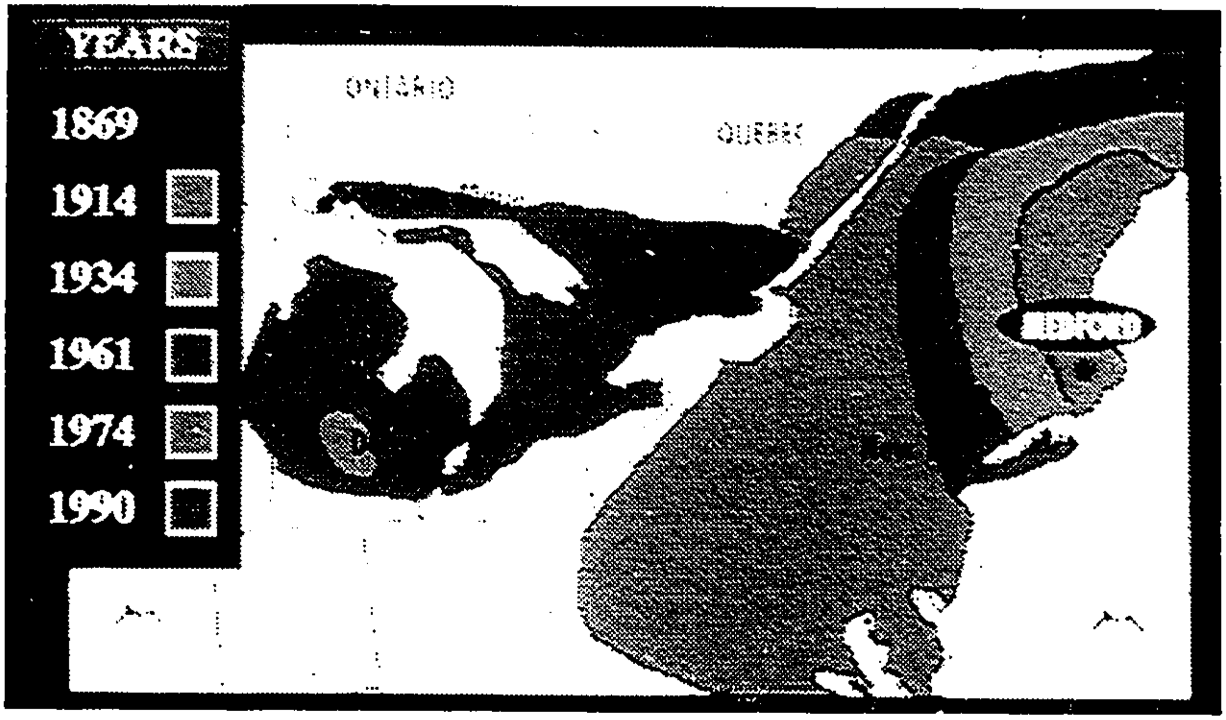
An accidental introduction of an exotic insect herbivore occurred in the eastern United States in 1869. The European gypsy moth (*Lymantria dispar* L.) (henceforth GM) spread from its point of introduction throughout the eastern United States and Canada (Brown 1968, Talerico 1981, Mauffette et al. 1983, Constable et al 1992, Allen et al.1993) and has been responsible for severe defoliation of forests in these areas. The GM has recently become established in the Great Lakes states and defoliation has increased 10-fold over the years 1988-1991 (Arteel and Lindroth 1992).

The life cycle of GM in North America is well understood, and the following summary has been extracted from (Leonard 1981) and (Keena and ODell 1993). The GM emerge from pupae in mid-July, although the date may vary with latitude. Males generally emerge 1-2 days before the females. Females are flightless and begin emitting a powerful pheremone to attract males. Both sexes do not feed as adults and after mating the female will lay a number of eggs which varies from 100 to over 1,000. About 8 or 9 months is spent in the egg stage with the 1st instars emerging in spring. Growth of the larvae is affected by temperature and moisture as well as quality and quantity of food. The number of instars is variable. Males may have 5-7 instars while females can have 6-8 instars in the wild. For laboratory reared strains, in particular New Jersey Standard Strain (NJSS) which was used for my experiments, females may have between 5 and 8 instars, while males have 5-7 instars. For the strain of NJSS reared at FPMI and used in my experiments I found that males never had more than 5 instars and females generally had 6-7 instars. After the final larval stage is reached the larvae pupate, and remain as pupae for about 2 weeks. Several hours after emergence males and females are ready to mate.

In order to identify forest types susceptible to GM outbreak, the acceptablility of tree host species in the east and southeast of North America has been much studied in recent years. Forest stand susceptibility to attack by GM is determined primarily by the composition and abundance of tree species in the forest (Barbosa 1978a, Mauffette et al. 1983, Lechowicz and Mauffette 1986, Kleiner and Montgomery1994). For example, it has long been recognized that mixed forests containing oak (*Quercus* L. spp.), poplar (*Populus* L. spp.), maple (*Acer* L. spp.) and pines (*Pinus* L. spp.) are the most susceptible to GM outbreak (Lechowicz and Jobin 1983). Red oak (*Quercus rubra* L.) and white oak (*Quercus alba* L.) are the most preferred hosts for early instars (Barbosa and Capinera 1977), but red maple (*Acer rubrum* L.), American beech (*Fagus grandifolia* Ehrh.), sugar maple (*Acer saccharum* Marsh.), eastern hemlock (*Tsuga canadensis* L.) Carr. and grey birch (*Betula populifolia* Marsh.) were also acceptable hosts for 1st and 2nd instars (Hough and Pimental 1978, Barbosa and Greenblatt 1979). Early instars generally do not survive well on pines such as white pine (*Pinus strobus* L.), and for this reason only mixed forests composed of conifer and preferred deciduous tree species were considered at risk. For example, Barbosa et al. (1983, 1986) found that mortality of GM was 100% when reared exclusively on white pine but that later instars (3rd -6th) individuals reared on a preferred host and then switched to white pine, had greater fecundity than individuals reared only on the preferred host.

As GM continues to expand its range north and westward (Fig. 1) the dominant tree species composition of the forest changes from a mix of oak, maple, elm (*Ulmus* L. spp.), red pine (*Pinus resinosa* Ait.) and white pine to one dominated primarily by conifers such as pines and Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) although these may also be mixed with birch and poplar (Hosie 1990). GM will therefore likely encounter pine species more often in its diet. Concern for the susceptibility of Pacific western forests to gypsy moth which are composed of various pines mixed with Garry oak and red alder led to the discovery that these two western deciduous trees are also very suitable hosts for all instars of GM in western North America (Miller et al. 1991). Tree species composition of Pacific western forest may then support outbreaks similar to those in the east where white and red oak are the primary hosts for the early instars.

Fig. 1. Range expansion of gypsy moth in North America 1869-1990.


YEARS
1869
1914 ▨
1934 ▨
1961 ■
1974 ▨
1990 ■
ONTARIO
QUEBEC
BEDFORD

The suitability of various conifers as a host for GM and the well-documented host switching from deciduous trees in early instars to conifers in later instars have thus become the subject of recent study. First, it appears that not all conifers are unsuitable to early instars of GM. Miller and Hanson (1989) found that a number of conifers in the genera *Pinus*, *Larix*, *Cedrus* and *Pseudotsuga* provided a diet most suitable for development from 1st instars to adults. Similarly Joseph and Kelsey (1994) found that Douglas-fir was acceptable to all instars of GM larvae which meant that Douglas-fir alone in the Pacific Northwest could maintain GM populations without the presence of other hosts.

Despite the recent interest in GM feeding preferences with regard to conifers, we do not have much information on how all GM instars will fare on some of the common pine species in central and western pine forests. Of the 105 species of pines in the world there are four pine species common to central Canada: jack pine (*Pinus banksiana* Lamb.), red pine, scotch pine (*P. sylvestris* L.- introduced from Europe) and white pine. All of these are economically important species: jack, red and white pine are used in the timber industry; while scotch pine is grown wild and on plantations for use as Christmas trees (Mirov 1967). It is therefore important from a practical point of view that we assess the direct threat of defoliation to these species in order estimate potential economic losses of timber.

In addition to our lack of understanding of how all GM instars will fare on pines, it is not known with certainty why GM will switch to pine species from deciduous hosts preferred by early instars. Mauffette and Lechowicz (1984) found that hosts preferred by larvae had considerably fewer pupae on and near these trees than hosts such as pine. They suggested that this difference could result from host differences in larval mortality or late instar migration between hosts, and that this behaviour may contribute to the broad polyphagy of GM. Rossiter (1987) also suggested that secondary host use contributed to host range expansion, and that it also may convey a measure of protection from disease-causing NPV which is not as abundant on non-host plants.

In many cases host choice and feeding are at least in part determined by the secondary chemistry of the tree species. We know much about the secondary chemistry of some hosts for GM, but very little about the compounds found in pine species and how these interact with all GM instars. For example, in a number of unfavoured host plants alkaloids seems to deter feeding by GM (Barbosa and Krischik 1987, Barbosa et al. 1990ab). Iridoid glycosides, when included in artificial diet at concentrations of 3.6 and 7.2%, significantly reduced the weight of wild larval GM (Bowers and Puttick 1989) indicating that these compounds as well as alkaloids play a role in host use by GM. The quinone juglone found in leaves of trees in the family Juglandaceae may explain why GM fares poorly on these trees. The GM larvae fed diets with high levels of the quinone juglone had much poorer growth and development than those fed diets without juglone added (Lindroth et al. 1990).

The role tannins (a class of polyphenolics) play in the feeding ecology of GM is somewhat more complex than the compounds mentioned above. In early instars GM feeds on tree species which tend to have high levels of tannins. They may not affect GM greatly because the highly alkaline gut conditions (Schultz and Lechowicz 1986) and abundance of surface-active phospholipids (Ian de Veau and Schultz 1992) prevent association of tannin-protein complexes which reduce digestibility of the food. However, high levels of total phenolics and tannins due to defoliation of oak species correlated with decreased pupal mass and fecundity for GM (Rossiter et al. 1988). Tannins also have a negative effect on weight of surviving sons and daughters of GM females (Rossiter 1991). The effect that other phenolic compounds such as simple phenolics have on GM feeding has been investigated even less. Lindroth and Hemming (1990) found that tremulacin (from poplar), a phenolic glycoside, when incorporated into diet at 3% reduced growth of 4th instar larvae. However, other phenolics such as flavonoids have not been examined for their effect on feeding by GM.

Flavonoids are a ubiquitous, abundant and diverse class of phenolics, but their interactions with herbivorous insects have not been well studied (Strong et al. 1984, Harborne 1989). It is estimated that almost 2% of all carbon photosynthesized by plants (1.0 x10^9 tons annually) is converted into flavonoids or closely related compounds (Markham 1982). Prior to 1992 the only flavonoids isolated from pines were pinocembrin (flavanone) and pinobanksin (flavonol) (Markham 1982), but recent work indicates that pines also have a rich flavonoid profile (Abou-Zaid and Nozzolillo 1991).

Flavonoids from pines may stimulate or deter feeding. Flavonoids isolated from elm bark are stimulants to adapted species such as *Scolytus multistratus* (Doskotch et al. 1973), and flavonoids derived from non-host plants are deterrent to the same species (Gilbert et al. 1967). Flavonoids act as feeding deterrents to *Bombyx mori* from mulberry leaves (Hamamura et al. 1962), and to *Agasicles* sp. from alligatorweed (Zielske et al. 1972). Flavonoids from pines have not been tested as feeding deterrents but there are many examples in the literature that demonstrate that flavonoids as a class deter feeding by insects (Elliger et al. 1980, Roy & Bergeron 1990, Dreyer et al. 1981, Todd et al. 1971, Dreyer & Jones 1981, Fisk 1980, Shaver & Lukefahr 1969).

How flavonoids and other plant secondary compounds interact with the third trophic level is also not well understood. We know little about how compounds, in particular compounds from a secondary host such as pine, interact with pathogens, parasites and predators of insects in general and GM in particular. This interaction between secondary chemistry of host species and the third trophic level has become increasingly important since parasites (Reardon 1981), pesticides (White et al. 1981), microbials such as *Bacillus thuringiensis* (Dubois 1981) and viruses are now all being employed to control GM populations in central and western Canada. If host secondary chemistry can harm or increase efficacy of other biological control agents, then conceivably some populations of GM may be more or less susceptible to biological controls based on the chemical profile of the tree species they are feeding on.

For example, tree species (and the secondary chemicals in each species) such as white oak can support high levels of GM parasitoid survival (Werren et al. 1992). Phenolic glycosides found in host trees act along with a bacterial pathogen, *Bacillus thuringiensis*, negatively and additively affect growth and survival of larval GM (Arteel and Lindroth 1992). Host plant foliage can also inhibit fungal infection of GM Hajeck (1989a). In addition, Keating and Yendol (1987), and Keating et al. (1988) found that GM larvae fed preferred host foliage infected with nuclear polyhedrosis virus (NPV) suffered significantly less mortality than those fed alternate host foliage inoculated with the same concentration of NPV. Keating et al. (1989) and Keating et al. (1990) found that phenolics, and in particular, hydrolysable tannins in preferred host plants inhibit NPV infection in GM. Similarly, Felton et al. (1987) noted that the flavonoid rutin, and chlorogenic acid inhibited infectivity of NPV, and prolonged NPV-infected larval survival time in *Helicoverpa zea* (Boddie).

To summarize, this study was initiated to further our understanding of the chemical feeding ecology of GM larvae on secondary hosts such as pine species. Phenolic extracts and pure flavonoids isolated from pines and incorporated into artificial diet were tested against early and late instar GM which have and have not been dosed with NPV. It was hoped that this research will provide a better understanding of why a broadly polyphagous host such as GM feeds on a secondary host such as pine species. This should also help to assess the defoliation risk to conifer forests that lie in the path of spread of GM, and provide important information on the efficacy of microbial controls when GM feeds on pine secondary compounds.

Theoretical Framework

1. General Scenario. The GM feeds on over 400 host tree species (Lechowicz and Jobin 1983) with varying degrees of preference. Feeding on different tree species may in part be determined by the nature of secondary compounds found in those species and by the effect those compounds have on pathogens, predators, and parasites of GM larvae. Recent work implicates viruses (Rossiter 1987, Myers 1993) as a driving force behind the population cycles of most outbreak species of forest Lepidoptera, including GM. Thus the compounds present in tree species, the tree-species composition of a forest, and the interaction of these compounds with a viral pathogen are all factors that may determine whether conditions favour an outbreak of GM. Feeding on pine species by GM is of particular interest because early instars generally do not prefer pine species which later instars readily consume. Therefore, secondary compounds from pine species may affect early instars but not later instars.

Feeding on pine species by later instars may convey benefits to virus-infected later instars if there are compounds present in pine that inhibit viral infection, but do not adversely affect GM larvae. One class of phenolics, tannins, are known to reduce mortality of virus-infected GM larvae feeding on hosts such as oak and maple species (Keating et al. 1988, 1989, 1990). However, other classes of phenolics such as simple phenols and flavonoids have not been assessed for their activity against GM NPV. However, Felton et al. (1987) found that rutin (a flavonol) and chlorogenic acid inhibited infectivity of a NPV that infects *Helicoverpa zea* (Boddie) and prolonged larval survival time.

Central Question: Do pines vary in overall phenolic composition and do these phenolics affect growth and performance of GM early and middle instar GM larvae? Do they also inhibit virulence of GM NPV in late instars?

Hypothesis 1: Phenolics (which include flavonols and flavonol glycosides) are compounds which inhibit herbivory by insects in a number of tree species and are responsible for inhibition of growth of early GM instars feeding on pines. Pine species vary significantly in the type and concentration of phenolics that are present, and these have adverse effects on the growth of early instar GM larvae. These same compounds can have little effect on growth of later instars, or they increase or decrease growth.

Objective 1: Establish the effect of phenolic extract from pine on growth rates and mortality of early GM (2nd) instars.

Objective 2: Establish the effect of phenolic extract from four pine species on growth rates and mortality of later GM (3rd and 4th) instars .

Objective 3: Separate, isolate, purify and identify the phenolic compounds present in the four pine species and test the effect of selected pure phenolics against GM 2nd, and 3rd instars to establish which compound may be responsible for effects on growth of GM.

Hypothesis 2: Flavonoids and phenolics isolated from pine species have third trophic level effects on pathogens of GM such as Nuclear Polyhedrosis Virus (NPV) and therefore enhance or inhibit infectivity of NPV in later instar GM larvae.

Objective 4: To obtain a stock of purified NPV and determine its efficacy. This will determine what concentrations (PIBs/larvae) should be used to test whether pine extracts and phenolics have an inhibitory effect on infectivity.

Objective 5: Dose 3rd and 4th instars with concentrations of virus in combination with extract containing phenolics and monitor growth and mortality. This will test what effect extract and virus together have on GM larvae.

Objective 6: Test pure flavonoids identified from pine and test against 3rd and 4th instars of GM infected with NPV and monitor growth and mortality to establish if these affect infectivity of NPV.

CHAPTER TWO: Extraction, Isolation and Identification of Phenolics and Flavonoids From Four Pine Species

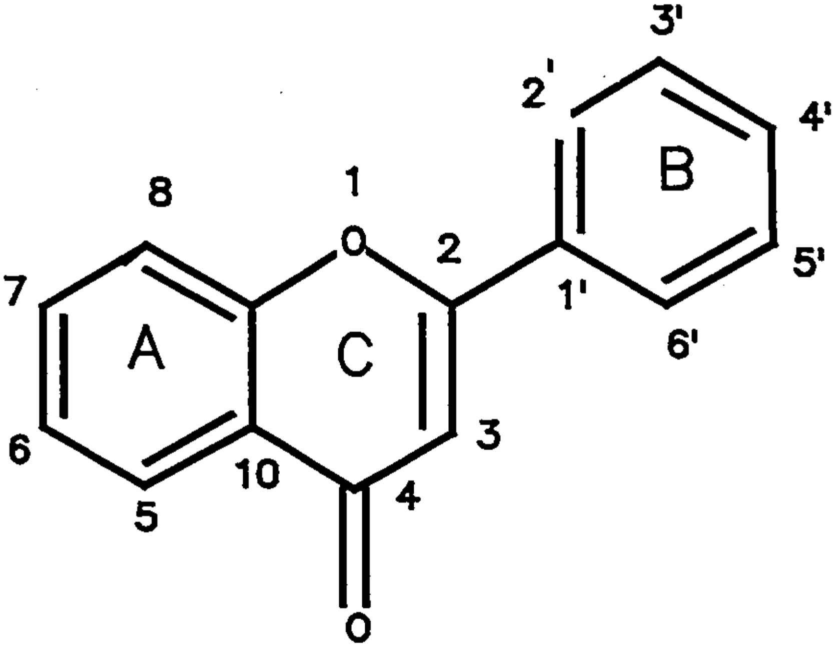
Introduction

The information on the effect of flavonoids on herbivorous insects continues to grow. Host choice in some cases is strongly determined by the flavonoid content of the plant. For example, Furth and Young (1988) found that host choice for several species of leaf feeding beetle (*Rhus*) correlated strongly with the flavonoid composition of the different hosts. Flavonoids are also feeding stimulants to the European elm bark beetle (*Scolytus multistriatus* Marsham) (Doskotch et al. 1973), silkworm (*Bombyx mori* L.) (Hamamura et al. 1962), and *Agasicles* sp. nov. (Zielske et al. 1972). Flavonoids can also be feeding deterrents to different insect species. Echeverri et al (1991) found the flavonid ermanin deterrent to *Dione juno* Cramer larvae, while Dryer et al. (1981) found a number of flavones, flavanones, and flavonols deterrent to various aphid species.

Elliger et al. (1980) found that quercetin inhibited larval growth of *Helicoverpa zea*, but kaempferol did not. These two flavonols may be a constitutive defense against generalist herbivores which may defoliate new leaves. Another flavonol, rutin (quercetin-3-rutinoside) has been found to inhibit growth of a generalist but not a specialist herbivore. Abou-Zaid et al. (1993) also found that quercetin and rutin significantly reduced growth and increased mortality of the European Corn Borer (*Ostrinia nubilalis* L.). Figure 2 demonstrates the general structure of flavonoids and the ring numbering system (Harborne 1984, 1989, Harborne et al. 1975, Markham 1982).

Leaves of pine species have been found to contain flavonols such as quercetin, kaempferol and myricitin (Niemann 1979, Markham and Whitehouse 1984, Abou-Zaid and Nozzollilo 1991). Heartwood, sapwood and bark of pines also contain flavanones such as pinocembrin, pinobanksin and pinoquercetin (Mirov 1967, Lindstedt and Misiorny 1951).

Figure 2. General flavonoid structure (after Markham 1982).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8
1
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7
1'
A
C
6'
6
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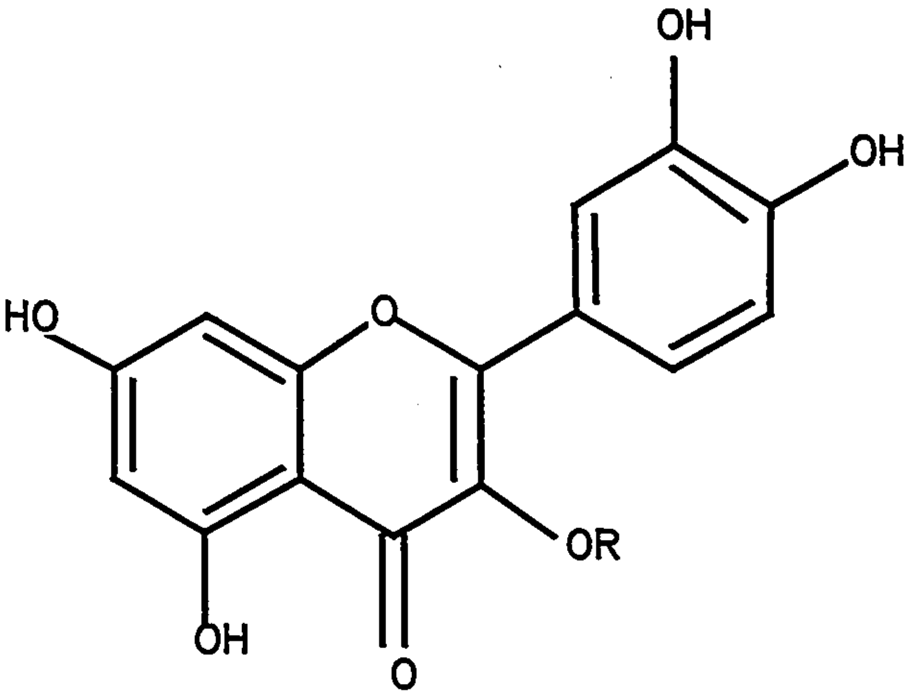
10
4
5
O

Figure 3. Structure of three of the flavonoids isolated from pine. Quercetin R=H. Rutin R=glucose-O-rhamnose. Quercetin-3-O-glucoside R=O-glucose.


