Running Head: Alkenylphenols and fruit interactions

Title: Secondary metabolites in a neotropical shrub: spatiotemporal allocation and role in fruit defense and dispersal

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ABSTRACT

Deciphering the ecological roles of plant secondary metabolites requires integrative studies that assess both the allocation patterns of compounds and their bioactivity in ecological interactions. Secondary metabolites have been primarily studied in leaves, but many are unique to fruits and can have numerous potential roles in interactions with both mutualists (seed dispersers) and antagonists (pathogens and predators). We described 10 alkenylphenol compounds from the plant species *Piper sancti-felicis* (Piperaceae), quantified their patterns of intraplant allocation across tissues and fruit development, and examined their ecological role in fruit interactions. We found that unripe and ripe fruit pulp had the highest concentrations and diversity of alkenylphenols, followed by flowers; leaves and seeds had only a few compounds at detectable concentrations. We observed a nonlinear pattern of alkenylphenol allocation across fruit development—increasing as flowers developed into unripe pulp then decreasing as pulp ripened. This pattern is consistent with the hypothesis that alkenylphenols function to defend fruits from pre-dispersal antagonists and are allocated based on the contribution of the tissue to the plant's fitness, but could also be explained by non-adaptive constraints. To assess the impacts of alkenylphenols in interactions with antagonists and mutualists, we performed fungal bioassays, field observations, and vertebrate feeding experiments. In fungal bioassays, we found that alkenylphenols had a negative effect on the growth of most fungal taxa. In field observations, nocturnal dispersers (bats) removed the majority of infructescences, and diurnal dispersers (birds) removed a larger proportion of unripe infructescences. In feeding experiments, bats exhibited an aversion to alkenylphenols, but birds did not. This observed behavior in bats, combined with our results showing a decrease in alkenylphenols during ripening, suggests that alkenylphenols in fruits represent a trade-off (defending against pathogens but reducing disperser preference). These results provide insight into the ecological significance of a little studied class of secondary metabolites in seed dispersal and fruit defense. More generally, documenting intraplant spatiotemporal allocation patterns in angiosperms and examining mechanisms behind these patterns with ecological experiments is likely to further our understanding of the evolutionary ecology of plant chemical traits.

Keywords: *Antagonism; Alkenylphenols; Defense trade-off hypothesis; La Selva Biological Station, Costa Rica; Mutualism; Optimal defense theory;* Piper sancti-felicis; *Specialized metabolites*

INTRODUCTION

One of the most extraordinary features of plants is their capacity to synthesize diverse secondary metabolites. Secondary metabolites, also referred to as specialized metabolites, are thought to function primarily in plant interactions with the abiotic and biotic environment. They can have broad consequences for the ecology and evolution of plants, consumers, and entire communities (Kessler and Kalske 2018). However, only a small fraction of secondary metabolites has been structurally elucidated, and an even smaller fraction has any ascribed function. A key step toward understanding the ecological roles of secondary metabolites is to describe the intraplant spatiotemporal patterns of secondary metabolite synthesis and relative concentrations (i.e. where and when are they occurring in the plant). These patterns may have crucial, yet often overlooked, consequences for plant fitness (reviewed in Moore et al. 2014). While most ecological studies of secondary metabolites have focused on leaves, many compounds are produced primarily in other organs, such as fleshy fruits.

Fleshy fruits function primarily to attract animal mutualists who will effectively transport seeds away from the parent plant. However, the same rewards that attract dispersers are a resource for many antagonists, including pathogens and seed predators. The high risk of attack of fruits, combined with the fact that fruits provide a direct link to plant fitness between generations, leads to predictions based on plant defense theory that fruits should be heavily defended (McKey 1974, Rhoades and Cates 1976, Zangerl and Rutledge 1996). Indeed, fruits often have higher diversity and concentrations of secondary metabolites compared to leaves (Herrera 1982, Cipollini and Levey 1997, Çirak and Radušienė 2007, Whitehead et al. 2013, Whitehead and Bowers 2014). Many secondary metabolites in fruits may also serve as frugivore attractants or function to mediate frugivore behavior and physiology (Thies et al. 1998, Cipollini 2000, Rodríguez et al. 2013, Baldwin and Whitehead 2015). Understanding the functional significance of fruit secondary metabolites could provide valuable insight into ecological processes, including seed dispersal—a critical ecological process that determines plant distribution and abundance (Cipollini and Levey 1997, Tewksbury 2002).