OH
OH
HO
O
OR
OH
O

There is evidence that turnover from a soluble to insoluble cell wall form for kaempferol glycoside (Barton 1972, Raske et al. 1989) occurs in at least one pine species. The two most common flavonols, quercetin and kaemferol are found in high concentrations in young leaves early in the growing season but are absent later in the growing season (Niemann 1979).

To determine the role pine phenolics play in feeding of early and late instar GM, and their effects on NPV, it was first necessary to collect and extract phenolics from the leaves of some common pine species which GM is known to feed on. First, four pine species (jack, red, scotch and white pine) were extracted for phenolics to give a crude, freeze-dried extract. The amount of phenolics in this extract was quantified so that it could be incorporated into artificial diet at known concentrations. This extract was then further fractionated and these fractions purified to identify flavonoids which might affect GM larvae and NPV infection. The following outlines the general and specific procedures used in the extraction, isolation and identification of phenolics and flavonoids from pines.

Materials and Methods

1. Plant Material

Collections of fresh needles of four pine species were made in the Kirkwood Forest approximately 80 km to the east of Sault Ste. Marie (lat. 46.34 N, long. 84.17 W) in June 1992. Using large shears mounted on pole-pruners, branches were cut approximately one metre from their termini, collected, placed into large garbage bags and immediately transported to the Natural Products Laboratory of the Forest Pest Management Institute at Sault Ste. Marie. All trees were sampled randomly from heights of 2-8 m. Trees in stands of scotch (*Pinus sylvestris*) and jack pine (*Pinus banksiana*) were 16-20 yrs old, whereas trees in stands of white (*Pinus strobus*) and red (*Pinus resinosa*) pine were approximately 30 years of age. In the lab, needles from the four species were removed from branches and twigs. If needles were not removed from the branches immediately upon return from the field, then the whole branches were frozen and needles removed as soon as possible. Stems, branches and current year (1992) foliage were separated from previous year growth and stored at -20^0 C. At least four kg of needles from previous year growth were obtained, two kg of which were frozen and stored for future extraction. The remaining two kg were subjected to extraction for phenolics and flavonoids for each species of pine (fig. 4).

2. Extraction

Fresh needle material (2 kg) was extracted in two steps. First needles were ground in a large Waring commercial blender with 100% EtOH and secondly with EtOH:H_2O (1:1) for 24 hr at 23^0 C each. The combined extracts were evaporated in a Buchi Rotavapor RE 120 under reduced pressure at 50^0 C until most of the EtOh and H_20 had been removed. The residue was then freeze-dried using an Edwards Supermodel freeze drier. This crude phenolic extract was then further fractionated using the general procedure given in figure 4. Details of the PVPP fractionation and subsequent procedures used to isolate and identify phenolics and flavonoids are given in figure 5.

Figure 4. Initial extraction of pine species for phenolics and flavonoids.

2.0 kg Fresh Leaf

Extract with 100% EtOH for 24 hrs
and then grind in blender

Drain and extract with EtOH:H_2O 1:1 for 24 hrs

Concentrate extract at 50°C under reduced pressure to reduced volumes
(concentrated extract)

Fractionate on PVPP
(100% H_2O--->100% EtOH)
(12 - 18 Fractions 2-4 L volumes each)

Rotavap fractions to 10-50 ml volumes

3. Fractionation and Separation Procedures Used For Red Pine

The procedure given below is essentially the same that was followed for the remaining three pine species except for slightly different amounts of extract being fractionated and minor differences in solvent systems. These differences will be described later.

The concentrated (153.52 g) extract was mixed with polyvinylpolypyrolidine (PVPP) powder (Sigma) and H_2O in a 400 ml beaker. This mixture was then applied to the top of a Buchner funnel packed with 200 g PVPP and eluted with combinations of EtOH and H_20 Elution was carried out at a slow rate using 2 L of H_2O followed by 2 L aliquots of increasing concentrations (20, 50, 80 & 100%) of EtOH. The following fractions were obtained: 3 fractions each for 100% and 80% EtOH, 4 fractions for 50% EtOH and one fraction each for 30% EtOH and 100% H_2O. These were then concentrated at 50^0 C and reduced pressure using a Rotavapor. The fractions were then spotted one dimensionally on sheets of Whatman #1 chromatography paper, placed in tanks containing BAW (*n*-butanol: acetic acid: water 4: 1: 5) and 15% acetic acid. They were removed and the chromatogram developed when the solvent front was approximately 2 cm from the top of the paper. Chromatograms were then examined with a hand-held UV lamp. Based on the paper chromatography fractions 1-12 were combined into fractions lettered A-E. Fractions D&E were further fractionated on a sephadex column with a mixture of 800 ml CH_2Cl_2, 400 ml EtOh, 100 ml 2-butanone and 20 ml acetone as a solvent system. 8 fractions were obtained from D and 7 fractions from E.

Further fractionation was carried out on PVPP columns using the following solvent systems: 1) CH_2Cl_2: EtOH: MeCOEt: Me_2CO (2: 2: 1: 1), 2) EtOH: MeCOEt: Me_2CO: H_20 (2: 1: 1: 1) and 3) EtOH: H_2O (1: 1). Final purification of compounds was achieved on a Sephadex LH-20 column (1 X 50 cm) using EtOH (95%), EtOh: H_20 (1: 1), EtOH: H_20 (7: 3) or EtOH: H_20: acetone (7: 2: 1) as eluting solvent.

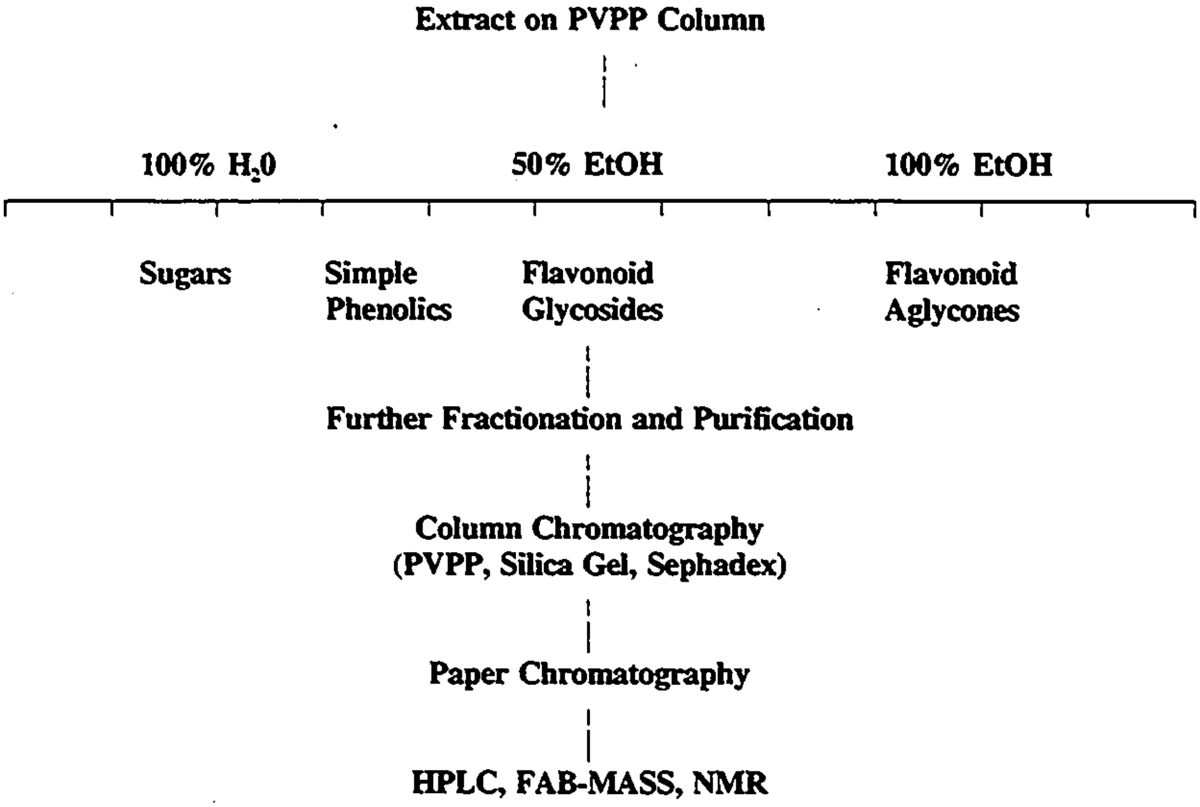
Fractions and pure compounds were injected into a Waters HPLC to determine retention times a Lichrosorb RP-18 (10 μm (Merck) reverse-phase column with a gradient technique, a Waters Delta Prep 400 pump, an autoscan photodiode array detector 996 and Millenium 2010 software. A gradient program developed by M. Abou-Zaid was used at room temp: solvent A=MeOH; B=5% aqueous HCOOH. The flow rate was set at 2 ml min^{-1} and two fixed detection wavelengths were used: 280 and 350 nm. Resolved peaks were scanned by the diode array detector from 250-400 nm.

Further purification followed and preliminary identification of the compounds using PC with various shift reagents and UV, ^1H-NMR,^{13}C-NMR and FAB-MS was verified by Dr. Abou-Zaid. For two of the pine compounds which I had chosen to test against GM all of this data is included in Appendix C for reference.

Jack Pine, Scotch Pine and White Pine

Methods were similar for jack, red and scotch pine. 250 ml of jack pine and 400 ml of scotch and white pine extract were fractionated on PVPP (above). Fractions were combined and further fractionated on Sephadex columns with a mixture of 400 ml CH_2Cl_2, 200 ml EtOH, 50 ml acetone and 50 ml H_20. All other procedures were as given for red pine.

Figure 5. Isolation and extraction procedures for phenolics and flavonoids.


Extract on PVPP Column
100% $H_2$0
50% EtOH
100% EtOH
Sugars
Simple Phenolics
Flavonoid Glycosides
Flavonoid Aglycones
Further Fractionation and Purification
Column Chromatography (PVPP, Silica Gel, Sephadex)
Paper Chromatography
HPLC, FAB-MASS, NMR

4. Estimation of Total Phenolics

The procedure for estimation of total phenolics was a modification of Swain & Hillis' (1959) method (Fig. 6). In this procedure gallic acid was used as a phenolic standard to contruct a standard curve, maximum absorption was measured between 250-350 nm where phenolics absorb strongest. Known weights of extracts were then dissolved in EtOH and subjected to the same procedure as the gallic acid standard with absorbance measured between 250 and 350 nm. A quadratic equation was found to fit the standard curve best (Fig. 7) and the absorbances measured for the extracts were then substituted into the equation to estimate total phenolic content.

Figure 6. Procedure for estimating total phenolics in extract of four pine species.

6 weights of gallic acid dissolved in 0.5ml EtOH & diluted with 7 ml H_2O

0.5ml Folin-Denis reagent added

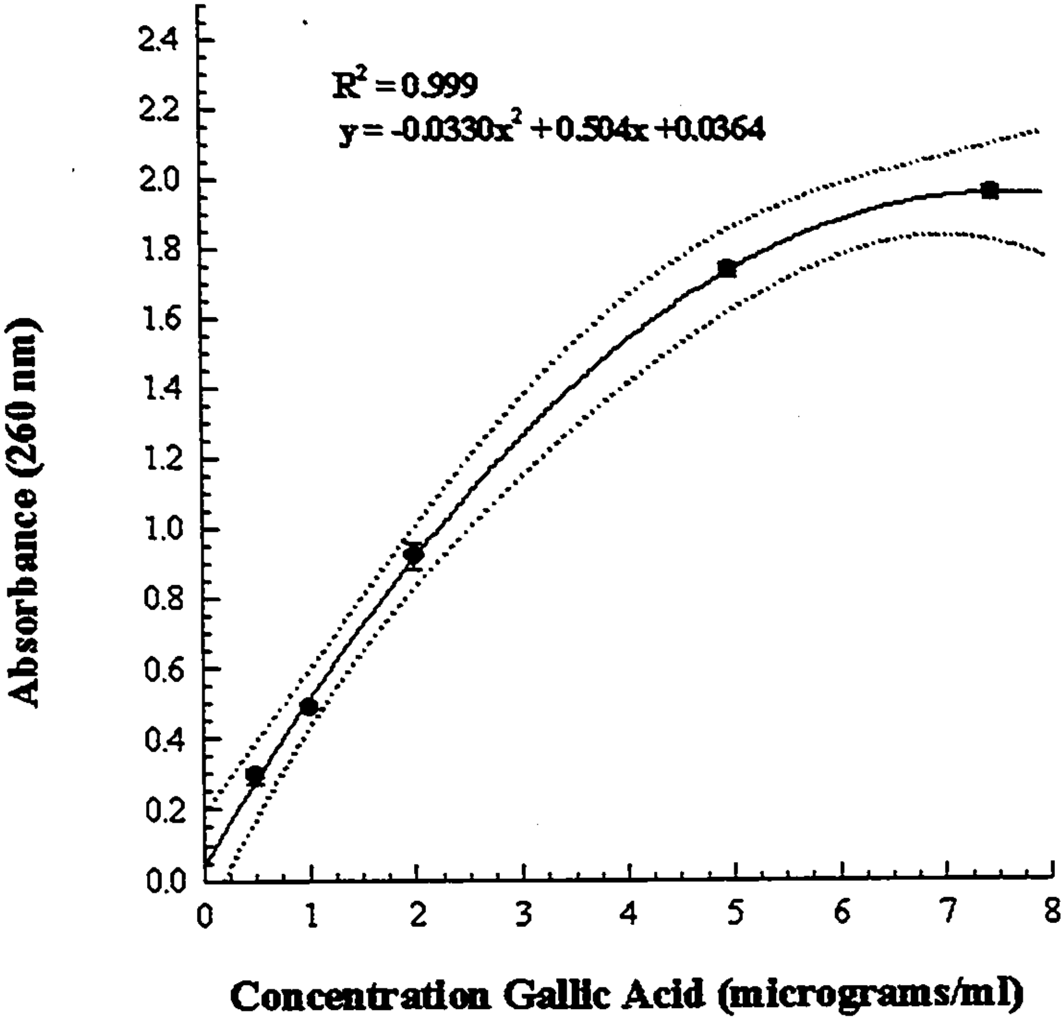
After 3 min 1 ml saturated $NaCO_3$ added

Mixture made up to 10 ml with H_2O & after 1 hr. peak absorption measured at 200-800 nm wavelengths

Plot standard curve for gallic acid

Follow same procedure with known weights of extract and calculate concentration of total phenolics based on equation of line for standard curve.

Figure 7. Standard curve with gallic acid as phenolic standard to determine level of total phenolics in extract. Dotted lines indicate 95% confidence limits.



$R^2 = 0.999$
$y = -0.0330x^2 + 0.504x + 0.0364$
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
7
8
Absorbance (260 nm)
Concentration Gallic Acid (micrograms/ml)

5. Identification of Purified Compounds

Structures of purified compounds were determined according to the following methods. Acid and enzymatic hydrolysis removed the sugar moiety from flavonoid glycosides and allowed identification of the aglycones and sugar residues. Acid hydrolysis with 2 M and 0.1 M HCl (mild hydrolysis) was carried out at 100° C for 60 min. Enzymatic hydrolysis was carried out with β-glucosidase (Sigma) using an acetate buffer (pH 5). Sugars released by hydrolysis were identified by co-chromatographing them with paper chromatography with standard sugars. The aglycones obtained by hydrolysis of isolated compounds were identified through co-chromatography with authentic samples (Apin and Extrasynthase) using paper chromatography and HPLC.

Results and Discussion

Estimation of Total Phenolics

An initial scan of jack pine extract at 0.1 mg/ml showed a strong peak in the 300 nm area where phenolics are known to absorb most strongly. Extract from each species was then scanned at 200-400 nm following Swain and Hillis' (1959) procedure and the peak absorption measured for five replicates. The mean peak absorption was then substituted into the equation for the gallic acid standard curve to give the approximate concentration of phenolics in concentrated extract from each pine species. Concentration of total phenolics in the extract (g phenolics/g extract) and as a percent of fresh weight are given in Table 1.

Table 1. Amount of total phenolics extracted and level of total phenolics as % of fresh weight for four pine species. Numbers in brackets are standard errors.

	Fresh Weight Needles Extracted (kg)	Total Weight Freeze-Dried Phenolic Extract (g)	Amount of all Phenolics Present in Extract (g)	Level of Total Phenolics in Needles (% of Fresh Weight)
Pine Species				
Jack	2.0	300.12	84.63 (1.08)	4.23 (0.005)
Red	2.0	307.02	116.74 (3.45)	5.80 (0.002)
Scotch	2.0	449.60	113.30 (2.31)	5.67 (0.001)
White	2.0	463.20	124.14 (4.16)	6.21 (0.002)

These results are consistent with the results of other studies for total phenolics in conifers. For example Joseph et al. (1993) and Joseph and Kelsey (1994) found levels of phenolics in Douglas-fir 1.58 - 4.0 % of dry needle weight. Wagner et al. (1990) found concentrations of 3.0, 4.0 & 6.0% dry weight in July for white fir (*Abies concolor* Gord & Glenn) Douglas-fir (*Pseudotsuga menziesii* (Mirb) Franco), and Engelmann spruce (*Picea engelmani* Perry ex Engelm), respectively. However, in Wagner et al. (1990) concentrations of phenolics could have been as high as 60% of dry weight if tannins were included. Jack pine can have a total phenolic content that varies between 0.5 and 1.5% fresh weight during summer and fall (Nozzolillo et al. 1990).

The identification of individual compounds which make up this value of total phenolics are given in the following section. The strength or weakness of the compound is a rough estimate only of how much is present relative to the other compounds.

Isolation and Identification of Phenolics and Flavonoids

Complete identification of all phenolic compounds found in the four pine species is underway, but is beyond the scope of this thesis. The following (Table 2) gives general information on how the compounds were identified (UV spot colour, HPLC retention times) as well as the distribution of flavone, flavonols and phenolics found in the extract from the four pine species. Two compounds, rutin (quercetin-3-O-rutinoside), and quercetin-3-O-glucoside were found in all species.

Table 2. Phenolic composition of four pine species with relative amounts of each compound.

	UV Spot Colour		HPLC R_t	Pine Species			
	- NH_3	+ NH_3	(min)	Jack	Red	Scotch	White
Flavonols							
Kaempferol-3-O-glucoside	brown	yellow	19.0	+++	--	--	++
Kaempferol-3-O-galactoside	brown	yellow	18.6	++	--	--	--
Kaempferol-3-O-coumaroylglucoside	brown	yellow	21.7	+++	--	--	--
Quercetin-3-O-glucoside	brown	yellow	17.6	++	++	++	++
Quercetin-3-O-galactoside	brown	yellow	17.4	++	--	--	+
Quercetin-3-O-p-coumaroylglucoside	brown	yellow	15.8	++	--	--	--
Quercetin-3-O-rutinoside	brown	yellow	24.5	tr	tr	+	++
6-Methylkaempferol-3-glucoside	dark brown	dark brown	20.2	+	+	+	--
Methylated flavonol (1)	dark brown	dark brown	25.2	--	++	--	--
Methylated flavonol (2)	dark brown	dark brown	32.5	--	--	++	--
Methylated flavonol glycoside	dark brown	dark brown	27.6	--	++	+	--
Flavones							
Methylated flavone (1)	dark brown	dark brown	29.9	--	+++	++	--
Methylated flavone (2)	dark brown	dark brown	25.7	--	+++	--	+
Methylated flavone (3)	dark brown	dark brown	19.2	--	+++	++	--
Flavone glucoside (1)	dark brown	dark brown	21.2	--	++	--	--
Flavone glucoside (2)	dark brown	yellow	22.2	--	+	+	--
Methylated flavone glycoside (1)	dark brown	yellow	22.8	--	++	+	--
Methylated flavone glycoside (2)	dark brown	yellow	23.6	-	++	--	--

Table 2. Phenolic composition of four pine species with relative amounts of each compound.

<u>**Phenolic Acids**</u>							
Phenolic Acid (1)	blue	blue	3.9	++	++	+	++
Phenolic Acid (2)	pink-blue	pink-blue	4.4	+	++	+	+
Phenolic Acid (3)	blue	blue	4.6	--	++	--	--
Diphenolic acid (1)	blue	blue	5.6	+	+++	+++	--
Diphenolic acid (2)	blue	blue	6.2	--	++	--	--
Phenolic glucoside	blue	blue	8.7	--	+++	+	--
Phenolic rhamnoside	blue	blue	11.2	?	++	+	--

+++ very strong; ++ strong; + present; **tr** traces; -- not detected

*Note: All pure compounds were identified by chemical and physical analysis with the help of Dr. M. Abou-Zaid. Chemical analyses include acid, mild acid and enzymatic hydrolysis. Physical analyses included UV spectra and chemical shifts, ^{1}H-NMR, ^{13}C-NMR and FAB-MS. FAB-MS and ^{1}H-NMR traces are given for rutin and quercetin-3-O-glucoside in Appendix C.

From Table 2 it is clear that each pine species has its own distinct phenolic profile. Jack pine is rich in flavonol compounds, in particular flavonol glycosides such as kaempferol-3-O-glucoside and quercetin-3-O-glucoside. In new foliage of Norway spruce (*Picea abies* [L.] Karst), the flavonol kaempferol-3-O-glucoside was found at high concentrations in early summer and was still present in the fall but at a much reduced concentration. These flavonols are essentially the same as those identified by Abou-Zaid and Nozzolillo (1991) for jack pine, although quercetin 3-O-rutinoside has not been found before in this species, and there are several as yet unidentified phenolic acids. Flavones are completely absent and there are low concentrations of only three of a possible total of seven phenolic acids found in these four pine species are present. In contrast, red pine does not have many flavonols, and not in any great concentration. However, red pine is rich in flavones and phenolic acids. In particular there are many methylated flavones and flavone glycosides for which the exact structures have yet to be determined. Scotch pine has strong to low concentrations of all three groups of phenolics but is not nearly as diverse or as strong as those of red pine. Finally, white pine has only seven of the 25 possible phenolics which are found in the other three pine species, and none of these is represented strongly.

The flavonoid profile of other pines does not appear to vary intraspecifically, but in a study of European mountain pine (*Pinus uncinata* Ram.) the concentration of quercetin was found to vary depending on the population (Lauranson and Lebreton 1991). Niemann (1979) reviewed the literature prior to 1979 on the constituents of pine needles, however the chemistry of these species is still not well studied. However, the main flavonoid constituents common to 42 pine species were kaempferol, quercetin, myricitin and dihydroquercetin. A variety of phenolic acids such as caffeic-, ferulic and chlorogenic-acids were found (Niemann 1979). Since that time Strack et al. (1988) found that most Pinaceae species had kaempferol-3-O-glucoside, but quercetin-3-O-glucoside and 3-*O*-arabinoside were found only in *Pinus*. Shen and Theander (1985) found several new flavonoids in *Pinus massoniana* Lamb. including taxifolin and its glycoside, but these were not found in the four pine species I looked at. Finally, Markam and Whitehouse (1984) found glycosides of quercetin, myricetin and tricetin in New Zealand white pine, *Dacrycarpus dacrydioides* (A. Rich) de Laub.

CHAPTER 3: Effect of Pine Extracts and Pure Phenolics on Growth and Survivorship of Gypsy Moth Larvae

Introduction

A great deal of work in recent years has been undertaken to determine host range and factors governing host choice for GM. These studies have followed three main approaches - 1) observational data and field studies involving manipulation of various instars of GM, 2) laboratory studies with foliage from selected hosts, and 3) laboratory studies incorporating extracts and secondary chemicals from various host species into artificial diet. I will summarize the current literature within each of these areas and also demonstrate how my study fits into this overall pattern.

1. Observational data

In North America observational data first began to be collected intensively by Barbosa (1978 ab). This author found the largest numbers of GM on red and white oak but also found significant numbers on birch, hemlock and red maple trees. Barbosa also found significant numbers of all instars on white pine, as well as pupae, indicating that they completed development on this tree species. Field studies such as that of Rossiter (1987) indicated that GM populations fed preferentially on pitch pine in non-outbreak conditions (conditions where gypsy moth populations are relatively low) and suggested that pines contained compounds that make the larvae more resistant to mortality caused by NPV.

Mauffette et al. (1983) found that GM in Québec generally preferred oak and other hardwoods, but avoided most pine and maple species with a number of tree species classed as intermediate hosts. Lechowicz & Mauffette (1986) summarized the available literature and found a large number of tree species classified as preferred, acceptable or avoided in various instars. Scotch pine was found to be acceptable and in fact, heavily consumed in Europe, whereas jack, red and white pine were favoured tree species by later instars. Lechowicz and Jobin (1983) also determined that red oak (*Quercus rubra* L.) and sugar maple (*Acer saccharum* Marsh) were among the most susceptible to attack by GM while red maple (*Acer rubrum* L.), yellow birch (*Betula lutea* Michx. f.) and others were generally avoided. Finally, Mauffette and Lechowicz (1984) were first to suggest that later instars migrate to host trees not preferred in earlier instars because of the large numbers of pupae found on supposedly non-preferred hosts.

Gross et al. (1990) sampled larvae and pupae from five different tree species - three oak species, red maple and black gum (*Nyssa sylvatica* Marsh) and found that sampling replicate trees within a host species is more important than including multiple study sites. This means that factors such as soil type, light availability etc., for host plants are less variable than individual genetic variability. Kleiner and Montgomery (1994) did an extensive study of host and site effects on GM larval growth parameters. These authors found that while foliage from xeric sites contains higher levels of phenolics, this did not affect females and that only males had slightly higher pupal weights. They also found that since dry sites supported the most preferred species, chestnut oak (*Quercus prinus* L.) and that therefore tree species composition of dry sites was more important than differences in foliage quality.

Cook et al. (1994) found that vertebrate predation was heavier on oak than on pine species and that this may account for the higher survival rates of pupae on pines. Finally, Rossiter et al. (1988) examined the effects of defoliation and red oak phenolics on GM growth and reproduction and found that variation in defoliation and in phenolic concentrations were also related to variation in fecundity and size of egg mass for GM.

2. Laboratory studies with selected host foliage

The studies with selected host foliage are more numerous than observational data and field studies. Miller et al. (1991) found that garry oak (*Quercus garryanna* Dougl.) and red aldner (*Alnus rubra* Bong.) were equally suitable hosts for GM. Barbosa & Greenblatt (1979) found that larvae fed grey birch (*Betula populifolia* Marsh.) had the most rapid development and largest pupae. Larvae fed American beech (*Fagus grandifolia* Ehrh.) and red maple foliage had the smallest pupae and longest development, whereas larvae reared on white oak (*Quercus alba* L.) and red oak foliage had intermediate developmental times and pupal weights. The GM survivorship to pupation was high for oaks and other preferred hosts but mortality was 100% for larvae reared exclusively on white pine, loblolly pine (*Pinus taeda* L.), and Virginia pine (*Pinus virginiana* Mill.) (Barbosa et al. 1983). In a later study Barbosa et al. (1986) examined two plant species diets for GM larvae. These authors found that when GM fed first on a favourable and then on an unfavourable species that larval mortality was higher on the two species diet, but that when one species is a conifer, the individuals had greater fecundity than on either species alone. Miller and Hanson (1989) studied GM feeding on 39 species in 18 genera of conifers and found that the most suitable species of conifers were in the family Pinaceae. In particular, several species of *Pinus* were suitable to complete development from 1st instar to pupation.

Relative growth rates (RGRs) for GM were found to be higher on oak than pine early in the growing season, but the reverse seemed to be true later in the season (Sheppard and Friedman 1990). The efficiency of conversion of ingested food (ECD) was also lower for two instars of larvae fed on pine as opposed to oak, and dietary history of the larvae also had significant effects on nutritional indices. However, Hajek (1989b) found no effect on mortality of GM 4th instars which were transferred between artificial diet and three ages of red oak leaves. Joseph and Kelsey (1994) examined the acceptability of a single conifer species, douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) to various GM instars. They found that while early instars may not do as well as later instars on douglas-fir, this species can support GM populations without the presence of other host species.

The relationship between leaf age and its quality for cottonwood (*Populus deltoides* Marsh.) was investigated by Meyer and Montgomery (1987). They found that high concentrations of phenolics present in younger leaves reduced growth and increased mortality of GM larvae. This contrasts with GM feeding on oak in which the older leaves contain higher concentrations of tannins and are less palatable to GM (Feeny 1970). Talhouk et al. (1990) examined the effects of drought and defoliation of birch clones on GM performance. For one clone they found that increased sucrose levels in wilted plants increased relative growth rate for GM, but that this did not hold true for another clone which was a more suitable host. Relative growth rates for GM were lower on partially defoliated than undefoliated plants and that this effect was independent of clone type. In host choice experiments Smitley et al. (1993) found that GM were influenced by the trunk species as well the foliage species and that preferred trees also tended to have larger canopy sizes. Finally, Berry et al. (1993) examined the effect of foliage from two host species, Douglas fir and white aldner (*Alnus rhombifolia* Nutt.) on the toxicity of diflubenzuron. They found that GM larvae reared on Douglas fir foliage were significantly more tolerant of diflubenzuron than those reared on white alder.

3. Laboratory studies where secondary chemicals are incorporated into artificial diet

The most numerous of these type of studies are those that incorporate extract and pure compounds from various preferred and non-preferred host species. I will avoid mention here of work already discussed in the general introduction, and in terms of interaction of compounds with pathogens I will focus only on effects of secondary compounds on non-NPV pathogens, since these will be introduced and discussed in the following chapter. A survey of the literature turned up the following studies, in addition to those reviewed previously.

Doskotch et al. (1977) were perhaps the first to incorporate extracts of plant leaves into an early artificial diet for GM composed of cellulose, agar and water. They examined extract from 30 plant species from a wide range of plant families and identified a number of potential of host species. In addition to Sheppard and Friedman's (1992) study mentioned earlier, Joseph et al. (1993) examined the effect of foliar nitrogen, terpenes, and phenolics from Douglas-fir on the development of GM larvae. They found that neonate larvae grew faster and were larger on high nitrogen control diet, but fourth instars did better on low nitrogen diets. Foliar phenolics incorporated into diet, alone, or in combination with terpenes excessively reduced growth of early instars and none survived to fourth instar. They concluded that while foliar nitrogen is a key element in determining growth of GM on Douglas-fir that this was influenced to some degree by the phenolics present in foliage. Stockhoff (1993ab) studied the effect of mixed foods containing various levels of nitrogen on protein intake of GM and found that larvae benefitted from eating a variety of foods to obtain a mean concentration of 2.9-3.25% nitrogen. Finally, Hajek and Renwick (1993) incorporated red oak foliage into artificial diet and found that this inhibited growth of a fungal pathogen of GM, *Entomophaga mimaga* Humber, Shimazu & Soper, but did not affect the fungus when larvae ate diet or red oak foliage alone.

My study falls into the above framework (3), in that phenolic containing extracts and pure compounds from host pine species were incorporated into artificial diet and then growth and development of GM were examined. Like other secondary metabolites, phenolics and flavonoids affect the growth and development of insects (Cody et al. 1988, Harborne 1989, Harborne 1991) although these compounds have generally received less attention in plant-insect studies than some others. Despite recent studies such as Larsson et al. (1992) we still know little of the ecological importance of these ubiquitous plant compounds.

Material and Methods

1. Artificial Diet

Diet prepared in the insect rearing department at FPMI was a modification of Odell et al. (1985). To make a one gallon batch (3600 ml), 2600 ml of distilled water was divided in two, placed in 3000 ml beakers and microwaved on high for approx. 23 min. and 60 g of agar was added. In a blender 600 ml of water, 100 g of casein, 24 g of salt mixture, 40 g of vitamin premix, 4 g of methyl paraben, and 8 g of sorbic acid were prepared and added to the hot agar solutions. 480 g of toasted wheat germ was then added to the final mixture and it was well stirred. For details on the source and composition of these constituents see Appendix B.

2. Larval Rearing

Larvae were reared from eggs in environmental chambers at FPMI at 25°C, 18:6 hr L:D, and 80% relative humidity to 2nd instars. The GM colony at FPMI is the New Jersey Standard Strain (NJSS) and was established in April 1989, from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service. If 2nd instars were to be used in an experiment then these were obtained from the insect rearing department at FPMI and put directly onto control and treated diets in the experiments. If 3rd or 4th larval instars were to be used these were obtained as 2nd instars and reared up personally in a standup Conviron environmental chamber to the instar needed under the same temperature and control regimes used above.

3. Preparation of Extract for Diet Incorporation

The following procedure for diet incorporation of extracts from needles of pine tree species was worked out originally by Abou-Zaid et al. (1993). Extracts or fractions to be incorporated were first freeze-dried to a powder using an Edwards Super-Modular 18 litre freeze drier. After the initial freeze drying, these were ground to a powder (using a mortar and pestle), freeze dried again, and then stored under vacuum until further use. To incorporate the extracts into diet, the freeze-dried powder was first weighed to give the needed concentration per amount of fresh diet. For example, a concentration of 0.1% extract in diet was obtained by weighing 200 mg of freeze-dried extract and adding it to 200 g of fresh liquid diet in a 300 ml mason jar. Prior to addition to diet, the 200 mg of extract was dissolved completely in approximately 4 ml of acetone and 1 ml of H_20 in a hot water bath. Liquid diet was allowed to cool to approximately 50^0 C before the dissolved extract was added. Liquid diet and extract were then completely mixed together on an Osterizer #8 blender. The diet was then poured into small petri dishes, allowed to cool, and if not used immediately, sealed with parafilm ("M" laboratory film) and placed in a Kenmore 63 series refrigerator at 2^0 C. Stored diet was replaced with fresh diet every two weeks. Diet cubes to be given to the larvae were then removed from the petri dishes using various sizes of cork borer (#1-5 bore).

4. Incorporation of Pure Compounds Into Artificial Diet

Pure compounds were incorporated into diet in the same way as extracts, except there was no need for freeze-drying (compounds were in a powder, or crystalline form after isolation). Procedures followed were after Beninger et al. (1993). Powdered pure compounds were dissolved first in acetone and H_2O as above and then added to artificial diet also as mentioned above. Three phenolic compounds found in pines were tested for growth reducing/enhancing effects against various gypsy moth instars: quercetin, rutin (quercetin-3-O-rutinoside) and quercetin-3-O-glucoside (Q3O). Rutin was found at low concentrations in all pine species, whereas Q3O was present at strong concentrations in all pine species. Quercetin itself was not found but it is an intermediate to the formation of the two glycosides chosen for testing which were found in the four pine species. Quercetin also is the basic flavonoid skeleton for many of the flavonoid glycosides found in the four pine species. Finally, quercetin itself may be produced in the gut of GM by the enzymatic cleavage of the sugar moiety (M. Abou-Zaid, pers. comm.).

5. Bioassays

First instars were not used in diet-based assays for two reasons. First, the larvae are extremely small and are easily subject to damage from handling. Second, first instars (or neonates) need to feed, at least in part, on the egg shells left after eclosion, and this would have been an added variable to account for in this instar. Feeding on contaminated egg shells by 1st instars is also an important means of transfer for the virus from one generation to the next (Doane 1969, 1970).

1. Long term studies: These studies examined growth and mortality of various instars exposed to different concentrations of extract or pure compound incorporated into diet over a period of three weeks. Weight (measured on a Sartorius 2004 MP scale, accurate to 5 significant digits) and mortality were monitored every 3-5 days. Larvae (of a selected instar) were placed individually into plastic Solo® P100, 1 oz. cups with treated diet cubes (treated diet cubes were replaced every 3-5 days). Larvae were kept in a standup Conviron chamber at 25° C, 85% relative humidity (RH) and 18:6 hr L:D for the duration of experiments. The light sources in these chambers were 8 cool white, 60 watt, 1500 milliampere, flourescent bulbs, and 4 regular incandescent 60 watt bulbs. Neither of these types of bulbs produces any appreciable UV light (M. Laporte, FPMI, pers. comm).

2. Short term studies: These studies examined the effect of various concentrations of extracts within a single instar. Nutritional indices were not calculated for 2nd instars because at most concentrations feeding was greatly reduced and the larval instar prolonged by several days. Since nutritional indices are usually calculated within an instar, and given the added variables of a significant reduction in feeding and lengthening of the instar due to extract or compounds, only 3rd and 4th instars were used. These instars fed on the diet treated cubes with little or no lengthening of the instar. Larvae were placed individually into plastic Solo® P100, 1 oz. cups with treated diet cubes and growth, amount of diet consumed, and frass produced after two days were all recorded. Larvae were kept in a standup Conviron chamber at 25° C, 85% RH and 18:6 hr L:D for the duration of experiments. Lighting was identical to that described previously. 2nd instars were reared to 3rd or 4th instar on control diet and then placed on diet containing concentrations of the extract incorporated. Larvae were allowed to feed for two days (or until they began to molt to the next instar) and if fresh weights were used, moisture loss was estimated from control diet cubes and frass to be 6.9% and 15% respectively. Moisture loss from the larvae was not estimated.

From the starting and final larval weights, frass and amount of diet consumed, the nutritional indices, defined by Waldbauer (1968) and modified by Stoyenoff et al.1994ab & Lindroth et al.1991, were calculated (see Appendix A for definitions of abbreviations) and then subjected to statistical analyses. All calculations of dry and wet weights, nutritional indices, correction factors and statistics were analyzed in a SAS® program written specifically for that purpose.

Results

Effect of Phenolic Extract From Pines on Growth of GM Larvae

1. Long Term Experiments

Results on the effect of various concentrations of pine extracts on 2nd instars are shown in tables 3-6. Jack pine extract had the greatest effect on reducing growth of 2nd instars continuously fed diet containing extract. At a concentration of 0.1 % extract in diet, larval growth was significantly reduced to 3.5, 28.2, 49.0% and 25.9% of the control weight for jack, red, scotch and white pine extracts, respectively. Probit analysis (Table 7) quantitatively shows that the pine phenolic extract reduced growth in the following order from most to least growth reduction: jack > red > white >scotch pine. All concentrations of jack pine greatly reduced growth of 2nd instars and therefore the fiducial limits could not be calculated. Cumulative mortality also increased with increasing concentration of extract, although mortality did not seem to be as dose dependent as weight. For example, all concentrations of jack pine extract above 0.075% caused almost 90% percent mortality while no concentrations of red pine extract caused more than 50% mortality (varying between 15 and 40%). A 2 x 2 contingency table analysis of mortality for the four species was then calculated for control mortality at 0 and 21 days and for mortality at the highest extract concentration tested. Mortality for jack pine was significantly different than the control at 0.15% ($\chi^2 = 27.03$, $P < 0.001$), but there was no significant difference for red ($\chi^2 = 1.09$, $P = 0.296$), scotch ($\chi^2 = 3.43$, $P = 0.065$), or white pine ($\chi^2 = 1.25$, $P = 0.264$).

Table 3. Effect of jack pine extract (concentration as % fresh weight of diet) on growth and survivorship of GM 2nd instars. Means (weight of larvae in milligrams/log transformed) followed by the same letter in the same column are not significantly different (Student Newman-Keuls test, SAS® Institute 1989). Standard Error in brackets, *n* = number of surviving larvae.

	Age of Larvae (days)				
	4	9	14	19	24
Conc'n 0	1.83^{a} (0.01) $n = 60$	3.44 ^{a} (0.05) $n = 57$	4.73^{a} (0.01 $n = 54$	5.61^{a} (0.12) $n = 52$	6.26^{a} (0.14) $n = 50$
0.050	1.80^{ab} (0.01) $n = 60$	3.10^{bc} (0.07) $n = 57$	3.88^{b} (0.19) $n = 44$	3.87^{b} (0.31) $n = 38$	4.21^{b} (0.46) $n = 29$
0.075	1.78^{b} (0.02) $n = 60$	3.19^{b} (0.09) $n = 54$	3.58^{c} (0.14) $n = 46$	3.71^{b} (0.12) $n = 38$	3.84^{b} (0.18) $n = 29$
0.100	1.78^{b} (0.01) $n = 60$	3.83^{d} (0.11) $n = 41$	2.79^{d} (0.15) $n = 27$	2.89^{b} (0.14) $n = 8$	2.87^{b} (0.22) $n = 5$
0.125	1.76^{b} (0.02) $n = 60$	2.83^{d} (0.09) $n = 41$	2.95^{d} (0.19 $n = 26$	3.20^{b} (0.17) $n = 12$	3.25^{b} (0.15) $n = 6$
0.150	1.84^{a} (0.01) $n = 60$	3.00^{c} (0.07) $n = 52$	2.97^{d} (0.19) $n = 22$	3.16^{b} (0.21) $n = 9$	3.13^{b} (0.21) $n = 5$
ANOVA statistics	$F = 4.65$ $P = 0.0004$ df = 5	$F = 33.80$ $P < 0.0001$ df = 5	$F = 140.45$ $P < 0.0001$ df = 5	$F = 169.12$ $P < 0.0001$ df = 5	$F = 163.20$ $P < 0.0001$ df = 5
Repeated Measures ANOVA			$F = 357.43$ $P < 0.0001$ df = 5		

$F = F$ statistic, P = probability, df = degrees of freedom

Table 4. Effect of red pine extract (concentration as % fresh weight of diet) on growth and survivorship of GM 2nd instars. Transformations, units and statistics as in Table 3.

	Age of Larvae (Days)				
Conc'n	4	9	14	19	24
0	1.73^b (0.02) $n = 60$	4.00^a (0.23) $n = 53$	5.56^a (0.13) $n = 53$	6.35^a (0.11) $n = 52$	6.83^a (0.26) $n = 51$
0.025	1.73^b (0.02) $n = 60$	3.66^{bc} (0.22) $n = 52$	4.94^b (0.22) $n = 50$	5.90^b (0.13) $n = 49$	6.63^a (0.19) $n = 49$
0.050	1.73^b (0.02) $n = 60$	3.49^c (0.23) $n = 50$	4.80^b (0.24) $n = 46$	5.63^c (0.21) $n = 47$	6.36^b (0.17) $n = 44$
0.075	1.80^{ab} (0.03) $n = 60$	3.53^c (0.15) $n = 50$	4.43^c (0.16) $n = 48$	5.16^d (0.18) $n = 48$	5.90^c (0.23) $n = 47$
0.100	1.85^a (0.03) $n = 60$	3.75^b (0.06) $n = 49$	4.35^c (0.11) $n = 49$	4.94^e (0.21) $n = 47$	5.50^d (0.46) $n = 45$
0.125	1.75^b (0.04) $n = 60$	3.44^c (0.21) $n = 53$	4.10^c (0.15) $n = 49$	4.38^f (0.33) $n = 44$	4.57^e (0.23) $n = 38$
ANOVA Statistics	$F = 5.77$ $P = 0.0001$ df = 5	$F = 12.27$ $P < 0.0001$ df = 5	$F = 85.28$ $P < 0.0001$ df = 5	$F = 124.76$ $P < 0.0001$ df = 5	$F = 117.77$ $P < 0.0001$ df = 5
Repeated Measures ANOVA			$F = 49.99$ $P = 0.0001$ df = 5		

Table 5. Effect of scotch pine extract (concentration in % fresh weight of diet) on growth and survivorship of GM 2nd instars. Transformations units and statistics as in Table 3.

	Age of Larvae (days)				
	4	9	14	19	24
Conc'n 0	1.67^b (0.01) $n = 60$	3.82^a (0.13) $n = 54$	5.16^a (0.18) $n = 54$	6.04^a (0.17) $n = 52$	6.57^a (0.34) $n = 47$
0.050	1.70^b (0.01) $n = 60$	3.57^b (0.11) $n = 56$	4.77^b (0.18) $n = 54$	5.69^b (0.15) $n = 51$	6.37^{ab} (0.24) $n = 51$
0.075	1.68^b (0.01) $n = 60$	3.50^b (0.14) $n = 57$	4.49^c (0.12) $n = 52$	5.35^c (0.13) $n = 52$	6.17^b (0.16) $n = 52$
0.100	1.65^b (0.01) $n = 60$	3.45^b (0.19) $n = 56$	4.31^{cd} (0.12) $n = 53$	4.94^d (0.32) $n = 51$	5.88^c (0.19) $n = 49$
0.125	1.76^a (0.02) $n = 60$	3.29^c (0.11) $n = 54$	4.21^{de} (0.13) $n = 45$	4.65^e (0.24) $n = 41$	5.27^d (0.17) $n = 40$
0.150	1.74^a (0.02) $n = 60$	3.09^d (0.17) $n = 58$	3.97^e (0.32) $n = 47$	4.44^e (0.31) $n = 40$	4.92^e (0.55) $n = 34$
ANOVA Statistics	$F = 8.71$ $P = 0.0001$ df = 5	$F = 29.53$ $P < 0.0001$ df = 5	$F = 79.83$ $P < 0.0001$ df = 5	$F = 100.49$ $P < 0.0001$ df = 5	$F = 64.20$ $P < 0.0001$ df = 5
Repeated Measures ANOVA			$F = 170.11$ $P < 0.0001$ df = 5		

Table 6. Effect of white pine extract (concentration in % fresh weight of diet) on growth and survivorship of GM 2nd instars. Transformations, units and statistics as in Table 3.