Fruits are complex organs, and different tissues and developmental stages likely experience an array of selective pressures and constraints that may shape their chemical traits. Studies that have compared the within-fruit spatial distribution of secondary metabolites (e.g. pulp vs seeds) have shown that the composition of secondary metabolites in these tissues can be highly variable and tissue-specific (Cappelletti et al. 1992, Barnea et al. 1993, Whitehead and Bowers 2013, Whitehead et al. 2013, Beckman 2013, Kolniak-Ostek 2016, D'Abrosca et al. 2017). For example, capsaicin in chilies occurs only in fruits and is highly concentrated in the placental tissue surrounding seeds (Iwai et al. 1979, Fujiwake et al. 1982). Secondary metabolite composition, at least in domesticated fruits, can also change dramatically during development (Hall et al. 1987, Kulkarni 2005, Zhang et al. 2010, Tohge et al. 2014), but these patterns, and the potential to inform our understanding of ecological function, are less explored in wild fruits.

There are different hypothesized adaptive functions of fruit secondary metabolites that generate different predictions for changes in phytochemical investment during fruit development. Here, we offer three hypotheses that explain the allocation patterns for suites of secondary metabolites in fruit development based on function: manipulation of mutualists, defense allocated by risk, and defense allocated by fitness (Fig. 1). First, a number of adaptive hypotheses explaining the patterns of biosynthesis of fruit secondary metabolites (e.g. gut retention time hypothesis, directed toxicity hypothesis, and attraction/repulsion hypothesis; Cipollini and Levey 1997) are united by the idea that certain suites of secondary metabolites may function primarily to mediate interactions with vertebrate consumers of ripe fruits. For example, the gut retention time hypothesis (Cipollini and Levey 1997, Baldwin and Whitehead 2015) posits that certain secondary metabolites in fruits could function to mediate the passage rate of seeds in frugivore guts, thereby impacting dispersal distance and the exposure of seeds to gut conditions. If manipulation of disperser behavior or physiology is the primary adaptive function driving the patterns for particular suites of secondary metabolites, we would predict maximum allocation to those compounds in ripe fruits: the stage of fruit development with the greatest amount of interaction with vertebrate frugivores.

Still, a particular suite of secondary metabolites may function primarily in defense against insect pests and microbial pathogens, as posited by the defense trade-off hypothesis (Cipollini and Levey 1997, Dyer et al. 2001, Cazetta et al. 2008, Whitehead and Bowers 2014, Whitehead et al. 2016). Our second and third hypotheses both explain phytochemical investment in fruits based on this idea. In both cases, the same secondary metabolites that defend fruits may also deter beneficial dispersers, leading to costly trade-offs when they are produced in ripe fruit pulp. Thus, if a certain secondary metabolite or suite of secondary metabolites function primarily in defense, we would expect the allocation to those secondary metabolites to decline with final ripening. However, the overall patterns during development may depend on the costs and benefits of defense.

Optimal defense theory predicts that plants allocate chemical defenses across different tissues based on the cost of defending that tissue, the relative risk of attack, and the fitness consequences of tissue loss (McKey 1974, Rhoades and Cates 1976). Depending on the relative importance of these factors, this could lead to various predictions for defense allocation during fruit development. If risk of attack is the main driver of allocation, we would predict that defenses are highest in immature fruits, which are composed of rapidly expanding and highly nutritious tissues that are not yet protected by physical defenses (e.g. a tough exocarp). The same general pattern is seen in leaves, where young leaves experience much higher rates of damage compared to mature leaves and are often more highly-defended (Kursar and Coley 1991, McCall and Fordyce 2010, Barton et al. 2019). If instead the fitness consequences of tissue loss are the main driver of allocation for particular metabolites, we would predict a non-linear change in the biosynthesis of those secondary metabolites during development. Early in development, the metabolic investment in a fruit is still minimal, but as fruits mature, their fitness value increases. The negative fitness costs of consumption would peak immediately before fruit maturity: the plant has invested heavily in producing fruit, but the seeds are not yet viable. Once seeds mature, the fitness consequences of consumption can shift from a net fitness loss to a net gain (depending on the consumer), and maintaining high levels of defenses could limit dispersal. Thus, in this scenario, we would predict that the phytochemical investment will increase over development, peak before maturation, and then decrease as fruits enter the final ripening stage and are ready for dispersal (Fig. 1).