	Age of Larvae (days)				
	4	9	14	19	24
Conc'n 0	1.77^{ab} (0.02) $n = 60$	3.68^{a} (0.09) $n = 60$	4.92^{a} (0.09) $n = 60$	5.70^{a} (0.06) $n = 53$	6.36^{a} (0.03) $n = 53$
0.050	1.77^{ab} (0.01) $n = 60$	3.49^{b} (0.1) $n = 54$	4.58^{b} (0.13) $n = 51$	5.32^{b} (0.22) $n = 50$	6.05^{b} (0.22) $n = 49$
0.075	1.75^{b} (0.01) $n = 60$	3.46^{bc} (0.09) $n = 54$	4.21^{c} (0.22) $n = 50$	4.74^{c} (0.34) $n = 44$	5.31^{c} (0.49) $n = 44$
0.100	1.79^{ab} (0.01) $n = 60$	3.41^{bcd} (0.09) $n = 55$	4.01^{d} (0.30) $n = 52$	4.31^{d} (0.37) $n = 47$	4.81^{d} (0.47) $n = 40$
0.125	1.76^{ab} (0.02) $n = 60$	3.33^{d} (0.08) $n = 58$	3.87^{de} (0.25) $n = 54$	4.03^{e} (0.24) $n = 44$	4.41^{e} (0.28) $n = 41$
0.150	1.81^{a} (0.01) $n = 60$	3.29^{d} (0.09) $n = 53$	3.81^{e} (0.17) $n = 46$	3.72^{f} (0.16) $n = 36$	3.91^{f} (0.28) $n = 31$
ANOVA Statistics	$F = 2.58$ $P = 0.0283$ df = 5	$F = 12.00$ $P < 0.0001$ df = 5	$F = 54.72$ $P < 0.0001$ df = 5	$F = 116.69$ $P < 0.0001$ df = 5	$F = 133.32$ $P < 0.0001$ df = 5
Repeated Measures ANOVA			$F = 277.17$ $P < 0.0001$ df = 5		

Table 7. Probit analysis to calculate EC_{50} (% fresh weight in artificial diet) for 2nd instars for all pine extracts calculated for 24 day old larvae.

Extract	EC_{50}	Fiducial Limits	
		Lower	Upper
Jack	0.00402	N/A	N/A
Red	0.05247	0.02954	0.07705
Scotch	0.08995	0.08291	0.09720
White	0.07477	0.05245	0.09245

The extracts (at 0.1%) reduced growth of 3rd instars (Table 8) to some extent but the reduction was not as pronounced as that for 2nd instars. For example, on day 21, all extracts reduced the growth of larvae relative to control (Table 8), but there was no difference between jack and red pine extracts, or between scotch and white pine extracts. However, jack and red pine extracts reduced growth of third instars relative to the control by 55.5% and 51.3 % respectively, compared to a growth of only 3.5 and 28.2% for 2nd instars fed extracts from these two host species at that concentration. Similarly, 3rd instars fed scotch and red pine extracts in diet grew 70.6 and 68.5% relative to the control, compared to growths of 49.0 and 25.9% relative to the control for 2nd instars. Mortality did not differ greatly between jack, red and scotch pine, but mortality was higher for third instars on white pine.

There was little effect on 4th instars fed extracts from pine species at 0.1% (Table 9). Interestingly, jack and red pine extracts did not significantly reduce growth of 4th instars relative to the control but scotch and white pine extracts did. This appears to be the reverse of the situation for 2nd and 3rd instars. Figure 12 summarizes the effect of extracts from the four pine species at 0.1% on growth of 2nd, 3rd and 4th instars of GM relative to the control. This figure shows that growth of 2nd instars was reduced greatly, 3rds less so, and 4ths very little and the extract from species which reduced growth of 2nd and 3rd instars now produce growth approximately equal to the control.

2. Short Term Experiments - Nutritional Indices

Due to the short duration of the experiment (48 hrs) and length of time necessary to collect data for nutritional indices, I could only use extracts from three of the pine species: jack, red and scotch pine. Table 11 illustrates the results for 3rd instars fed extract at 0.1% in diet. The efficiency of conversion of digested material (ECD) and the efficiency of conversion of ingested (ECI) material were significantly lower for jack pine but white and scotch pine did not differ from the control. Relative growth rate (RGR) for jack pine was also lower than the control and the other two species of pine but this difference was not significant at the 0.05 level. Interestingly, the approximate digestibility (AD) of the control was significantly lower than the diet with extract from pines added. Finally, there was no significant difference between the control and extract treated diets in relative consumption rates (RCR).

Table 8. Effect of pine extracts (0.1%) on growth and survivorship of GM 3rd instars. Transformations, units and statistics as in Table 3.

Extract	Age of Larvae (days)				
	9	12	15	18	21
Control	4.29^{a} (0.03) $n = 39$	5.53^{a} (0.03) $n = 38$	6.24^{a} (0.04) $n = 38$	6.47^{a} (0.05) $n = 38$	6.52^{a} (0.07) $n = 34$
Jack	4.29^{a} (0.04) $n = 39$	5.34^{b} (0.05) $n = 39$	5.70^{b} (0.06) $n = 38$	5.93^{c} (0.07) $n = 36$	5.87^{c} (0.08) $n = 35$
Red	4.23^{a} (0.04) $n = 38$	5.09^{c} (0.06) $n = 38$	5.37^{c} (0.06) $n =38$	5.70^{d} (0.08) $n = 36$	5.84^{c} (0.08) $n = 33$
Scotch	4.33^{a} (0.03) $n = 40$	5.31^{b} (0.04) $n = 40$	5.84^{b} (0.04) $n = 39$	6.23^{b} (0.04) $n = 39$	6.17^{b} (0.06) $n = 37$
White	4.32^{a} (0.03) $n = 37$	5.37^{b} (0.06) $n = 37$	5.84^{b} (0.08) $n = 37$	6.05^{bc}(0.09) $n = 36$	6.13^{b} (0.08) $n = 29$
Total ANOVA Statistics	$F = 1.26$ $P = 0.287$ df = 4	$F = 10.79$ $P < 0.001$ df = 4	$F = 28.90$ $P < 0.001$ df = 4	$F = 17.61$ $P < 0.001$ df = 4	$F = 14.85$ $P < 0.001$ df = 4
Repeated Measures ANOVA			$F = 21.26$ $P < 0.0001$ df = 4		

Table 9. Effect of four pine extracts (0.1%) on growth and survivorship of GM 4th instars. Transformations, units and statistics as in Table 3.

Extract	Age of Larvae (days)		
	12	17	21
Control	5.14^{a} (02) $n = 40$	5.97^{a} (0.04) $n = 37$	6.68^{a} (0.06) $n = 37$
Jack	5.16^{a} (0.03) $n = 40$	5.98^{a} (0.05) $n = 39$	6.65^{a} (0.04) $n = 34$
Red	5.20^{a} (0.02) $n = 40$	5.94^{a} (0.03) $n = 35$	6.59^{ab} (0.04) $n = 33$
Scotch	5.12^{a} (0.02) $n = 40$	5.84^{a} (0.04) $n = 36$	6.54^{ab} (0.04) $n = 35$
White	5.04^{b} (0.02) $n = 40$	5.92^{a}(0.03) $n = 38$	6.48^{b} (0.04) $n = 38$
Total ANOVA Statistics	$F = 6.46$ $P = 0.001$ df = 4	$F = 2.19$ $P = 0.072$ df = 4	$F = 3.48$ $P = 0.0093$ df = 4
Repeated Measures ANOVA		$F = 4.29$ $P = 0.0025$ df = 4	

Table 10. Effect of jack pine extract (1.0%) on growth and survivorship of gypsy moth **4th** instars. Transformations, units and statistics as in Table 3.

Extract	Age of Larvae (days)		
	15	18	21
Control	5.40^{a} (0.03) $n = 40$	5.84^{a} (0.03) $n = 39$	6.46^{a} (0.04) $n = 39$
Jack	5.39^{a} (0.04) $n = 40$	5.56^{b} (0.04) $n = 40$	5.95^{b} (0.08) $n = 33$
ANOVA Statistics	$F = 0.07$ $P = 0.80$ df = 1	$F = 27.95$ $P < 0.0001$ df = 1	$F = 45.53$ $P < 0.0001$ df = 1
Repeated Measures ANOVA		$F = 64.47$ $P < 0.0001$ df = 1	

Figure 8. Growth of 2nd, 3rd and 4th instar gypsy moth as a percentage of control when fed extract at 0.1% from four pine species.

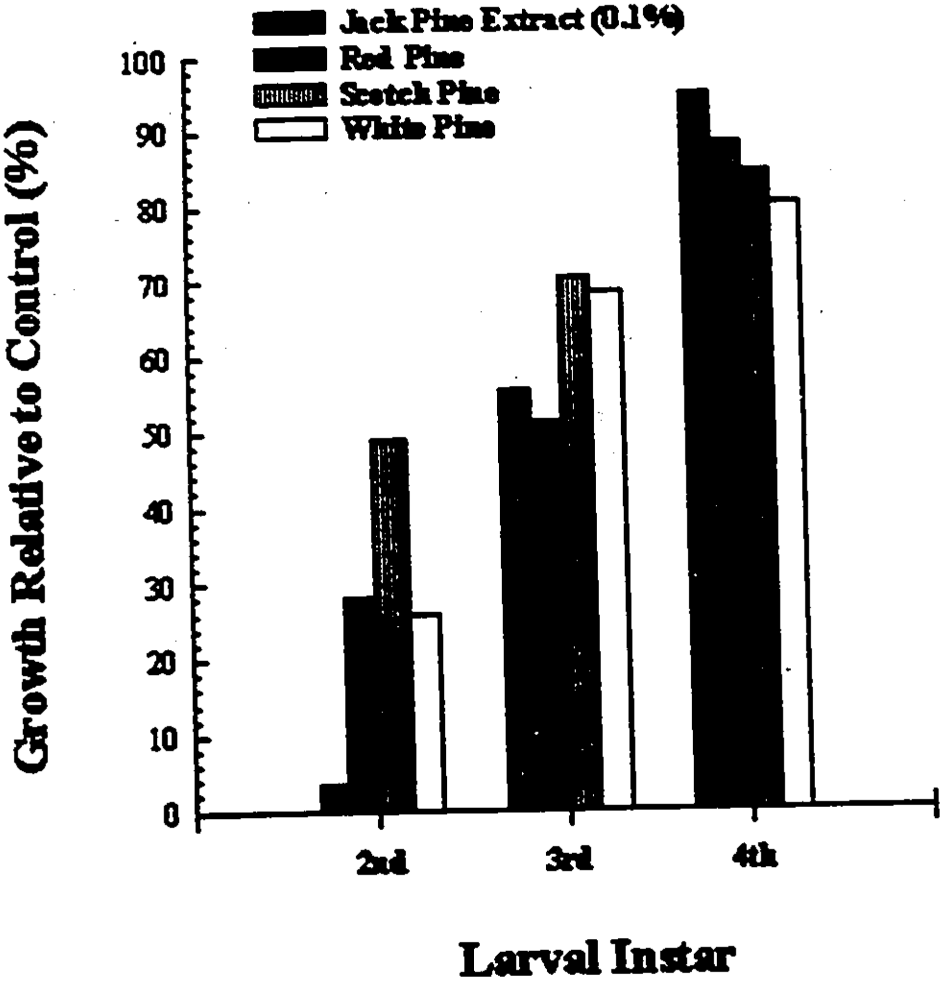

Jack Pine Extract (0.1%)
Red Pine
Scotch Pine
White Pine
Growth Relative to Control (%)
0
10
20
30
40
50
60
70
80
90
100
2nd
3rd
4th
Larval Instar

Table 11. Nutritional indices (fresh weights) for GM 3rd instars fed extract 0.1% in diet. Means followed by same letter in the same column are not significantly different (Student Newman-Keuls test, SAS® Institute 1989). Standard Error in brackets, n = number of larvae.

	ECD (%)	AD (%)	ECI (%)	RGR (mg/mg day)	RCR (mg/mg day)
Control	35.36^{a} (2.8) $n = 36$	74.10^{b} (1.7) $n = 36$	24.95^{a} (1.5) $n = 36$	0.295^{a} (0.03) $n = 36$	1.10^{a} (0.06) $n = 40$
Jack	26.15^{b} (1.9) $n = 37$	78.56^{a} (1.3) $n = 37$	19.94^{b} (1.3) $n = 37$	0.224^{a} (0.02) $n = 37$	1.03^{a} (0.06) $n = 40$
Red	35.49^{a} (2.6) $n = 35$	75.96^{a} (1.8) $n = 35$	25.73^{a} (1.7) $n = 35$	0.309^{a} (0.03) $n = 35$	1.09^{a} (0.07) $n = 35$
Scotch	31.57^{ab} (1.8) $n = 36$	79.34^{a} (1.5) $n = 36$	24.51^{a} (1.2) $n = 36$	0.284^{a} (0.02) $n = 36$	1.11^{a} (0.05) $n = 40$
Total ANOVA Statistics	$F = 3.60$ $P = 0.015$ df =3	$F = 2.33$ $P = 0.077$ df =3	$F = 3.41$ $P = 0.019$ df =3	$F = 2.02$ $P = 0.114$ df =3	$F = 0.41$ $P = 0.748$ df =3

Table 12 gives the calculated nutritional indices for 4th instars of GM. There was no significant difference in the ECD or ECI values between the control and extract treated diet. However, unlike the results for 3rd instars, the control diet had a significantly higher AD, and also a significantly higher RCR. Only red pine extract significantly reduced the RGR for 4th instars.

The effect of phenolics from pine at a concentration of 0.1% in diet seemed to have less of a negative effect on growth and mortality of GM larvae with increasing larval age (Fig. 10). Second instars fed 0.1% pine extract continuously in diet had very low growth relative to the control. However, 2nd instars which had been reared on control diet to 3rd before placing on treated diet cubes were not affected as severely as 2nd instars which had been placed on treated cubes immediately. Also, 2nd instars reared to 4ths on control diet before placement on treated cubes appeared little affected, if at all. In contrast to 2nd and 3rd instars which did consistently poorly on jack pine diet, 4th instars grew significantly better on jack pine than scotch or white pine. Since 4th instars are more likely to encounter higher phenolic levels in leaves later in the season a single experiment was designed to examine the effect of extract from jack pine at a higher concentration (1.0%, a tenfold increase) which would simulate phenolic levels in foliage in mid-summer. The results are given in Table 10, above. At a concentration of 1.0% jack pine extract in diet, growth of GM was significantly reduced and mortality increased relative to the control. This is in contrast to 4th instars fed only 0.1% extract, which did as well as the control larvae.

Table 12. Nutritional indices for GM **4th** instar fed extract 0.1% in diet. Units and statistical tests as in Table 11.

	ECD (%)	AD (%)	ECI (%)	RGR (mg/mg day)	RCR (mg/mg day).
Control	41.59^{a} (3.9) *n* = 25	62.31^{a} (3.9) *n* = 25	25.77^{a} (2.9) *n* = 25	0.208^{a} (0.03) *n* = 25	0.790^{a} (0.06) *n* = 25
Jack	54.50^{a} (4.3) *n* = 19	43.62^{b} (3.7) *n* = 19	22.59^{a} (2.1) *n* = 19	0.143^{a} (0.02) *n* = 19	0.646^{a} (0.09) *n* = 19
Red	47.57^{a} (5.1) *n* = 20	45.28^{b} (5.8) *n* = 20	19.82^{a} (2.76) *n* = 20	0.139^{a} (0.03) *n* = 20	0.624^{a} (0.09) *n* = 20
Scotch	45.95^{a} (3.7) *n* = 17	58.06^{ab} (5.8) *n* = 17	25.96^{a} (3.0) *n* = 17	0.143^{a} (0.03) *n* = 17	0.577^{a} (0.09) *n* = 17
ANOVA Statistics	$F = 1.62$ $P = 0.192$ df =3	$F = 3.89$ $P = 0.012$ df =3	$F = 1.11$ $P = 0.349$ df =3	$F = 1.92$ $P = 0.133$ df =3	$F = 1.38$ $P = 0.254$ df =3

Effect of Pure Compounds On Growth of Gypsy Moth Larvae

Quercetin 3-O-glucoside occurred at high concentrations in all four pine species (Table 2). In addition, quercetin 3-O-rutinoside (rutin) was found in all four pine species at various concentrations but was most abundant in white pine. I therefore decided to test two of the compounds: the aglycone quercetin and a flavonol glycoside rutin which were available commercially or isolated and purified in a sufficient quantity against GM 2nd and 3rd instars. The only source for quercetin-3-O-glucoside was the relatively small amount which had been isolated from the four pine species, and only allowed testing of one concentration (0.1%) against GM 2nd and 3rd instars.

The aglycone quercetin was the most active of the three pure compounds against GM 2nd instars (Table 13). At a concentration of only 0.005% in diet growth of 2nd instars was significantly reduced relative to the control on days 9 and 14. Concentrations of 0.01 and 0.05% reduced growth significantly more than the control or a concentration of 0.005%. Mortality was also increased at all concentrations but was only around 20-30% compared to control mortality of 10%. This compound also inhibited growth of larvae which had been reared to 3rd instar and then placed on treated diet, however the effect was not as great as that seen for 2nd instars (Table 16). At the highest concentrations (0.01 and 0.05%) growth was reduced significantly but there was only a 20% reduction relative to the control. There did not seem to be any effect on mortality at these concentrations.

Rutin at a concentration of 0.05% reduced growth of 2nd instars significantly by days 14, 19 and 24 (Table 14). There was no significant differences relative to control between concentrations of 0.005% and 0.01%. Mortality for the two highest concentrations of rutin was only 20% compared to a control mortality of 7.5%. Growth was also reduced for rutin fed 3rd instars (Table 17). At concentrations of 0.01 and 0.05% growth was reduced by 17 and 33% respectively relative to the control. However this was not as much as for 2nd instars and mortality did not seem to be affected.

Only enough quercetin-3-O-glucoside was available for a test of one concentration (0.1%) against GM 2nd and 3rd instars. This flavonol glycoside had little effect on growth of 2nd instar larvae until the larvae were 19 days old (Table 15). By the final day of the experiment growth was reduced by approximately 50% to the control. As seen in the extracts and pure compounds previously discussed the growth of 3rd instars was not as affected by the same concentration of quercetin-3-O-glucoside (Table 18). At a concentration of 0.1% growth was only reduced by some 15% relative to the control by day 21 compared to 50% by day 21 for 2nd instars. Again mortality was not increased due to the presence of the compound (control mortality was consistently higher).

Table 13. Effect of pure quercetin on growth and survivorship of GM 2nd Instars. Transformations, units and statistics as in Table 3.

Conc'n (%)	Age of Larvae (Days)				
	4	9	14	19	24
0.000	1.86^a (0.03) $n = 40$	3.66^a (0.02) $n = 38$	5.24^a (0.05) $n = 38$	6.04^a (0.05) $n = 37$	6.46^a (0.08) $n = 37$
0.005	1.87^a (0.02) $n = 40$	3.53^b (0.02) $n = 39$	5.09^b (0.06) $n = 36$	5.98^{ab} (0.06) $n = 30$	6.32^a (0.08) $n = 30$
0.010	1.84^a (0.03) $n = 40$	3.53^b (0.03) $n = 40$	5.02^b (0.03) $n = 40$	5.84^b (0.07) $n = 39$	6.06^b (0.08) $n = 34$
0.050	1.88^a (0.03) $n = 40$	3.54^b (0.03) $n = 40$	5.0^b (0.04) $n = 38$	5.65^c (0.06) $n = 37$	5.86^b (0.07) $n = 34$
Total ANOVA Statistics	F =0.36 P=0.784 df =3	F = 6.74 P = 0.0003 df =3	F = 6.39 P = 0.0004 df =3	F = 8.51 P<0.0001 df =3	F = 13.14 P<0.0001 df =3
Repeated Measures ANOVA			F = 27.24 P < 0.0001 df = 3		

Table 14. Effect of pure rutin on growth and survivorship of GM **2nd** instars. Transformations, units and statistics as in Table 3.

Conc'n (%)	Age of Larvae (Days)				
	4	9	14	19	24
0.000	1.86^a (0.03) $n = 40$	3.66^a (0.02) $n = 38$	5.23^a (0.05) $n = 38$	6.04^a (0.06) $n = 37$	6.46^a (0.08) $n = 37$
0.005	1.85^a (0.02) $n = 40$	3.60^a (0.03) $n = 40$	5.22^a (0.03) $n = 37$	5.85^a (0.08) $n = 37$	6.28^a (0.10) $n = 35$
0.010	1.86^a (0.02) $n = 40$	3.65^a (0.02) $n = 40$	5.18^a (0.03) $n = 37$	6.01^a (0.06) $n = 39$	6.45^a (0.04) $n = 32$
0.050	1.85^a (0.02) $n = 40$	3.60^a (0.03) $n = 40$	4.92^b (0.04) $n = 36$	5.47^b (0.06) $n = 34$	5.7^b (0.08) $n = 32$
ANOVA Statistics	$F = 0.13$ $P = 0.942$ df = 3	$F = 1.86$ $P = 0.139$ df = 3	$F = 14.42$ $P < 0.001$ df = 3	$F = 16.23$ $P < 0.001$ df = 3	$F = 19.25$ $P < 0.001$ df = 3
Repeated Measures ANOVA			$F = 50.56$ $P < 0.0001$ df = 3		

Table 15. Effect of pure quercetin-3-O-glucoside on growth and survivorship of GM 2nd instars. Means compared using PROC GLM (SAS®) and with *t* -tests, otherwise units etc. as in Table 3.

Conc'n (%)	Age of Larvae (Days)				
	4	9	14	19	24
0.00	2.00^a (0.02) $n = 40$	4.87^a (0.05) $n = 39$	5.36^a (0.04) $n = 37$	6.25^a (0.07) $n = 37$	6.43^a (0.04) $n = 33$
0.10	2.00^a (0.05) $n = 40$	4.43^a (0.03) $n = 40$	5.24^a (0.05) $n = 39$	5.57^b (0.07) $n = 36$	5.86^b (0.068) $n = 32$
T-statistic	$T = -1.50$ $P = 0.167$ df = 9.0	$T = 1.37$ $P = 0.18$ df = 77	$T = 4.12$ $P = 0.046$ df = 74	$T = 8.96$ $P < 0.000$ df = 60	$T = 7.27$ $P < 0.001$ df = 63
ANOVA Statistics	$F = 2.06$ $P = 0.173$ df = 1	$F = 0.95$ $P = 0.332$ df = 1	$F = 3.34$ $P = 0.072$ df = 1	$F = 48.36$ $P < 0.0001$ df = 1	$F = 49.32$ $P <0.0001$ df =1
Repeated Measures ANOVA			$F = 85.13$ $P < 0.0001$ df = 1		

Table 16. Effect of pure quercetin on growth and survivorship of GM 3rd Instars. Statistics, transformations and units as in Table 3.

Conc'n (%)	Age of Larvae (Days)				
	9	12	15	18	21
0.000	4.31^{a} (0.01) $n = 39$	5.35^{a} (0.03) $n = 39$	6.12^{a} (0.05) $n = 36$	6.31^{a} (0.06) $n = 36$	6.65^{a} (0.07) $n = 33$
0.005	4.18^{b} (0.03) $n = 40$	5.30^{a} (0.04) $n = 38$	6.04^{a} (0.04) $n = 38$	6.29^{a} (0.05) $n = 37$	6.52^{ab} (0.05) $n = 33$
0.010	4.20^{b} (0.03) $n = 39$	5.21^{b} (0.03) $n = 38$	5.88^{b} (0.06) $n =32$	6.17^{a} (0.05) $n = 31$	6.25^{bc} (0.07) $n = 30$
0.050	4.07^{c} (0.03) $n = 40$	5.07^{c} (0.03) $n = 39$	5.87^{b} (0.04) $n = 37$	6.15^{a} (0.04) $n =37$	6.37^{c} (0.06) $n =37$
ANOVA Statistics	$F = 17.32$ $P < 0.0001$ df = 3	$F = 14.73$ $P < 0.0001$ df = 3	$F =7.13$ $P = 0.0002$ df = 3	$F = 2.59$ $P = 0.056$ df =3	$F = 7.44$ $P = 0.0001$ df = 3
Repeated Measures ANOVA			$F = 26.9$ $P < 0.0001$ df = 3		

Table 17. Effect of pure rutin on growth and survivorship of GM 3rd instars. Statistics, transformations and units as in Table 3.

Conc'n (%)	Age of Larvae (Days)				
	9	12	15	18	21
0.000	4.47^{a} (0.04) $n = 40$	5.29^{a} (0.04) $n = 38$	5.90^{a} (0.05) $n = 38$	6.38^{a} (0.05) $n = 35$	6.38^{a} (0.05) $n = 34$
0.010	4.23^{b} (0.04) $n = 40$	5.06^{b} (0.03) $n = 39$	5.64^{b} (0.04) $n = 39$	6.21^{b} (0.04) $n = 38$	6.49^{a} (0.03) $n = 38$
0.050	4.30^{b} (0.03) $n = 40$	5.05^{b} (0.03) $n = 40$	5.65^{b} (0.04) $n = 40$	5.97^{c} (0.05) $n = 38$	6.21^{b} (0.05) $n = 37$
Total ANOVA Statistics	$F = 11.00$ $P < 0.001$ df =2	$F = 18.25$ $P < 0.001$ df =2	$F = 11.08$ $P < 0.001$ df =2	$F = 21.59$ $P < 0.001$ df = 2	$F = 18.82$ $P < 0.001$ df =2

Table 18. Effect of pure quercetin-3-O-glucoside on growth and survivorship of GM 3rd instars. Means compared using PROC GLM (SAS®) and with t -tests, otherwise units etc. as in Table 3.

Conc'n (%)	Age of Larvae (Days)				
	9	12	15	18	21
0.000	4.11 (0.03)^{a} $n = 40$	4.85 (0.05)^{a} $n = 35$	5.67 (0.07)^{a} $n = 33$	6.16^{a} (0.07) $n = 33$	6.47^{a} (0.08) $n = 32$
0.10	4.02 (0.02)^{b} $n = 40$	4.91 (0.04)^{a} $n = 40$	5.56 (0.04)^{a} $n = 40$	6.09^{a} (0.04) $n = 40$	6.35^{a} (0.04) $n = 40$
T-statistic	$T = 2.72$ $P = 0.008$ df = 70.2	$T = -0.97$ $P = 0.336$ df = 70	$T = 1.26$ $P = 0.212$ df = 54	$T = 0.87$ $P = 0.39$ df = 53	$T = 1.41$ $P = 0.166$ df = 49
ANOVA Statistics	$F = 7.39$ $P = 0.0081$ df = 1	$F = 0.94$ $P = 0.335$ df = 1	$F = 1.72$ $P = 0.194$ df = 1	$F = 0.82$ $P = 0.368$ df = 1	$F = 2.19$ $P = 0.143$ df = 1
Repeated Measures ANOVA			$F = 3.86$ $P = 0.053$ df = 1		

Discussion

Role of Phenolics in Plant Defense

The severity of outbreaks and damage that forest feeding lepidoptera cause has led to research toward understanding their population cycles and development of potential control mechanisms. The GM, in particular, has been studied rather intensively in recent years because it is an introduced species which has spread, and continues to spread westward throughout the forests of the Nearctic. This intense research has resulted in a large number of papers published recently on host choice, range expansion, pathogens, control measures and population dynamics. I have tried to summarize these as much as possible in the general introduction and in the introduction to this chapter. Since there has been so much published on GM alone, I will try to limit my discussion in this chapter and refer only to those studies which are directly relevant to my study and how these fit into the larger scheme of plant-insect interactions.

As mentioned in the introduction to this chapter, my study agrees with others that attempt to answer questions about the feeding ecology of GM in the laboratory. The advantage of laboratory studies is that many of the environmental variables that affect field studies, such as temperature, moisture and diet can be controlled for. Indeed, the development of artificial diet such as that of Odell et al. (1985) for GM has made it possible to manipulate dietary constituents such as protein, sugar, fat, water, secondary chemicals etc. in such a way that very useful quantitative data on the nutritional ecology can be obtained.

Many of these laboratory studies have sought to answer, questions generated by field observations. Studies such as those of Mauffette et al. (1983), Gross et al. (1990) and Miller et al. (1991), have tried to answer questions of host range and choice for GM. For the data presented and analyzed in this chapter, I have attempted to test the hypothesis stated at the beginning of this thesis that feeding on pine by GM is, at least in part, determined by the sensitivity of different instars to allelochemicals present in pines. In particular, I have tested a class of secondary chemicals, phenolics, in both extract and in pure form against three instars of GM. Where does such a broad class of compounds fit in with the theories of plant defense?

Phenolics, and polyphenolics such as tannins, in general are considered to be "quantitative" defenses according to Feeny's (1976) plant apparency theory. These phenolics have long been viewed as digestibility reducing substances (note: tannins are considered phenolics, but are actually polyphenolics, whose monomers are chalcones, gallic acid). However, the role of polyphenolics such as tannins as digestibility reducing substances to larval insects has been questioned in the recent literature (Martin & Martin 1983, Karowe 1989). In addition, except for a few studies such as those of Elliger et al. (1980) and Abou-Zaid et al. (1993), the role of other phenolics such as flavonoids play in plant-insect interactions has not been well elucidated. In this chapter, the role of phenolics in extract from pines and their effect on feeding of different instars of GM was examined. In addition, key phenolics and phenolic glycosides from pine have been identified and then examined as to how they affect growth of early and later instars of GM.

Effect of Pine extracts on growth of 2nd, 3rd and 4th Instars of GM

The first experiments with extract containing the phenolic fraction from pines indicated that 2nd instars were very sensitive to phenolics isolated from the four pine species (Tables3-6). At most concentrations tested, the extract reduced growth and increased mortality to second instars that were fed extract in diet continuously. For example, jack pine and red pine had the lowest EC_{50} values of approximately 0.0042 and 0.0525% (Table 7), respectively. These results suggest that 2nd instar GM may not feed on pines because they are sensitive to the range of phenolics found in pines. However, the reports of pine feeding by early instars of GM are conflicting. For example, Barbosa & Greenblatt (1979) found that mortality was 100% for larvae reared only on a diet of white, loblolly and Virginia pine. However, Joseph & Kelsey (1994) found that douglas-fir foliage could support GM populations without the presence of other preferred host species. In Ontario, severe defoliation of white pine and eastern hemlock (*Tsuga canadensis* [L.] Carr.) has been reported in a 1992 survey (Jones et al. 1993). However, records indicate that, in instances, of severe damage, the stands of trees consisted of a mixture of pine and oak species.

Seasonal and Interspecific Variation in Phenolic Content of Pine Needles and Potential Effects on Gypsy Moth

The data from the laboratory studies suggest that 2nd instars at least would not survive on pines with fresh leaf phenolic concentrations of 0.05% or greater since their growth is greatly reduced and mortality increased when fed phenolic extract at this concentration. In my study of the chemistry of the four pine species, the total phenolic content (gallic acid equivalents GAE) varied from 4.23 % of fresh weight for jack pine to 6.21% of fresh weight for white pine for the period the needles were collected (late June 1992). Joseph et al. (1993) and Joseph & Kelsey (1994) found levels of phenolics in Douglas-fir varied between 1.58 - 4.0 % of dry needle weight; with the highest concentrations occurring in both new flush and 1-year old needles during the spring and early summer. Wagner et al. (1990) have also documented seasonal changes in phenolics for three conifer species Douglas-fir, white fir (*Abies concolor* Gord & Glenn) and Engelmann spruce (*Picea engelmani* Perry ex Engelm). These authors found that concentrations of total phenols were highest in early June at just over 7.0% of dry weight, but that these compounds dropped to 3.0, 4.0 and 6.0% for white fir, Douglas-fir, and Engelmann spruce respectively by the end of July. It would therefore not be unusual for pine species to show seasonal variation in amount of total needle phenolics. Jack pine (two year old seedlings) at least, has a total phenolic content that varies between 0.5 and 1.5%. (Nozzolillo et al. 1990). In new foliage of Norway spruce (*Picea abies* [L.] Karst), the concentration of the flavonol kaempferol-3-O-glucoside dropped from a high of 18 nmol/needle in May of 1987 to a low of 2 nmol/needle in August of 1987 (Strack et al. 1989). The flavonoid profile of pines does not appear to vary intraspecifically, but in a study of European mountain pine (*Pinus uncinata* Ram.), quercetin was found at concentrations of 9 to 17% (of total flavonoid content) depending on the locale (Laurenson and Lebreton 1991).

While it is clear that 2nd instars are harmed by low concentrations of phenolics in artificial diet, it is also apparent that later instars are less affected by a concentration of 0.1%

extract. After finding that the extract was effective at a very low concentration, I decided to see if later instars were as sensitive to this concentration. It is clear that for third instars, growth was not reduced as much as that of 2nd instars, whereas 4th instars did not appear to be affected at all by low concentrations of phenolics in diet. However, when the extract was incorporated into diet at a concentration of 1.0% (jack pine extract), a more realistic concentration given the amount in needles, 4th instar growth was severely reduced and mortality increased.

Role of P-450-Dependent Polysubstrate Monooxygenases in Resistance

The ability of GM to tolerate low levels of phenolics in diet by the fourth instar may be due to increased activity of cytochrome P-450-dependent mixed-function oxidases (MFOs, which are recently described as polysubstrate monooxygenases PSMOs, however I will cite the original author's terminology) in later instars. Ahmad (1986) found greater MFO specific activity as the larvae advanced to higher instars so that they may process dietary allelochemicals with a similar efficiency as younger larvae. However, other factors such as nutrient deficiency and their effect on MFOs must be taken into account on insects feeding on nutrient deficient diet (Lindroth et al. 1991). In addition, an understanding of the effect of allelochemicals is complicated by some recent publications. Lindroth et al. (1993) looked at activity of cytochrome P-450 monooxygenase, esterase, glutathione transferase, carbonyl reductase enzymes and found that each of these enzymes was affected by plant species (maple or aspen), CO_2 or NO_3^-, or by an interaction of these factors. In contrast, Sheppard and

Friedman (1989) reared gypsy 3rd to 6th instars on oak and pine foliage but did not find any correlation between two monooxygenases (NADPH oxidase, and O-demethylase) and the host plant on which the larvae fed. They concluded that monooxygenases played little or no role in the ability of GM's ability to feed and develop on pine foliage. The role of monooxygenase activity will be discussed more thoroughly in relation to its effect on pure phenolics later in this chapter.

When phenolic containing extract from Douglas-fir was incorporated into diet at a concentration at 3.0%, (which reflects that found in the needles), Joseph et al. (1993) found that growth of neonate larvae was very reduced relative to the control. Survivorship after 11 days was unaffected, but none of the larvae survived to the 4th instar for calculation of nutritional indices. However, Joseph and Kelsey (1994) found that larvae could begin and complete development on Douglas-fir foliage itself. The reason for this difference may be due to the presence of tannins and flavonoid monomers and dimers of tannins such as catechin and epicatechin (Joseph et al. 1993). These tannins and tannin monomers could complex with proteins, amino acids etc., in artificial and natural diets (Bernays et al. 1989).

In my study, it is doubtful that tannins and tannin monomers and dimers binding to dietary constituents are responsible for the response of GM 2nd - 4th instars. After consultation with Dr. Abou-Zaid (pers. comm.) the extraction procedure for tannins and other polyphenolics is different from the one we employed for simple phenolics and flavonoids. For example, extraction of condensed tannins (catechins) should be done with ether or ethyl acetate, concentrated and then chromatographed 2-dimensionally in butanol-acetic and dilute aqueous acetic acid (Ribéreau-Gayon 1968). Careful study of the chromatograms from pine showed trace levels of tannins in the extract prepared from pines. We did not find any catechin or epicatechin, which are monomers of tannin (Table 2), in the extracts although these have been recorded previously from scotch pine. Pines are rich in simple phenolics and flavonoids but do not appear to have tannin. It has been suggested that dihydroxyphenols may form quinones under oxidative conditions, and then go on to bond with amino acids in proteins, which, in turn, affect their digestibility (Felton et al. 1992), and this may be one reason for the sensitivity of early instars to pine phenolics in the extract.

Effect of Extracts (0.1%) on 3rd and 4th Instar Nutritional Indices

Significant short term effects of pine extract on growth of third and fourth instars was observed in my study. For third instars extract containing phenolics significantly lowered the ECD for those fed jack pine, but AD was lower for the control than for any of the pine species tested, which did not differ statistically from one another. This inverse relationship between ECD and AD has been noted before but there is no clear explanation why this occurs (Scriber and Slansky 1981). The different components of metabolism such as basal metabolic rate, activity, and growth need further study. ECI for jack pine was also significantly reduced, but RGR and RCR were unaffected for larvae feeding on the three pine species. Even though RGR appeared to be unaffected after only two days, it is clear that there must be long term effects since 3rd instars fed extract at 0.1% weigh significantly less after several days. ECD for 4th instars was unaffected by extract at a concentration of 0.1% in diet, but AD was significantly lowered for larvae fed jack and red pine extract. ECI was unaffected, but RGR was significantly lower for red pine while RCR was significantly lower than the control for all pine species. Stoyenoff et al. (1994 ab) noted differences in performance indices for GM when switched from one host species to another. They found that RGRs, mortality, and pupal weights were strongly affected by the second host type eaten, while larval weights and development were affected by the first host. It is uncertain how this relates to my study since the experimental designs were very different, and the first "host" for GM in my study was simply artificial diet. The nutritional indices do, however, indicate that at low levels phenolics in diet do not have significant negative short-term effects on 4th and perhaps later instars of GM.

Effect of Pure Flavonoids on Growth of 2nd and 3rd Instars

Rutin, quercetin and quercetin-3-O-glucoside all significantly reduced growth relative to the control, but mortality was not strongly affected. This indicates that some of the pure flavonoid compounds isolated from pines are at least in part responsible for the reduced growth observed in the phenolic-containing extracts discussed above. In addition there were still significant negative effects on growth of 3rd instars at concentrations as low as 0.01% (rutin and quercetin) and 0.1% (quercetin-3-O-glucoside).

A number of flavonoids are known to inhibit growth of other insect larvae. Elliger et al. (1980) found that, in general, the greater the degree of substitution (hydroxylation, methylation, glycosylation) of the flavonoid molecule, the greater the reduction in growth of larval *H. zea* after 12 days. Elliger et al. (1980) also found rutin, quercitrin, and quercetin to be very effective at reducing growth of *H. zea* (EC_{50} = 4.0, 4.5 and 3.5 mmol/kg respectively). However, Lindroth and Peterson (1988) found that quercetin at a dietary concentration of 0.1% did not affect growth and development of *Spodoptera eridania* (Cramer). Quercetin may therefore differ in its toxicity to different lepidopteran species. Finally, Lindroth and Peterson (1988) found rutin to be very effective at reducing growth and increasing mortality at a concentration of 2.2%. However, in my study, 2nd and 3rd instars of GM larvae were affected by all three flavonoids tested when incorporated into diet in concentrations as low as 0.01%. In this regard, the effect of flavonoids on GM appears to be somewhat intermediate between *H. zea* and *S. eridania* mentioned above.

Other studies have found that the relationship between plant phenolic glycosides and performance of GM is complex. Tremulacin, an aspen phenolic glycoside, at a concentration of 2.0% had no effect, but at 3% reduced growth of 4th instar larval GM (Lindroth and Hemming 1990). They also suggested that high levels of these compounds may overwhelm the detoxification capacity of GM. In a later paper Lindroth and Weisbrod (1991) argued that genetic variation in the esterase activity of GM larvae may account for the variable response. However, Mitchell et al. (1993) recently found that flavonoids such as quercetin and kaempferol inhibit cytochrome P-450-dependent ecdysone 20-monooxygenase activity of another lepidopteran larvae, *Manduca sexta*. Clearly, more physiological studies are needed to determine how flavonoids and flavonoid glycosides act in insects.

Other studies that examine effects of flavonoids are those of Stamp (1994ab), who examined the effects of rutin, potassium and temperature on performance of *Manduca sexta* caterpillars. Stamp et al. (1991) also found that the effect rutin has on a predator is dependent on temperature and that *Manduca sexta* larval growth was reduced by rutin (Stamp and Scrobola 1993). Waiss et al. (1979) found that maysin (rhamnsyl 6-C-(4-ketofucosyl) 5,7,3',4'-tetrahydroxyflavone) isolated from corn silk severely retarded growth of *H. zea* when incorporated into diet . Ermanin (7,4'-di-O-methylkaempferol) isolated from *Passiflora foetida* L. is a strong feeding deterrent to *Dione juno* (Cramer) larvae at a concentration of 40.0 ppm (Echeverri et al.1991). Finally, flavonoids from cotton *Gossypium arboreum* L. are a potential source of resistance to the tobacco budworm, *Helicoverpa virescens* Boddie. (Hedin et al. 1992).

From the above review it is clear that phenolics and flavonoids do play a number of roles in plant-insect interactions. From my study and others it is also clear that phenolics and flavonoids may in part determine host choice for a variety of insects including GM. The results obtained here with phenolics in extract and pure compounds suggest that one of the reasons pines are not fed on by early instars is due to their overall phenolic content, and in particular, may be due to compounds such as quercetin, quercetin-3-*O*-glucoside, and rutin that act as deterrents. In the following chapter I will present results to test the hypothesis that these same compounds in pine may benefit later instars by providing protection from a viral pathogen.

CHAPTER FOUR: Effect of Pine Extracts and Pure Phenolics on Gypsy Moth Larvae Infected with Nuclear Polyhedrosis Virus

Introduction

To date, our theoretical understanding of how plants and insects interact has essentially been based only on the studies of two trophic level systems, that is, direct effects of host plants on herbivorous insects (Price et al.1980). However, terrestrial communities with plants as primary producers at the first trophic level, also have herbivores at the second trophic level, and these herbivores in turn have predators, parasites, and pathogens etc., at the third trophic level. Therefore defensive compounds in plants may have direct effects not only on herbivores, but may also indirectly affect predators and pathogens at the third trophic level. The need to study the effects of plant defenses against herbivores over three trophic levels is practical as well as theoretical. For example, host plant compounds such as alkaloids which convey resistance to herbivores, reduce survivorship of parasitoids of the herbivore (Campbell and Duffey 1979).

Most studies of generalist herbivores such as the gypsy moth (GM) have dealt with primary and secondary host use, and the effect of secondary chemicals from various hosts on feeding deterrence and growth reduction (see review in general introduction). However, little has been published on the effect of host plant chemistry on various pathogens, parasites and predators of GM. It is known that compounds (probably tannins) from red oak foliage inhibit sporulation and increase incubation time for *Entomophaga maimaiga* Humber, Shimazu &

Soper, a fungal pathogen of GM (Hajek and Renwick 1993). Survival of a GM parasitoid, *Cotesia melanoscela* (Ratz.) was higher for GM larvae reared on white oak than for those reared on red maple, hickory, or American beech. Aspen phenolic glycosides had negative and additive effects on larval mortality when GM was infected with a bacterial pathogen, *Bacillus thuringiensis* Berliner (*Bt.*) (Hwang et al. 1995, Arteel and Lindroth 1992), but tannins from oaks and aspen appear to reduce infectivity of *Bt* (Appel and Schultz 1994). Host-plant chemistry also affects infectivity of another pathogen, GM nuclear polyhedrosis virus (NPV) (Keating and Yendol 1987, Keating et al. 1990).

The GM NPV has generated considerable research regarding its potential use as a control agent (Doane 1970, Cunningham et al. 1991,1993, Webb et al. 1994) and its importance in affecting its host's feeding behaviour (Rossiter 1987, 1991, Rossiter et al. 1988). In general, the effect viruses such as NPV have on their insect hosts may be the most important factor behind the "boom and bust" population dynamics typical of GM and other forest lepidoptera (Myers 1993). This type of population cycle is evident in Ontario where severe GM defoliation rose to approximately 250,000 hectares in 1985 but by 1987 had dropped to only 11,564 hectares (Keizer 1989). In 1991 defoliation again rose to a high of 347, 415 hectares (Jones and Bolan 1992) but then dropped once more within two years to only 9,784 hectares by 1993 (Evans et al. 1994).

Transmission of NPV from one generation to the next may be trans-ovum (Doane 1969) or environmental due to death and release of NPV from infected larvae (Murray and Elkinton 1989,1990). Strains of NPV isolated from natural GM populations vary in their infectivity (Doane 1967, Magnoler 1974) perhaps due to geographic location of collection, but generally, the number of polyinclusion bodies (PIBs)/larvae necessary to induce infection increases with larval age. Infectivity of NPV strains generally drops with length of time in storage.

The effect of plant chemistry on host/pathogen may affect host choice for GM. Late instar larval mobility, as well as the range and type of host eaten may in part be explained if larvae are protected to some extent from the effects of NPV (and other potential pathogens). Mobility and host switching by non-outbreak populations of gypsy larvae has been inferred by Mauffette and Lechowicz (1984), and documented by Rossiter (1987). Host-switching by non-outbreak populations may result from hormonal changes associated with molting. Mobility of late-instar larvae also serves to expose larvae to the diversity of host trees in a forest (Rossiter 1987). For example, Rossiter (1987) found that larvae that switched to pitch pine (generally a species not eaten by early instars) preferred it in later instars and that these larvae benefitted nutritionally while also gaining protection from egg parasitoids and pathogens such as NPV. Individuals that fed on pitch pine may have escaped infection from pathogens for the following reasons: 1) this behaviour removes them from areas of greater larval density therefore lowering the chance they will encounter infected conspecifics or substrates, 2) a diet of pitch pine may reduce the negative impact of NPV on individuals that are already infected or, 3) only individuals which are strong and healthy can afford to be mobile and therefore are innately more resistant to infection.

There have been no studies to support or discount the first and third hypotheses, however, if the second hypothesis is true then different types of host foliage should vary in their ability to protect GM from NPV infection. In support of this hypothesis, Keating &

Yendol (1987) found that virus-induced mortality was lower on preferred than non-preferred host plants. Dietary constituents such as proteins, sugars and secondary chemicals which vary from host to host should also affect NPV induced mortality. Additions to artificial diets of protein, salts, acid and tannins result in a significant decrease in larval susceptibility to NPV, but sucrose, surfactants, and gallic acid had no effect (Keating et al. 1989). The GM feeding on plants containing more hydrolysable tannins suffered lower NPV-induced mortality (Keating et al. 1990). However, foliage constituents from an exotic tree species, neem (*Azadiracta indica* Juss.) had no effect on infectivity of NPV (Shapiro et al. 1994).

Phenolic compounds such as rutin and chlorogenic acid found in host plants significantly inhibited NPV infectivity when tested against the tomato fruitworm (*Helicoverpa zea*: Noctuidae) (Felton et al. 1987). Phenolics are a large class of compounds which are found in all tree species and may have effects on GM larval nutrition as well as provide protection from NPV infection. Phenolics from most pine species are not well known, but flavonols, flavones, and flavonol glycosides have been isolated from jack pine (Abou-Zaid and Nozzolillo 1991). Differential sensitivity of early and late instars to these compounds may explain why pines are not consumed in early instars, but are readily consumed in later instars. Early instars are quite sensitive to low concentrations of phenolics in artificial diets (Chapter 3) but these compounds do not slow growth of later instars as strongly. We also know little of how these compounds affect 3rd and later instars of GM which have been infected with NPV. If the use of pitch pine depends on the mobility of late instars and in recognition of protective properties of this host against NPV infection, then perhaps other pine hosts such as jack, red, scotch and white pine may also provide protection. In particular, phenolics from pines may provide a measure of protection to GM.

The purpose of the following experiments therefore, was to determine if the phenolic extract and pure phenolics present in these four pine species provide some measure of protection from GM NPV. This was done by determining: 1) short term effect of extracts on nutrition of virus-infected larvae as measured by nutritional indices, 2) long-term effect of extracts and pure compounds on weight gain and mortality and, 3) effect of extracts and pure phenolics from pine on survivorship to adulthood.

Materials and Methods

Preparation of Stock Solution of NPV

The GM virus (Disparvirus- Gypcheck) was obtained from the virus production unit at Forest Pest Management Institute (Sault Ste. Marie, Ont.) as a crude preparation of whole, ground, freeze-dried 3rd instar larvae. This crude viral preparation was suspended in distilled water and centrifuged three times at 3,000 rpm for 30 minutes. The final pellet was re-suspended in distilled water and served as a purified stock solution.

To calculate virus concentration of the stock solution 100 µL were diluted in 100 ml of distilled water containing mouse albumin. Three aliquots of 5 microlitres of the diluted stock were then applied to two microscope slides and stained with Buffalo Black solution. After drying, PIBs were counted using Wigley's (1980) method. It was therefore determined that the stock solution contained $2.93 \pm 0.17 \times 10^9$ PIBs/ml. Stock solution was then stored in a refrigerator at 2^0 C for use in the experiments.

Procedure for Dosing Larvae

There are three methods for inoculating larvae with a known amount of NPV. The first is to deliver the required number of PIBs in several microlitres of distilled water directly into the larval midgut using a microsyringe (T. Wright FPMI pers. comm.). The second is to incorporate virus of known concentration directly into liquid diet (52° C) and allow the larvae to feed freely on the cooled diet (Lewis et al. 1981). The third is to surface treat a small amount of larval food with NPV which is subsequently completely consumed by the larva (Lewis et al. 1981). There are a number of problems with the first approach, such as damage to the larval digestive system (particularly in 1st - 3rd instars) and regurgitation of fluid. There can also be a reduction in viral infectivity due to the heat of the diet in the second method. Therefore, in this study it was decided to surface coat artificial diet as outlined below.

For determination of the LD_{50} and for dosing the larvae which would consume extract treated diet, the stock solution of 2.93×10^9 PIBs/ml was diluted to give 10 ml volumes containing the appropriate number of PIBs/ml using the formula $C_1V_1 = C_2V_2$. For example, to dose larvae with 60,000 PIBs, of NPV a 52 µl volume of stock solution (2.93×10^9 PIBs/ml) was added to 10 ml of distilled water to give a final concentration of 1.52×10^7 PIBs/ml. This solution was then drawn into a Yale 1/4 cc tuberculin syringe. The syringe was calibrated with a Houston-Atlas microdoser model 1010 and then placed in a Houston-

Atlas microjector model 1003. The microjector was set to deliver 4 µl droplets of NPV solution each time it was triggered either by foot pedal or switch. Each four µL droplet contained approximately 60,000 PIBs of NPV in distilled water. Small diet plugs were then surface treated with 4µl droplets of the solution, each droplet containing approximately 60,000 PIBs. These small plugs were completely consumed (usually after a 24 hr period) and each larva therefore received 60,000 PIBs of NPV.

60,000 PIBs/larva was close to the LD_{50} for 4th instars and close to the LD_{95} for 3rd instars. This dose was then used in most of the experiments involving nutritional indices and long term weight gain of the larvae. However, for survivorship to adulthood studies doses of 10, 30, and 60,000 PIBs were used, representing low to moderate doses of virus for 3rd instars.

Infectivity of the NPV Stock: LD_{50} for Second, Third and Fourth Instars

1) 2nd Instars: The LD_{50} for the virus preparation is known and has been calculated by Bill Kaupp (pers. comm.) to be 214 PIBs/larva.

2) 3rd Instars: Stock solution of virus was diluted to give final concentrations of 1000, 3000, 10,000, 20,000, 30,000 and 100,000 PIBs/4µl . Late 2nd instars were obtained from the insect rearing unit at FPMI and reared through to early 3rd instars. Larvae were starved overnight and the next day were given small (#1 bore) diet plugs that had been dosed with 4µL of the above viral solutions (forty larvae/treatment). Larvae were not fed again until they had consumed the entire diet cube, and then regular observations were made of weight and mortality.

3) **4th Instars:** Stock solution of virus was diluted to give stock solutions of 1,000, 3,000, 10,000, 30,000, 50,000, 70,000, and 100,000 PIBs/4µl. Early 4th instars were obtained from the insect rearing unit at FPMI, starved overnight and fed diet plugs (#1 bore) which had been treated with 4µL of the above solutions of virus. Larvae were not fed again until they had consumed the eritire diet cube and then regular observations (every three days) were made of weight and mortality.

Virus and Extract Experiments

1) Short Term Effects - Nutritional Indices: Small diet plugs were dosed with four µL applications of viral solution (60,000 PIBs/larva). Extract from four pine species (0.1%) had been incorporated into diet plugs using previously described techniques. Treatments were as outlined in table 19. Control diets did not contain extract but were treated with (+) or without (-) 60,000 PIBs diet cube. Diet containing extracts from four pine species was also treated with and without 60,000 PIBs diet cube.

Table 19. Treatments for calculation of 3rd instar nutritional indices. + = treated with virus, - = not treated with virus.

	Extract (0.1%)				
	Control	Jack	Red	Scotch	White
NPV	+	+	+	+	+
NPV	-	-	-	-	-

Third instar larvae (40 in each treatment) were then placed individually into Solo® cups with the treated diet cubes. After 24 hrs, most if not all of the larvae had completely consumed the diet plugs. These were then removed, weighed and placed on large diet plugs treated only with extract. After three days, larval, diet and frass weights were recorded. These experiments examined the effect of virus and virus plus extract over a short period of time (48 hrs) on the digestion and assimilation of food by this instar.

Sample sizes varied between the treatments for several reasons. First, larvae that did not consume the entire diet cube within 24 hr were rejected. Second, larvae which did not feed after being placed on treated cubes were rejected, since this led to problems in calculating the indices due to negative weight gains. Third, although every effort was made to choose larvae of the same age, in some cases larvae had begun to molt to the fourth instar at the end of the experiment. These were also rejected because indices need to be calculated within an instar and also because molting larvae can lose up to 40% of their weight gain within an instar through voiding of the gut and loss of old cuticle (Waldbauer 1968).

2) Long Term Growth and Mortality: In this series of experiments 3rd and 4th instar larvae which had been reared on control diet were dosed with 60,000 PIBs each and then placed on diet cubes containing extract (0.1%) or various concentrations of pure compound. Weight gain and mortality were then recorded at regular intervals over a fifteen day period (3rd instars) and nine day period (4th instars) from the beginning of the experiment.

3) Survivorship to Adulthood: 3rd instar larvae were reared on control diet and then were dosed with three concentrations of (10.000 30.000 and 60.000 PIBs) of NPV/larva by surface coating of small diet plugs with the appropriate concentration of virus (see above). Fourth instars were dosed only with 60.000 PIBs/ larva. These larvae were then placed on diet plugs containing 0.1% extract from the four pine species or 0.1% quercetin-3-O-glucoside. For each treatment. 6 repetitions of 20 larvae (120 larvae in total for each treatment) were used to obtain the mean mortality. Larvae were reared to adulthood and then sexed on adult eclosion. Each experiment took from 6-12 weeks to complete.

Results

LD_{50} for Third and Fourth Instars

The LD_{50} for the virus isolate was calculated 21 days after dosing using a probit analysis (SAS® Institute 1989) and the results presented in table 20. The LD_{50}s for 3rd and 4th instars were approximately 10.000 and 70.000 PIBs/ larva respectively.

Table 20. Probit analysis for 3rd and 4th instars. Mortality 21 days after dosing with virus.

	LD_{50}	95% Fiducial Limits	
		Lower	Upper
3rd Instars	10,431 PIBs/larva	6,522 PIBs/larva	16,466 PIBs/larva
4th Instars	70,265 PIBs/larva	47,112 PIBs/larva	131,043 PIBs/larva

Nutritional Indices for 3rd and 4th Instars Dosed with NPV and Fed Extract from Pines in Diet

Means and standard errors for the nutritional indices for third instars are presented in table 21. There was little effect of extract, or extract and virus on the ECD for the three extracts tested. However, jack pine extract alone did reduced the ECD value for third instars relative to the control with virus, but not relative to the control without virus. Virus alone also significantly reduced the AD for control larvae, as did red pine extract combined with virus compared to scotch pine extract alone, but there was no significant difference between the two. None of the treatments reduced AD greatly from the control with no virus. ECI for jack pine extract alone was reduced relative to red pine extract and virus, but the ECIs for jack pine and red pine combined with virus did not differ greatly from the controls, dosed or undosed with virus. The RGR for jack pine alone was reduced relative to the control with virus, but not greatly from the control which was not dosed with virus. Finally, none of the extracts, or extracts with virus, seemed to affect RCR relative to the controls.

Table 21. Nutritional indices (calculated from fresh weights) for GM 3rd instars fed extract 0.1% in diet and dosed with 60,000 PIBs (+) or undosed (-). Means followed by SE in brackets (see Appendix A for abbreviations).

	ECD (%)	AD (%)	ECI (%)	RGR (mg/mg day)	RCR (mg/mg day)
Control-	35.36 (2.8) *n* = 36	74.10 (1.7) *n* = 36	24.95 (1.5) *n* = 36	0.295 (0.03) *n* = 36	1.104 (0.06) *n* = 36
Control+	36.10 (2.4) *n* = 34	72.75 (1.4) *n* = 34	26.15 (1.5) *n* = 34	0.352 (0.03) *n* = 34	1.240 (0.07) *n* = 34
Jack-	26.65 (1.9) *n* = 38	78.75 (1.2) *n* = 38	20.43 (1.3) *n* = 38	0.022 (0.02) *n* = 38	1.024 (0.05) *n* = 38
Jack+	32.15 (2.9) *n* = 26	75.52 (1.8) *n* = 26	23.12 (2.2) *n* = 26	0.303 (0.04) *n* = 26	1.176 (0.08) *n* = 26
Red-	35.49 (2.6) *n* = 35	75.96 (1.8) *n* = 35	25.73 (1.7) *n* = 35	0.309 (0.03) *n* = 35	1.092 (0.07) *n* = 35
Red+	38.82 (2.0) *n* = 33	72.31 (1.4) *n* = 33	27.41 (1.2) *n* = 33	0.363 (0.03) *n* = 33	1.260 (0.05) *n* = 33
Scotch-	31.57 (1.8) *n* = 36	79.34 (1.5) *n* = 36	24.51 (1.2) *n* = 36	0.284 (0.02) *n* = 36	1.108 (0.05) *n* = 36
Scotch+	30.44 (2.6) *n* = 30	77.79 (1.6) *n* = 30	22.83 (1.8) *n* = 30	0.263 (0.03) *n* = 30	1.027 (0.07) *n* = 30

Table 22. Summary table of 2-way ANOVA performed on nutritional indices for **3rd** instars fed 4 pine extracts (0.1%) and dosed with 60,000 PIBs NPV. *F* and *P* values are for type III sum of squares, significant effects underlined.

		Nutritional Indices									
		ECD		AD		ECI		RGR		RCR	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Extract	3	4.33	0.005	4.59	0.004	5.76	0.004	3.07	0.028	1.39	0.245
Virus	1	1.24	0.266	4.23	0.039	2.19	0.150	4.25	0.040	4.13	0.043
Extract *Virus	3	0.90	0.443	0.33	0.807	0.74	0.532	1.12	0.340	1.66	0.018

2-way ANOVAs were then performed on the nutritional indices (Table 22). Extracts from all three pine species alone significantly reduced all indices except RCR for 3rd instars, while virus alone reduced the AD and RGR but increased the RCR. The only significant interaction effect between virus and extract was to increase the RCR.

Effects of Extract and Virus on Growth and Mortality of Gypsy Moth 3rd and 4th Instars.

Long term exposure of larvae infected with virus to diet containing pine extracts was measured by recording weight and mortality at 3 day intervals over a period of 12 days. The 2-way repeated measures ANOVAs were then performed to determine the effect of extract, virus and virus-extract interactions on weight. By day 12 all treatments except for white pine alone had reduced the weight of 3rd instars relative to the controls with and without doses of virus (Table 23). Overall, mean weights for larvae fed extract ± virus (± indicates with or without virus inoculation) generally declined from days 9 -21 although the differences were not as great for days 18 and 21.

At the end of the experiment (day 21) survivorship between larvae in the controls ± virus and extracts ± virus approached significance (2-way contingency table, $\chi^2 = 8.01$, $P = 0.093$). Individual pairwise comparisons were then performed comparing survivorship with the four extracts ± virus individually and the control ± virus. Survivorship for larvae fed jack pine extract and dosed with virus did not differ significantly from survivorship for control dosed with virus (Fisher's Exact Test, $P = 0.190$), however survivorship was significantly higher for larvae dosed with virus which were fed red (Fisher's Exact Test, $P = 0.009$), scotch (Fisher's Exact Test, $P = 0.022$), and white (Fisher's Exact Test, $P = 0.035$) pine extract compared to control larvae dosed with virus only. Multiple pairwise comparisons between extracts ± virus did not show any significant differences in survivorship ($P = 0.097$ - 0.839, 6 comparisons among extracts and virus). In Table 24, a 2-way repeated measures ANOVA was performed on the weights in Table 23. Pine extracts alone had a significant effect on reducing growth of 3rd instars. Virus alone did not have any significant effect on weight of the 3rd instars, but the interaction between virus and extract did combine to reduce the weight of 3rd instars significantly. There were also significant time-virus and time-virus-extract interactions over the course of the experiment.

Table 23. The effect of pine extracts (0.1%) and virus on weight of GM **3rd** instars dosed with 60,000 PIBs (+) or undosed (-). Log-transformed means, with standard errors in brackets.

	Age of Larvae (Days)				
	9	12	15	18	21
Control-	4.29 (0.03) *n* = 39	5.53 (0.03) *n* = 38	6.24 (0.04) *n* = 38	6.47 (0.05) *n* = 38	6.51 (0.07) *n* = 34
Control+	4.26 (0.04) *n* = 40	5.52 (0.04) *n* = 38	5.87 (0.09) *n* = 35	6.74 (0.14) *n* = 8	6.53 (0.31) *n* = 4
Jack-	4.27 (0.04) *n* = 40	5.32 (0.05) *n* = 40	5.68 (0.07) *n* = 39	5.91 (0.07) *n* = 37	5.83 (0.08) *n* = 36
Jack +	4.32 (0.03) *n* = 40	5.17 (0.05) *n* = 40	5.46 (0.05) *n* = 39	5.67 (0.09) *n* = 14	5.74 (0.12) *n* = 9
Red-	4.23 (0.04) *n* = 40	5.04 (0.07) *n* = 40	5.37 (0.06) *n* = 38	5.70 (0.08) *n* = 36	5.82 (0.08) *n* = 33
Red+	4.24 (0.03) *n* = 35	5.32 (0.05) *n* = 35	5.67 (0.07) *n* = 35	6.04 (0.13) *n* = 19	6.24 (0.11) *n* = 17
Scotch-	4.33 (0.03) *n* = 40	5.31 (0.04) *n* = 40	5.84 (0.04) *n* = 39	6.23 (0.04) *n* = 39	6.16 (0.06) *n* = 37
Scotch+	4.31 (0.03) *n* = 40	5.34 (0.03) *n* = 40	5.81 (0.05) *n* = 36	6.26 (0.08) *n* = 18	6.19 (0.08) *n* = 16
White	4.32 (0.03) *n* = 37	5.37 (0.06) *n* = 37	5.84 (0.08) *n* = 37	6.05 (0.09) *n* = 36	6.11 (0.08) *n* = 29
White+	4.05 (0.02) *n* = 40	5.08 (0.05) *n* = 40	5.39 (0.08) *n* =37	5.97 (0.12) *n* = 15	6.12 (0.13) *n* = 12

Table 24 2-way repeated measures analysis of the larval weights in table 23. **3rd** instars fed 4 pine extracts (0.1%) and dosed with 60,000 PIBs NPV.

Source	df	Type III SS	Mean Square	*F*	*P*
Extract	4	13.78	3.45	10.27	< 0.001
Virus	1	0.024	0.024	0.07	0.789
Extract*Virus	4	5.44	1.36	4.06	0.0034
Error	216	72.47	0.336		
Time	4	344.63	86.16	1806.10	< 0.001
Time*Extract	16	7.27	0.454	9.52	< 0.001
Time*Virus	4	0.344	0.086	1.80	0.126
Time*Extract* Virus	16	1.88	0.117	2.46	0.0012
Error	864	41.22	0.048		

Long term growth and mortality were also recorded for 4th instar larvae fed pine extracts and dosed with virus (Table 25). There were significant differences between the mean weights on days 15, 18 and 21. On day 15, weights for larvae fed white pine extract and control larvae dosed with virus were significantly reduced relative to the control which had not been dosed with virus. However, by day 21 these larval weights did not differ significantly from the control with no virus. This may indicate that as the larvae mature they become resistant to the growth reduction effects of both extract and virus. By day 21, jack, red and white pine extract in combination with virus all reduced growth of the larvae relative to control with no dose of virus. Weights of larvae fed scotch pine whether dosed with virus or not did not differ significantly from the control with no virus.

Mortality at day 21 was expressed as a percentage and arcsin transformed. Mortality was then summed for all larvae which had not been dosed with virus (n = 5), and for all larvae which had been dosed with virus (n = 5) to determine whether the virus had caused significant mortality by day 21. There was no significant mortality (Kruskal-Wallis test, χ^2 = 2.21, P = 0.138) by day 21 for fourth instars which had been dosed with virus (extracts and control) compared to larvae which had not been dosed with virus (extracts and control). This is in contrast to larvae dosed with virus in the 3rd instar which did have a significant increase in mortality relative to those larvae which had not been dosed with virus.

To determine if there were significant differences in survivorship of larvae between the different treatments (extracts ± virus and controls ± virus) a 2 x 5 contingency table analysis on survivorship to day 21 was performed. There was no significant difference on day 21 in survivorship between larvae fed extracts and dosed with virus and control larvae dosed with virus (Contingency Table Analysis, $\chi^2 = 0.28$, $P = 0.991$). Multiple pairwise comparisons for survivorship between the controls ± virus, and extracts ± virus failed to show any significant differences in survivorship for 4th instars.

A 2-way repeated measures ANOVA was then performed on the weights from table 25 (Table 26). Extracts alone had a significant negative effect on weight. Virus alone also had a significant negative effect on weight, greater than that of extract alone. The effect of the extract-virus interaction was also significant, although not as significant as extract and virus alone. Finally there was a significant time-extract and time-extract-virus interaction for this instar. Virus alone did not increase or decrease larval weight over time, but both extract as well as extract and virus together reduced larval weight over time.

Survivorship to Adulthood for 3rd and 4th Instars Dosed with NPV and Fed Extract

3rd instars dosed with concentrations of NPV from a low of 10,000 PIBs/larva to a high of 60,000 PIBs and fed pine extracts had very low survivorships to adulthood (Table 27). The calculated LD_{50} for 3rd instars 21 days after dosing was approximately 10,000 PIBs/larva, however survivorship to adulthood was only 2-7%. Previous standardized bioassays that utilize surface treatment of the diet (Lewis et al. 1981) recommend recording mortality for 14 days after dosing since highest mortality occurs between 10-15 days. Recording mortality at 21 days is perhaps a more conservative way of estimating the LD_{50}. However, it is apparent that late instar and pupal mortality contribute significantly to a reduction in survivorship to adulthood.

Table 25. The effect of pine extracts (0.1%) and virus on weight of GM **4th** instars dosed with 60,000 PIBs (+) or undosed (-). Log-transformed means with standard error in brackets)

	Age of Larvae (Days)		
	15	18	21
Control-	5.15 (0.02) *n* = 40	5.97 (0.04) *n* = 37	6.68 (0.06) *n* = 37
Control+	5.00 (0.04) *n* = 40	5.77 (0.06) *n* = 38	6.53 (0.06) *n* = 36
Jack-	5.16 (0.03) *n* = 40	5.98 (0.05) *n* = 39	6.65 (0.04) *n* = 34
Jack +	5.05 (0.02) *n* = 40	5.73 (0.05) *n* = 37	6.40 (0.05) *n* = 35
Red-	5.20 (0.02) *n* = 40	5.94 (0.03) *n* = 35	6.58 (0.04) *n* = 32
Red+	5.19 (0.03) *n* = 30	5.77 (0.04) *n* = 28	6.38 (0.05) *n* = 28
Scotch-	5.15 (0.02) *n* = 40	5.84 (0.03) *n* = 36	6.54 (0.04) *n* = 35
Scotch+	5.13 (0.03) *n* = 40	5.84 (0.04) *n* = 38	6.61 (0.04) *n* = 36
White-	5.04 (0.02) *n* = 40	5.92 (0.03) *n* = 38	6.48 (0.04) *n* = 38
White+	5.12 (0.02) *n* = 40	5.69 (0.05) *n* = 40	6.22 (0.07) *n* =38

Table 26. 2-way repeated measures analysis of the larval weights in table 25. **4th** instars fed 4 pine extracts (0.1%) and dosed with 60,000 PIBs NPV.

Source	df	Type III SS	Mean Square	*F*	*P*
Extract	4	2.43	0.61	5.18	< 0.005
Virus	1	3.78	3.78	32.23	0.0001
Extract*Virus	4	1.57	0.39	3.35	0.0104
Error	339	39.72	0.117		
Time	2	331.17	165.58	6085.60	< 0.001
Time*Extract	8	1.59	0.20	7.30	< 0.001
Time*Virus	2	0.84	0.42	15.48	< 0.001
Time*Extract* Virus	8	1.08	0.13	4.95	< 0.001
Error	678	18.45	0.03		

At the NPV concentrations used there was no significant increase or decrease in survivorship to adulthood for larvae dosed with 10,000 and 60,000 PIBs/larva between the treatments. However, at 30,000 PIBs/larva mortality was significantly reduced for those larvae which fed on diet containing jack pine extract. All surviving individuals were males.

To evaluate whether concentration of virus and extract fed on interacted to affect larval survivorship, a 2-way ANOVA was performed on NPV concentration and extract (Table 28). Extract alone did not affect survivorship, and there was no extract-NPV interaction that affected survivorship. However, virus alone did significantly decrease survivorship to adulthood for larvae dosed in the 3rd instar.

One final experiment using extracts from four pine species and viral dosing was conducted using 4th instars. These were dosed with a single concentration of virus (60,000 PIBs/larva) and fed pine extracts at 0.1% in diet and mortality was monitored until adulthood (Table 29). This dose was close to the calculated LD_{50} and the control larvae which had been dosed with virus did have approximately 50% mortality. Larvae which had been fed jack, red, scotch and white pine extract all had significantly higher mortality than control larvae at adult eclosion.

Table 27. 1-way ANOVA on mortality to adult eclosion (data arcsin transformed) for gypsy moth **3rd** instars dosed with different virus concentrations. Means (SE in brackets are based on 6 repetitions with 20 larvae per repetition. Means followed by the same letter within a column are not significantly different.

Extract (0.1%)	Virus Concentration (PIBs/larva)		
	10,000	30,000	60,000
Control	1.244^{a} (0.08)	1.571^{a} (-)	1.571^{a} (-)
Jack	1.410^{a} (-)	1.359^{b} (0.07)	1.486^{a} (0.07)
Red	1.368^{a} (0.09)	1.571^{a} (-)	1.571^{a} (-)
Scotch	1.385^{a} (0.13)	1.518^{a} (0.05)	1.518^{a} (0.05)
White	----	1.465^{a} (0.07)	1.465^{a} (0.07)
ANOVA Statistics	$F = 0.59$ $P = 0.63$ df = 4	$F = 3.33$ $P = 0.026$ df = 4	$F = 1.19$ $P = 0.34$ df = 4

Table 28. 2-way ANOVA of larval mortality to adulthood in table 27. **3rd** instars fed control, jack red, and scotch pine extracts (0.1%) and dosed with 10-, 30-, and 60,000 PIBs NPV.

Source	df	Type III SS	Mean Square	*F*	*P*
Extract	3	0.078	0.026	1.00	0.3980
Virus	2	0.447	0.223	8.65	0.0005
Extract*Virus	6	0.248	0.041	1.60	0.1620

Table 29. 1-way ANOVA on mortality to adult eclosion (data arcsin transformed) for gypsy moth **4th** instars dosed with different virus concentrations. Means are based on 6 repetitions with 20 larvae per repetition. Means followed by the same letter within a **row** are not significantly different.

Virus Conc'n	Extract				
	Control	Jack	Red	Scotch	White
60,000	0.637^{b} (0.09)	1.199^{a} (010)	1.297^{a} (0.09)	1.337^{a} (0.08)	1.297^{a} (0.09)
ANOVA Statistics			$F = 10.48$ $P < 0.001$ df = 4		

Long Term Effects of Pure Phenolics From Pines on Growth and Mortality of 3rd Instars Dosed with Virus

The effect on long term growth and mortality for GM larvae dosed with virus in the 3rd instar and then fed three concentrations of quercetin is presented in table 30. For days 9, 12, and 15 larvae fed all concentrations of quercetin that were inoculated with virus had reduced growth relative to the undosed control larvae. Larvae fed quercetin at 0.05% with and without virus had the most reduced growth on days 9 and 12 but by day 15 larvae fed quercetin at 0.01% and 0.05% dosed with virus had the greatest reduction in growth relative to the undosed and dosed control larvae. By days 18 and 19 only those larvae fed quercetin at 0.05% and dosed with virus had growth reduced to any extent from the other treatments and controls.

Differences in larval survivorship between the different treatments (quercetin ± virus and controls ± virus) were determined with a 2 x 4 contingency table analysis. There was no significant difference on day 21 in survivorship between larvae fed extracts and dosed with virus and control larvae dosed with virus ($\chi^2 = 0.48$, $P = 0.941$). Multiple pairwise comparisons for survivorship between the controls ± virus and quercetin ± virus also failed to show any significant differences in survivorship.

A 2-way repeated measures ANOVA was conducted on the weights below (Table 31) and from this it is clear that there was a very significant reduction in larval weights over time for those larvae fed quercetin (all concentrations). There was also a significant reduction in growth due to the virus alone, but there was no significant interaction between quercetin and the virus. However, there was a slightly significant time-virus interaction.

Rutin at two concentrations (0.01%, 0.02%) was also tested against third instars with and without a dose of 60,000 PIBs of virus, and growth was monitored regularly over a period of days (Table 32). Initially all treatments (control with virus, rutin with and without virus) significantly reduced growth (days 9, 12) but by day 12 and through to days 18 and 21 the control treatment with virus was not significantly different from the control without virus. Larvae given rutin (0.05%) in diet and virus had reduced growth relative to the controls throughout the experiment whereas larvae fed rutin (0.01%) with or without virus did not differ significantly in growth from the controls by day 21. Mortality due to virus for control and rutin-fed larvae was not as pronounced by days 18 and 21 as it was for those fed quercetin.

To determine the effects of rutin and virus as well as whether there were any effects on weight due to rutin-virus interactions, a 2-way ANOVA was conducted on the weights from above (Table 33). Rutin alone had a significant negative effect on growth of larvae. Virus alone also had a significant negative effect, and the interaction between rutin and virus was also very significant in reducing growth of the larvae. There were also significant time-rutin and time-virus interactions over the experimental period.

Table 30. The effect of quercetin and virus on weight of GM **3rd** instars dosed with 60,000 PIBs (+) or udosed (-). Log-transformed means with standard errors in brackets.

	Age of Larvae (Days)				
	9	12	15	18	21
Control-	4.31 (0.01) *n* = 40	5.35 (0.03) *n* = 40	6.12 (0.05) *n* = 36	6.31 (0.06) *n* = 36	6.65 (0.07) *n* = 33
Control+	4.10 (0.06) *n* = 40	5.30 (0.04) *n* = 38	5.99 (0.06) *n* = 35	6.31 (0.06) *n* = 22	6.58 (0.06) *n* = 14
Querc- 0.005%	4.18 (0.03) *n* = 40	5.30 (0.04) *n* = 38	6.04 (0.04) *n* = 38	6.29 (0.05) *n* =37	6.52 (0.05) *n* = 33
Querc + 0.005%	4.20 (0.02) *n* = 40	5.21 (0.03) *n* = 38	5.92 (0.06) *n* = 33	6.19 (0.10) *n* = 21	6.60 (0.11) *n* = 11
Querc- 0.01%	4.20 (0.03) *n* = 39	5.21 (0.03) *n* = 38	5.88 (0.06) *n* = 32	6.17 (0.05) *n* = 31	6.25 (0.07) *n* = 30
Querc+ 0.01%	4.15 (0.02) *n* = 40	5.10c (0.05) *n* = 36	5.74 (0.05) *n* = 33	6.22 (0.06) *n* = 19	6.36 (0.08) *n* = 13
Querc- 0.05%	4.06 (0.03) *n* = 40	5.07 (0.03) *n* = 39	5.87 (0.04) *n* = 37	6.15 (0.04) *n* = 37	6.37 (0.06) *n* = 35
Querc+ 0.05%	4.07 (0.03) *n* = 40	5.07 (0.03) *n* = 39	5.64 (0.05) *n* = 36	5.94 (0.06) *n* = 22	6.26 (0.06) *n* = 13

Table 31. 2-way repeated measures analysis of the weights in table 30. **3rd** instars fed pure quercetin (0.005, 0.05, 0.05%) and dosed with 60,000 PIBs NPV.

Source	df	Type III SS	Mean Square	*F*	*P*
Quercetin	3	7.84	2.61	35.36	< 0.001
Virus	1	0.72	0.72	9.81	0.002
Querc*Virus	3	0.10	0.03	0.45	0.720
Error	174	12.85	0.117		
Time	4	489.32	122.33	1646.40	< 0.001
Time*Querc	12	1.06	0.09	1.19	0.286
Time*Virus	4	0.72	0.18	2.41	0.048
Time*Querc *Virus	12	1.08	0.09	1.21	0.272
Error	696	51.71	0.074		

Table 32. The effect of rutin and virus on weight of GM 3rd instars dosed with 60,000 PIBs (+) or undosed (-). Log-transformed means with standard errors in brackets.

	Age of Larvae (Days)				
	9	12	15	18	21
Control-	4.47 (0.04) *n* = 40	5.29 (0.04) *n* = 38	5.90 (0.05) *n* = 38	6.38 (0.05) *n* = 35	6.55 (0.04) *n* = 34
Control+	4.09 (0.02) *n* = 40	4.85 (0.03) *n* = 37	5.78 (0.04) *n* = 37	6.05 (0.05) *n* = 32	6.42 (0.10) *n* = 14
Rutin- 0.01%	4.23 (0.04) *n* = 40	5.06 (0.03) *n* = 39	5.65 (0.04) *n* = 38	6.21 (0.04) *n* = 38	6.49 (0.03) *n* = 37
Rutin+ 0.01%	4.16 (0.04) *n* = 40	4.94 (0.05) *n* = 37	5.67 (0.07) *n* = 36	5.88 (0.09) *n* = 31	6.48 (0.03) *n* = 16
Rutin- 0.05%	4.31 (0.03) *n* = 40	5.05 (0.03) *n* = 40	5.65 (0.04) *n* = 40	5.98 (0.05) *n* = 38	6.21 (0.05) *n* = 37
Rutin+ 0.05%	4.24 (0.02) *n* = 40	4.87 (0.04) *n* = 40	5.57 (0.05) *n* = 39	5.78 (0.08) *n* = 30	6.11 (0.08) *n* = 30

Table 33. 2-way repeated measures analysis of the weights from table 32. **3rd** instars fed pure rutin (0.01, 0.05%) and dosed with 60,000 PIBs NPV.

Source	df	Type III SS	Mean Square	*F*	*P*
Rutin	2	4.16	2.08	31.73	< 0.001
Virus	1	3.23	3.23	49.15	< 0.001
Rutin*Virus	2	1.54	0.77	11.73	< 0.001
Error	150	9.85	0.066		
Time	4	382.87	95.72	1485.85	< 0.001
Time*Rutin	8	3.04	0.38	5.90	< 0.001
Time*Virus	4	0.81	0.20	3.16	0.014
Time*Rutin* Virus	8	0.42	0.05	0.81	0.593
Error	600	38.65	0.064		

A 2-way contingency table analysis (2 x 3) on survivorship (day 21) for all treatments in table 32 was performed. There was no significant difference in survivorship overall between any of the treatments ($\chi^2 = 4.0$, $P = 0.137$). In pairwise comparisons, survivorship for larvae dosed with virus and fed rutin at 0.01% ($\chi^2 = 0.01$, $P = 0.911$) and 0.05% ($\chi^2 = 2.88$, $P = 0.090$) did not differ significantly from the control dosed with virus. Similarly, larvae dosed with virus and fed rutin at 0.01% and 0.05% did not differ significantly from each other ($\chi^2 = 2.66$, $P = 0.103$).

The final pure compound to be tested against 3rd instars in combination with virus was quercetin-3-O-glucoside (Q3O) (Table 34). This compound was incorporated into diet at only one concentration (0.1%), because available quantities were limited. Initially (day 9) Q3O alone appeared to reduce growth of larvae more relative to the undosed control than Q3O and control dosed with virus. However, there was not much growth reduction from day 12 to day 21 for any of the treatments. Although mortality for virus infected larvae appeared to increase there was no significant difference between the controls or Q3O treated larvae at day 21 (2 x 2 contingency table analysis, $\chi^2 = 0.47$, $P = 0.624$).

A 2-way repeated measures ANOVA performed on the weights from table 34 (Table 35) showed that Q3O alone significantly reduced weight of larvae (at least initially) but virus alone did not appear to have any effect on weight, in contrast to rutin and quercetin. There was no significant Q3O-virus interaction effect on weight of the larvae.

Table 34. The effect of Q3O and virus on weight of GM **3rd** instars dosed with 60,000 PIBs (+) or undosed (-). Log-transformed means with standard errors in brackets.

	9	12	15	18	21
Control-	4.18 (0.03) *n* = 40	4.85 (0.05) *n* = 35	5.67 (0.07) *n* = 33	5.67 (0.07) *n* = 33	6.16 (0.07) *n* = 32
Control+	4.10 (0.02) *n* = 40	4.90 (0.03) *n* = 39	5.71 (0.04) *n* = 39	6.03 (0.05) *n* = 39	6.49 (0.06) *n* = 20
Q3O- 0.1%	4.02 (0.02) *n* = 40	4.91 (0.04) *n* = 40	5.56 (0.04) *n* = 40	6.09 (0.04) *n* = 40	6.35 (0.04) *n* = 40
Q3O + 0.1%	4.10 (0.03) *n* = 40	4.89 (0.04) *n* = 36	5.65 (0.04) *n* = 35	5.92 (0.08) *n* = 33	6.34 (0.09) *n* = 19

Table 35. 2-way repeated measures analysis of the data weights in table 34. **3rd** instars fed pure quercetin-3-O-glucoside (0.1%) and dosed with 60,000 PIBs NPV.

Source	df	Type III SS	Mean Square	*F*	*P*
Q3O	1	0.83	0.83	10.21	0.002
Virus	1	0.004	0.004	0.04	0.833
Q3O*Virus	1	0.07	0.07	0.83	0.365
Error	106	8.63	0.08		
Time	4	346.64	86.66	1068.42	< 0.001
Time*Q3O	4	0.39	0.10	1.19	0.316
Time*Virus	4	0.97	0.24	3.00	0.019
Time*Virus* Q3O	4	0.04	0.01	0.12	0.975
Error	424	34.39	0.08		

Discussion

The hypothesis that gypsy moth larvae may acquire enhanced resistance to disease through the consumption of plant secondary chemicals was first proposed by Rossiter (1987). However, enhanced resistance, or a reduction in viral infectivity, may be measured by a number of different criteria. For example, Keating et al. (1990) found that tannins inhibited infectivity of NPV when mortalities were assessed and compared 17 days after the 3rd instar larvae were dosed, whereas the length of time larvae survived was used as a measure of how plant secondary chemicals (Felton et al. 1987) or plant species (Rossiter 1987) protected GM larvae from NPV infection.

While mortality after a fixed number of days, and longevity of the larvae provide useful information as to the potential for plant secondary compounds to provide disease resistance, they do not indicate short-term physiological responses (within a single instar) of larvae to host plant chemicals and NPV. Physiological responses of larvae switching from one host to another may be an indication of the short-term cost or benefit to larvae which are infected with virus and switch to another host such as pine. To answer the question of whether pine compounds provided GM with protection from infection in my study, I looked at both short and long-term effects of pine extracts and pure compounds in artificial diet on GM larvae and NPV infection. It should be noted however, that lab studies such as this one are lacking to some extent from the actual situation in nature, in that infected larvae are moved from control diet to diet containing extracts or pure compounds from pine, rather than switching between actual hosts. However, lab studies do allow the concentration of extract or compound, and viral dose to be quantified.

Short Term Effects of Extract on Third Instars- Nutritional Indices

The fact that phenolic extract from pines and virus both affect short term physiological functions of GM is clear from Tables 21 and 22. Extract generally tends to reduce all indices except the RCR, which means that 3rd instars when switched to diet containing pine extract do not process food, convert it to biomass, or grow as efficiently as they do when fed control diet. NPV infection also tends to reduce the AD and RGR but increases the RCR, which means that food processing and growth are reduced by the virus but consumption of food is increased. The reduction in the AD and RGR for larvae fed virus was not unexpected since it is known that relatively high doses of NPV can reduce the growth rate of infected GM larvae as well as inhibit molting and pupation due to inactivation of ecdysone (molting hormone) (Burand and Park 1992). The growth reduction in infected larvae may result from viral replication which competes intracellularly with the larval host for energy and nutrients (Burand and Park 1992).

Overall the lack of virus-extract interaction on all nutritional indices except RCR means that extract and virus both reduce the growth of larvae but there is no interactive effect. However, the loss of efficiency in processing and converting food to biomass that occurs from NPV infection and feeding on pine extracts, results in a significant virus-extract interaction for consumption rate (Table 22). This shows that there are clear physiological costs to 3rd instars when they are switched to diet containing pine extract, but that in the short term, the three pine extracts tested do not interact with the virus in any way to increase or decrease growth, or the efficiency of conversion of food to larval biomass.

Effect of Extract and Virus on Growth and Mortality of Gypsy Moth Third and Fourth Instar Larvae

Virus and pine extracts together generally reduced the growth of third instars over the experimental period (Table 23). The significant virus-extract interaction also shows that extract and virus together affected weight of the larvae (Table 24). There was also a significant increase in survivorship by day 21 for those infected 3rd instars which were fed red, scotch and white pine extracts in artificial diet compared to control larvae. However, these results must be viewed with caution because the length of time a larvae lives is not necessarily an indication that the larva will survive to adulthood. Larval survival measured at a certain time post-inoculation may not be an accurate indication of the protective effect of different host foliage, or host foliage constituents, because these same larvae, reared for a longer period of time, may suffer high rates of final instar and pupal mortality.

For example, from Table 23 the higher rates of survival on day 21 may be interpreted to mean that larvae infected with 60,000 PIBs/larva which fed on red, scotch and white pine extracts were protected from viral infection by compounds in the extracts. However, Table 27 shows the opposite is true when infected larvae are reared to adulthood on diet containing the pine extracts. At both 10, and 60,000 PIBs there was no significant difference in mortality between the extracts and control for infected larvae. Therefore, assessing larval survival at a certain time post-inoculation may not truly indicate that the extracts are inhibiting infectivity of NPV. These larvae simply live longer, and do not reach adulthood to breed. Infected larvae that feed on extracts and survive a much longer time (in some cases over eight weeks, or twice the normal time to pupation) may actually be detrimental to gypsy moth populations since these larvae may be larger in the final instar and produce much more virus particles when they die than smaller larvae do. This in turn would result in more individuals within the population being exposed to NPV infection, and speed the spread of the virus.

The virus itself may have evolved a tactic to prolong GM larval life, and therefore maximize the production of viral progeny. O'Reilly (1995) reviewed the literature and found that all the baculoviruses studied to date (including GM NPV) have a gene, *egt*$^+$, that produces an enzyme which glucosilates ecdysone (moulting hormone), thereby inactivating it. This causes the insect to grow more slowly, have fewer molts and produce more PIBs in the final instar larva, than larvae infected with a mutant *egt-* strain of NPV (O'Reilly 1995). Therefore, it seems that if pine extracts act with *egt*$^+$ and prolong larval life, but in general (jack pine extract excluded) do not lower mortality at adult eclosion, then perhaps more viral progeny would be produced than if *egt*$^+$ were acting alone. We must therefore be cautious in interpreting previous studies which have assessed larval mortality at a fixed time post-inoculation. We must also not characterize a longer larval survival time when infected with NPV and fed plant extracts as an indication of reduced viral infectivity, since an increase in survival time may result in more PIBs released into the environment.

A significantly lower mortality at adult eclosion for third instar larvae dosed with

30,000 PIBs and fed jack pine is interesting. However, when the effect the three virus concentrations and extract have on mortality are analyzed together in a 2-way analysis, the virus-extract interaction is not significant. Keating and Yendol (1987) found that gypsy moth 3rd instars infected with three concentrations of virus (4, 10, and 25,000 PIBs/larva) when reared on a diet of pitch pine had significantly higher mortality at 10, and 25,000 PIBS/larva than larvae which had been fed a diet of red maple and red oak. However, these larvae were not reared to adulthood, and late instar and pupal mortality could have resulted in a different interpretation of the data.

To summarize, there seems little benefit for infected 3rd instars that feed on pine extracts in artificial diet. There are short-term physiological costs in switching from control diet to diet containing extracts as well as long term reductions in growth rate due to extract alone and the interaction of virus and extract. Survivorship 15 days post inoculation may have been higher for larvae fed diet containing some pine extracts which were inoculated with 60,000 PIBs, but survivorship to adulthood was not significantly greater for larvae fed pine extracts and dosed with 10,000, 30,000 and 60,000 PIBs.

Fourth instars infected with virus and fed extract also had significant weight reductions over the experimental period (Table 25). The extract-virus interaction was also significant (Table 26). Larvae fed jack, red and white pine had the most significant reduction in growth, but 7 days post-inoculation (age 21 days) there was no significant differences in mortality between the groups. Previous studies examining effect of host foliage (Keating and Yendol 1987) as well as nutrients and plant allelochemicals (Keating et al. 1988) on mortality dosed larvae which were in the early third instar. No work has been done to date on the effects of plant allelochemicals on larvae dosed in the 4rd instar. Gypsy moth males and females generally only have one more instar after the 4th, although females may have a sixth instar (Leonard 1981) The lack of significant mortality by day 21 for 4th instars which were dosed with virus whether fed extract or not, may be because the viral incubation time was insufficient to cause high mortality by the end of the experiment.

Final instar and pupal mortality are high when 4th instars are dosed with 60,000 PIBS and fed extract and reared to adulthood (Table 29). Control larvae had significantly lower mortality than infected larvae fed pine extracts, but the mortality for larvae fed different extracts did not differ significantly. Only a single 60,000 PIBs dose was given to these larvae, so it is not possible to say whether the effect holds over a range of viral concentrations.

It therefore does not seem likely that 4th instars, like 3rd instars benefit to any extent from feeding on the phenolic extract from pines in artificial diet. There were significant negative long term effects on weight due to the virus and extracts, and there was no significant difference in mortality between larvae fed extracts and virus, and the larvae just fed extracts. Survivorship to adulthood was higher for inoculated 4th instars when fed jack pine extract, but it is difficult to determine how significant this is since a range of virus concentrations was not used.

Effect of Pure Phenolics and Virus on Growth and Mortality of Gypsy Moth Third Instar Larvae

Tannins (Young et al. 1995), and in particular hydrolyzable tannins (Keating et al.

1988, 1989), in leaves have been shown to reduce infectivity of nuclear polyhedrosis viruses. However, the effect that simple phenolics and flavonoids may have on viral infectivity has not been examined in any detail. Felton et al. (1987) examined the effect of two phenolic glycosides, chlorogenic acid and rutin, on infecticity of a NPV that infects *Helicoverpa zea*, a noctuid larvae. Both of these compounds reduced the infectivity of NPV and prolonged the survival time of larvae.

In my study, quercetin, the phenolic aglycone of rutin and Q3O, reduced growth of third instars, as the virus itself did, but there was no significant virus-quercetin interaction on weight of third instars over time. Over time, the growth inhibiting effects of the virus were also significant. There also was no difference in mortality 15 days post-inoculation between the control±virus and treated larvae. It therefore does not seem that the aglycone quercetin is responsible for any inhibition in the infectivity of the virus, but does cause weight reduction. A phenolic glycoside of quercetin found in pine, rutin, caused significant weight reductions alone and synergistically with the virus over time. However, as with quercetin, there was no significant reduction in mortality between the control±virus and the rutin±virus treatments 15 days post-inoculation. Rutin, therefore, has negative physiological effects on GM 3rd instars and also does not seem to be responsible for a reduction in infectivity of NPV, unlike Felton et al's (1987) study with *Helicoverpa zea*. In Felton et al.'s (1987) study, two criteria for inhibition of NPV infection were used: differences between LD_{10-90} for control, rutin and chlorogenic acid fed larvae 10 days post-inoculation, and larval survival time to death. In the present study no significant difference was found in mortality between the treatments 15 days post-inoculation, although mortality to adulthood or death for pure compounds was not monitored. A higher LD_{50} value for rutin treated larvae which had been infected with NPV or a mortality count taken at a certain point post-inoculation may not necessarily indicate that a compound, or extract has reduced the ultimate infectivity of NPV. As indicated previously, if these larvae are reared to adults late instar and pupal mortality may be significant. It is also uncertain in Felton et al.'s (1987) study whether a lengthened larval survival time until death is an indication of the protective effects of rutin against NPV since none of these larvae survived to adulthood and thus reproduction. Selection for larval survival characteristics can operate if those selected larval characters allow the larvae to survive until adulthood and reproduction, i.e. increased survivorship on rutin fed diet to adult.

The final phenolic glycoside to be tested, which was found in each of the four pine species, was Q3O. There was a significant effect of Q3O on weight of GM 3rd instars, and no significant virus-Q3O interaction, and there was no significant difference in mortality between treatments 15 days post-inoculation. Unlike the above experiments, virus alone did not affect weight for these larvae, although there was significant time-virus interaction factor. These results also suggest that Q3O had no significant effect on infectivity of NPV and that, if anything Q3O acted to reduce long-term growth of the larvae.

To summarize, there seems little advantage to GM larvae infected with NPV when they consume pure phenolics and phenolic glycosides in extract. However, Keating et al. (1988, 1989) found that low *p*H, and polyphenolics such as tannic acid, and hydrolyzable tannins all reduced mortality for GM 3rd instars 17 days post-inoculation. These authors hypothesized that the phenolics (tannins) in the aqueous extract bind to PIBs which are largely composed of protein, thus preventing them from infecting the larvae. However, when plant extracts were aggregated with NPV PIBs and fed to the insects with diet there was no significant difference between aggregated and unaggregated NPV when fed to GM 3rd instars. Keating et al.(1988, 1989) suggest that one reason for this may be because lab diet is not as well buffered as fresh leaf tissue.

General Discussion

From the preceding chapters it is clear that of the four pine species for which needles were extracted and analyzed, their total phenolic content as well as the type and amount of individual phenolics varies between species. Both the phenolic extract from pine and individual flavonoid compounds found in pine inhibit growth of early instar GM, and to some extent 3rd and 4th instars. The 2nd instars are quite sensitive to the phenolic extract when incorporated into diet in amounts which are generally lower than the level of total phenolics reported for jack pine by Nozzolillo et al (1990). They are also sensitive to relatively low concentrations of pure flavonoids. Joseph et al. (1993) also noted that their phenolic fraction added to diet affected larval survival and development more than diets of Douglas-fir foliage containing comparable concentrations of phenolics. They suggest that monomers of tannin such as catechin and epicatechin, as well as flavonoid monomers, form complexes with protein in diet and therefore reduce its digestibility. No tannin monomers were found in the extract in this study (Table 2) but the flavonoid monomers as well as the simple phenols could be responsible for some protein binding with diet constituents and this may explain why early instars were so sensitive to phenolic extract and pure compounds in diet. However, the fact that 2nd instars are very sensitive, but that 4th instars are relatively unaffected by low concentrations of pine phenolics does lend support to the hypothesis that early instars of gypsy moth do not feed on pines in early instars at least in part because of the phenolics present. In addition, there are significant physiological costs (as judged by nutritional indices) to 3rd instars removed from control diet and then placed on diet containing phenolic extract.

The hypothesis that late instar GM are protected to some extent from NPV infection by phenolics from pines in 3rd and 4th instars is not supported in my study. There are short-term physiological costs to 3rd instars which are dosed with virus and placed on diet containing phenolic extract. There are also long-term, negative effects on growth and survivorship to 3rd and 4th instars when dosed with NPV and fed phenolic extract and pure flavonoids. Survivorship to adulthood is no different from the control for 3rd instars, and for 4th instars significantly more of the control larvae than larvae fed phenolic extracts from pines survive to adulthood. Therefore, given the above noted caution in interpreting results when phenolics are incorporated into artificial diet, this laboratory study does not support one aspect of Rossiter's (1987) theory that GM already infected with NPV benefit from feeding on the pines examined in my study. It may be that feeding on pines may remove larvae from areas of greater larval density, thus lowering the chance of infection, or that healthy individuals which are innately resistant to NPV infection are more mobile, and therefore encounter pines in their diet.

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Appendix A

Abbreviations Used in Text

Chemistry

Ac	Acetone
BAW	*n*- butanol: acetic acid (H_2O; 4: 1:5) upper phase
CC	Column Chromatography
CH_2Cl_2	Methylene Chloride
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
EtOH	Ethanol
FAB-MAS	Fast Atom Bombardment Mass Spectroscopy
HCl	Hydrochloric Acid
HCOOH	Formic Acid
^{1}HNMR	Proton Nuclear Magnetic Resonance
HOAc	Acetic Acid
H_20	Distilled Water
HPLC	High Pressure Liquid Chromatography
MeOH	Methanol
PC	Paper Chromatography

PVPP	Polyvinylpirolydine
TLC	Thin Layer Chromatography
UV	Ultraviolet Light

Statistical

ANOVA (one or two way)	Analysis of variance
EC_{50}	Effective concentration to reduce growth of test insect by 50%
df	Degrees of freedom
F	F- statistic
LD_{50}	Lethal dose required to kill 50% of population of test insect
n	Sample size and/or survivorship
P	Probability
R	R-value, a measure of association between a dependent (Y) and independent (X) variable
r^2	R-squared, the amount of variation explained by regressing Y on X.
SD	Standard Deviation
SE	Standard Error

General

AD	Approximate digestibility
ECD	Efficiency of conversion of digested material (wt. gain)/(wt. food eaten - wt frass) x 100
ECI	Efficiency of Conversion of Ingested Material (wt. gain)/(wt. food eaten) x 100
FPMI	Forest Pest Management Institute
Instar	Stage of larval development between successive molts - occurs in holometabolous insects
NPV	Nuclear Polyhedrosis Virus
RCR	Relative Consumption Rate (amount of food eaten)/(mean larval wt x days)
RGR	Relative Growth Rate (wt. gain)/(mean larval wt. x days)
PIBs	Polyinclusion Bodies
Secondary Chemical	Any chemical which is not involved in primary plant metabolism.

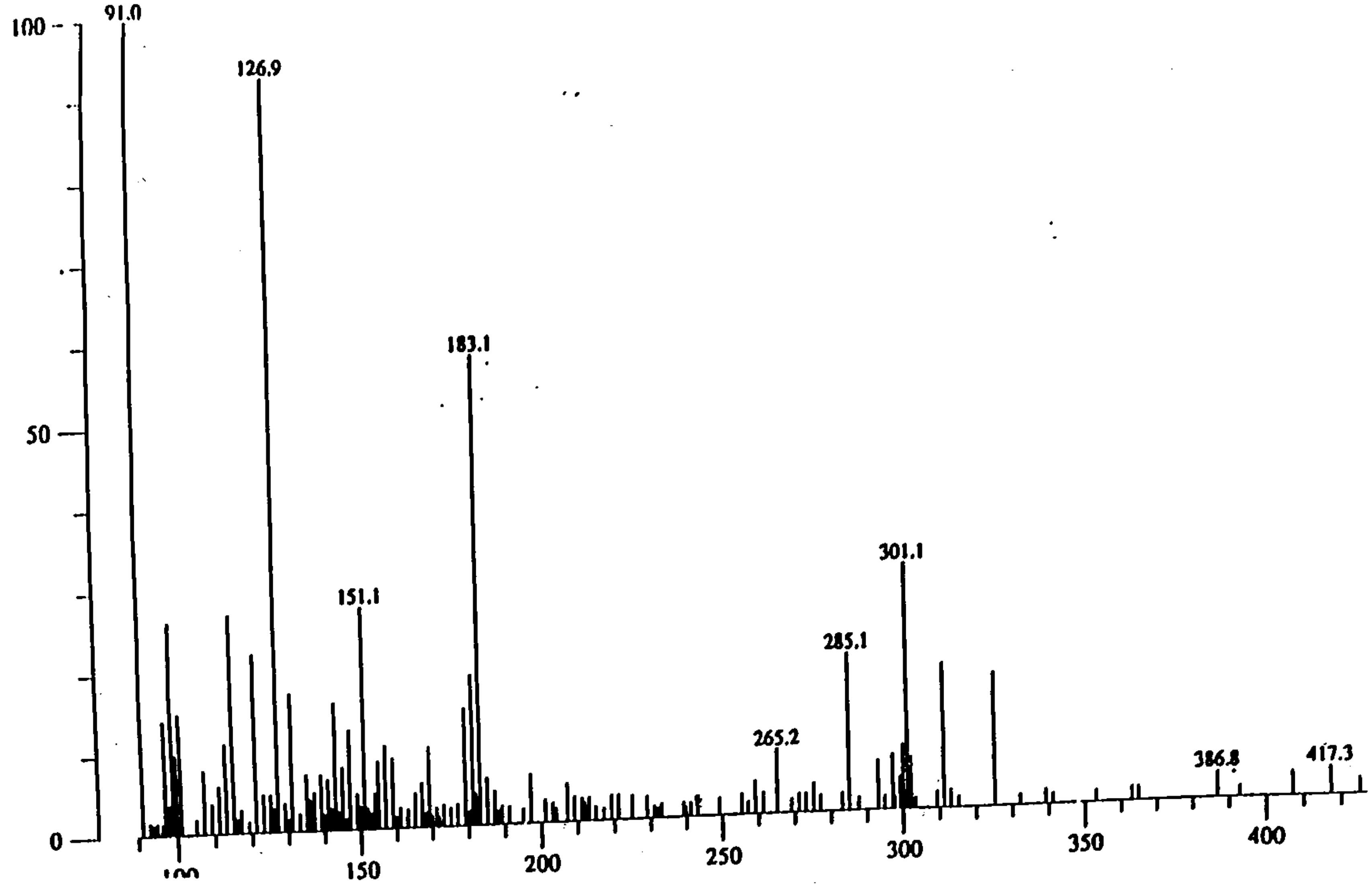
Appendix B

Gypsy moth bell diet

Ingredient	Source	Grams per Litre
Wheat Germ	Local health food store	120
Casein	ICN Biomedicals Canada Ltd.	25
Salt Mix, Wesson	ICN Biomedicals Canada Ltd.	8
Sorbic Acid	Bio-Serv, Frenchtown, NJ	2
Methylparaben	Bio-Serv, Frenchtown, NJ	1
Vitamin Premix	Bio-Serv, Frenchtown, NJ	10
Agar	Bio-Serv, Frenchtown, NJ	15
Water	Distilled at FPMI	800

Appendix C

Figure 9. FAB-MS results for rutin.



100
50
0
91.0
126.9
151.1
183.1
265.2
285.1
301.1
386.8
417.3
100
150
200
250
300
350
400

Figure 10. ^{1}H-NMR results for rutin.

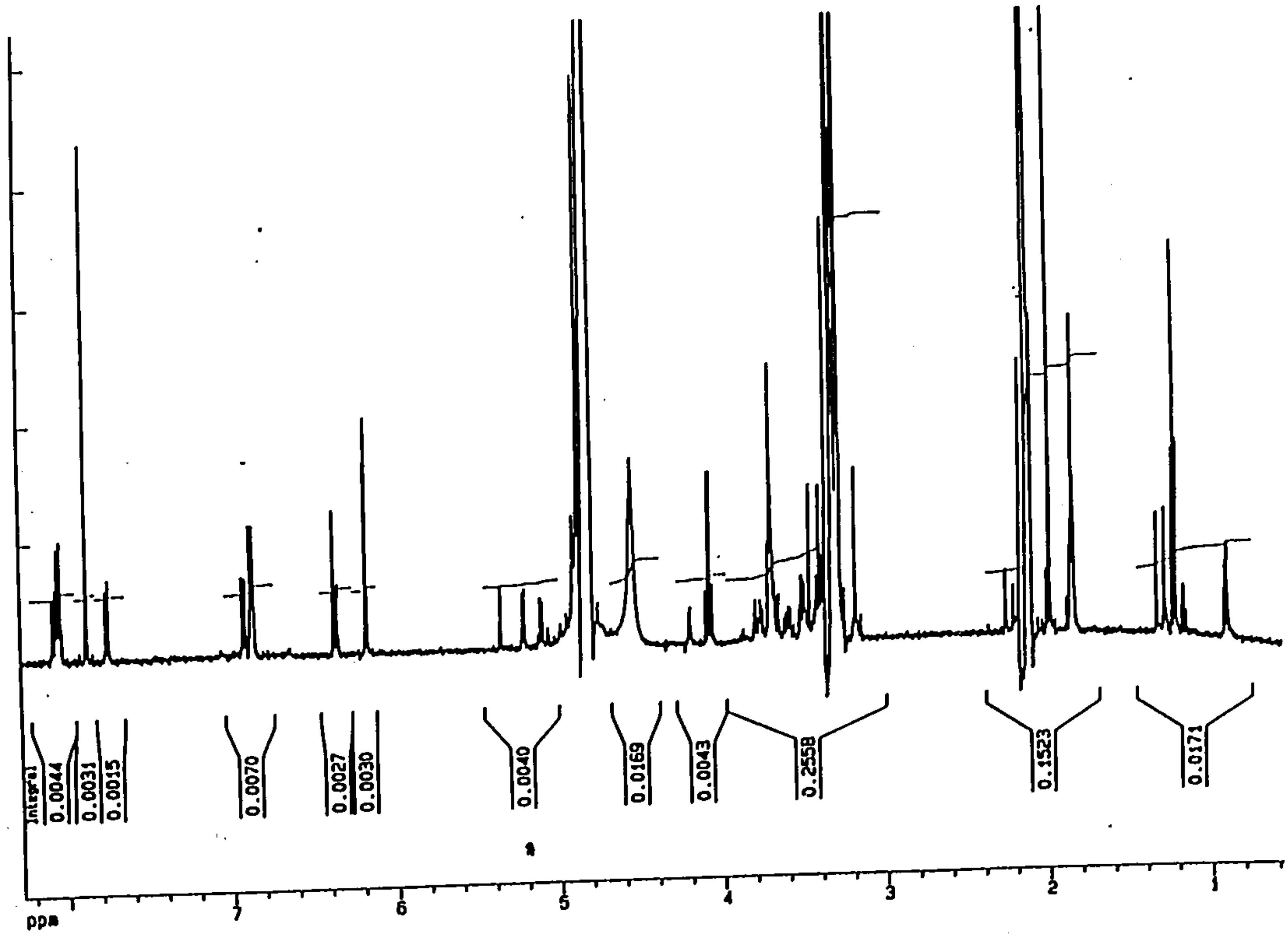

Integral
0.0044
0.0031
0.0015
0.0070
0.0027
0.0030
0.0040
0.0169
0.0043
0.2558
0.1523
0.0171
ppm
7
6
5
4
3
2
1

Figure 11. FAB-MS results for quercetin-3-O-glucoside.

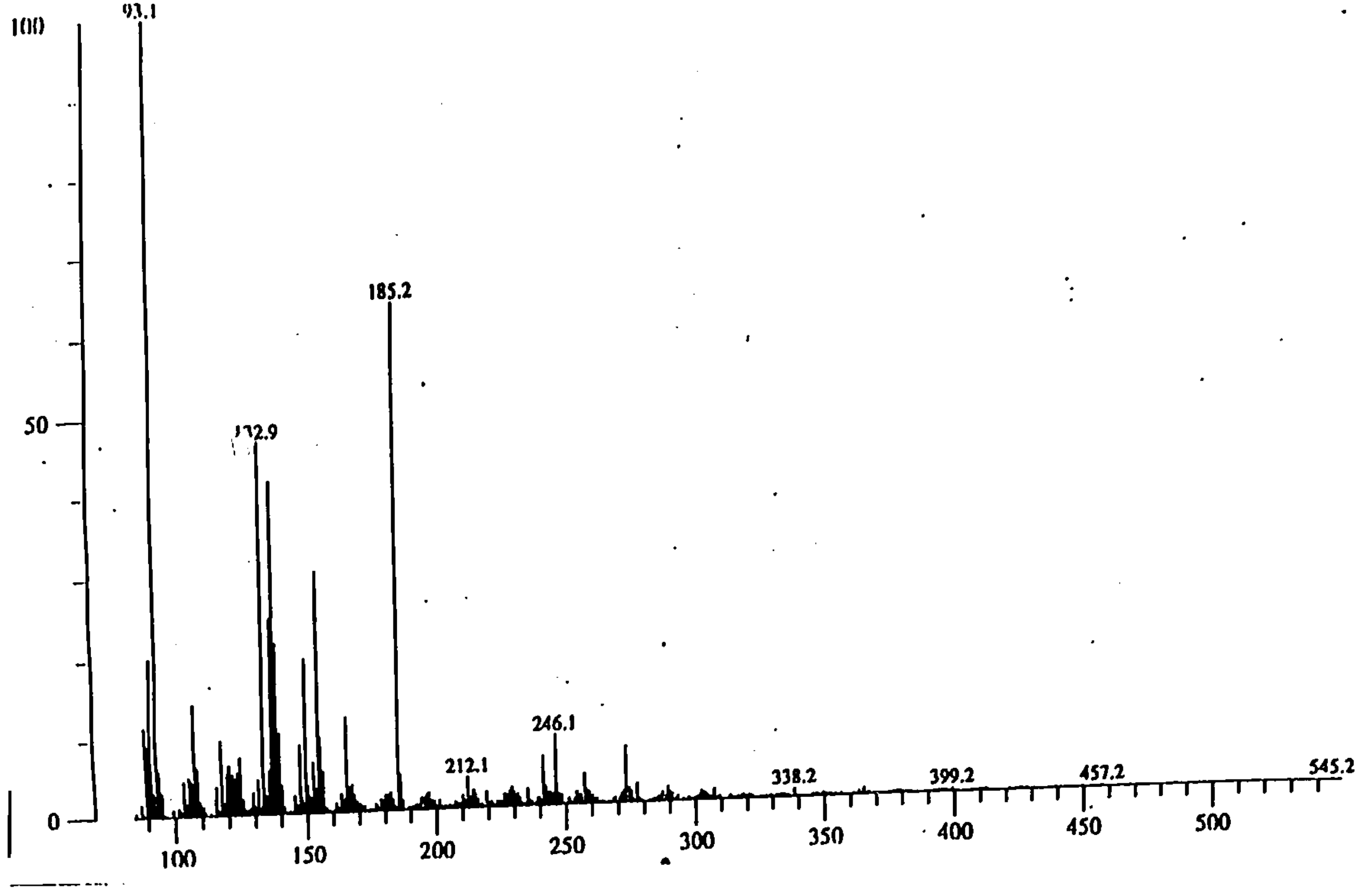
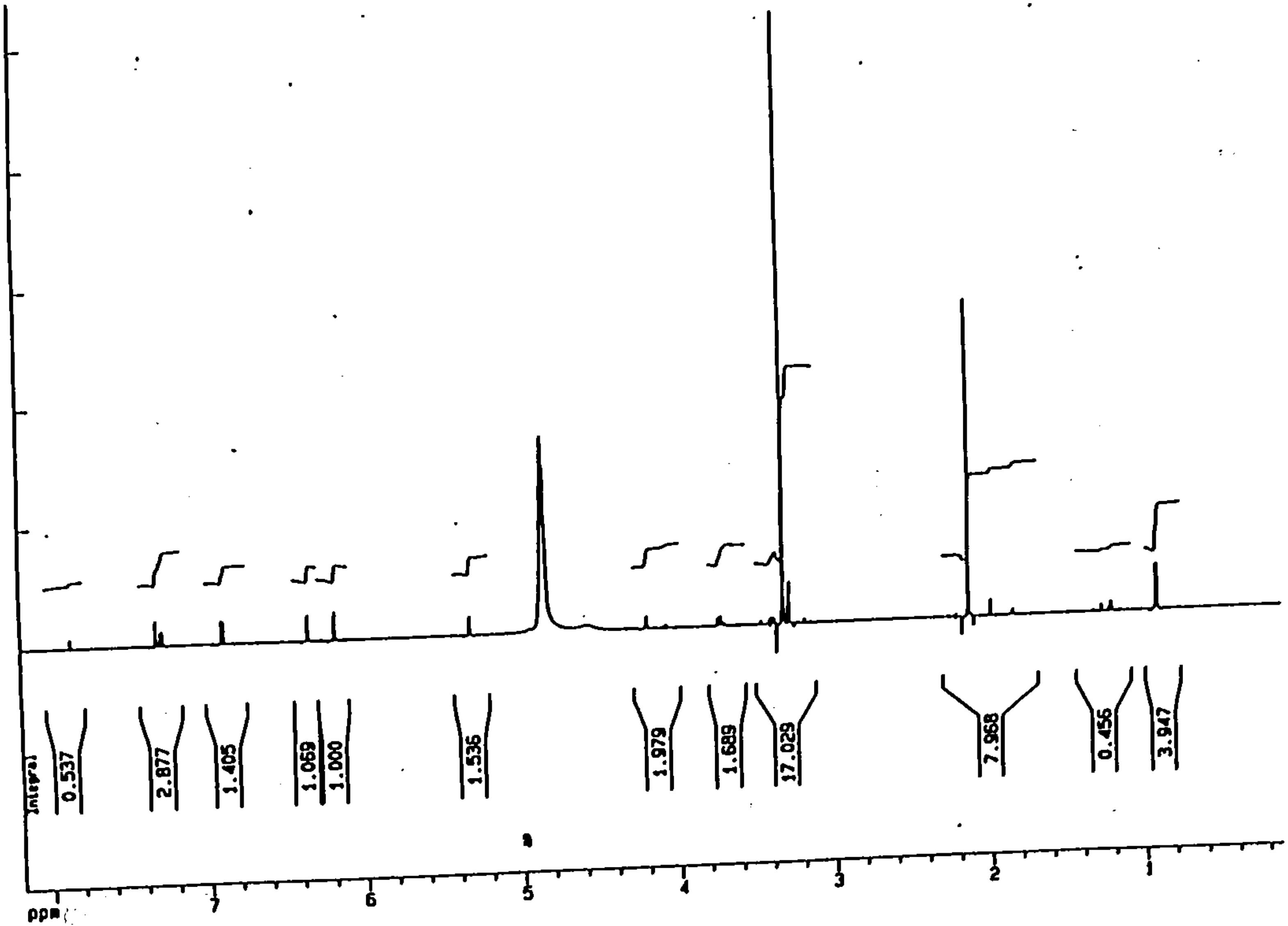

100
50
0
93.1
132.9
185.2
212.1
246.1
338.2
399.2
457.2
545.2
100
150
200
250
300
350
400
450
500

Figure 12. ^{1}H-NMR results for quercetin-3-O-glucoside.


Integral
0.537
2.877
1.405
1.069
1.000
1.536
1.979
1.689
17.029
7.968
0.456
3.947
ppm
7
6
5
4
3
2
1