The three hypotheses we offer are not mutually exclusive alternatives that cover all metabolites—different secondary metabolites or suites of metabolites within a plant may be expected to follow different patterns depending on their specific biological functions. Furthermore, the hypotheses described above rest on the assumption that secondary metabolites in fruits provide adaptive benefits in biotic interactions and are specifically regulated in plants according to their fitness costs and benefits. It is also critical to consider that the occurrence patterns of many secondary metabolites may be the result of neutral or non-adaptive processes. At least two non-adaptive processes may contribute to spatial and developmental patterns. First, certain secondary metabolites in fruits may be present due to strong selection for defense of leaves and other plant parts, combined with physiological constraints on their exclusion from fruit tissues (Swain 1977, Cipollini and Levey 1998, Eriksson and Ehrlén 1998, Cipollini et al. 2002). In this case, we might expect that: 1) secondary metabolites should be more diverse and abundant in leaves than in fruits, and 2) concentrations in fruits and leaves should be correlated. These predictions have not been supported in other systems comparing secondary metabolites between leaves and wild fleshy fruits (e.g. iridoid glycosides in *Lonicera*, Whitehead and Bowers 2013), but could be true for other plant species or classes of compounds. Second, temporal variation in secondary metabolite abundance during fruit development may occur as a passive consequence of other physiological processes, rather than the specific adaptive regulation of particular compounds. For example, a reduction in the concentration of a compound during ripening could be simply due to enzymatic degradation that occurs during fruit softening (Brady 1987). In this case, as with any non-adaptive scenario, there may be limited or neutral consequences of fruit secondary metabolites in fruit defense or seed dispersal. Thus, furthering our understanding of the evolutionary ecology of secondary metabolites in fruits requires a combination of descriptive documentation of spatiotemporal occurrence patterns and ecological experiments to examine the bioactivity of fruit secondary metabolites in interactions with fruit consumers—including both mutualists and antagonists.

In this study, we combine structure elucidation of secondary metabolites, quantitative descriptions of spatiotemporal chemical variation, field observations, fungal bioassays, and behavioral experiments with birds and bats to provide a broad overview of the evolutionary ecology of fruit secondary metabolites in *Piper sancti-felicis* Trel. (Piperaceae). *Piper sancti-felicis* is a widespread and abundant neotropical shrub and was chosen for a case study because it fruits abundantly throughout the year across much of the neotropics and represents a dietary staple for bats and birds (Fleming 2004, Thies and Kalko 2004). Little was known about the secondary chemistry of this species, and our initial analyses suggested infructescences were dominated by compounds structurally related to alkenylphenols described from other species of *Piper* (Orjala et al. 1998, Valdivia et al. 2008, de Oliveira et al. 2012, Yang et al. 2013, Varela et al. 2017, Yoshida et al. 2018). This study had four specific objectives: 1) to elucidate the structures of the major alkenylphenol compounds present in *P. sancti-felicis*; 2) to assess the extent to which spatial patterns of alkenylphenol occurrence across tissues (leaves, flowers, unripe fruit pulp, ripe fruit pulp, and seeds) and temporal patterns during fruit development are consistent with different hypothesized functions of fruit secondary metabolites (Fig. 1); 3) to test the effects of alkenylphenols in interactions with fruit-associated fungi (antagonists); and 4) to test the effects of fruit alkenylphenols in interactions with vertebrate seed dispersers (mutualists). Together, these investigations provide an overview of the ecological significance of a group of secondary metabolites, demonstrate the value of using intraplant spatiotemporal variation to understand ecological roles, and, more broadly, contribute a holistic understanding of the functions of secondary metabolites in biotic interactions, including fruit defense and seed dispersal.

METHODS

Study site and system

All plant collection and experiments were conducted at La Selva Biological Station (hereafter, La Selva), Heredia Province, Costa Rica. The station is managed by the Organization for Tropical Studies (OTS) and comprises approximately 1600 ha of tropical wet forest. The site has high diversity of the genus *Piper*, and hosts over 60 species (OTS 2020). *Piper* is one of the largest genera of flowering plants, containing approximately 1,000 species globally. The greatest diversity of *Piper* is found in the neotropics and lowland tropical forest sites, such as La Selva (Gentry 1990). The genus has distinctive inflorescences (spikes containing hundreds of small, reduced flowers along a rachis), and each flower matures into a single-seeded drupelet, creating an infructescence (Greig 2004; Fig. 2). For most species of *Piper*, all fruits on an infructescence mature simultaneously and are dispersed as a single unit. Previous investigations into the genus have described the presence of a broad range of secondary metabolites in leaves, including amides, alkaloids, lignans, terpenes, and steroids (Dyer et al. 2004, Richards et al. 2015). In our initial chemical investigations of *Piper santi-felicis*, we found that fruits were dominated by alkenylphenols. Structurally-related compounds have been described from several other species of *Piper* and have known antifungal, antimicrobial, and cytotoxic properties *in vitro* (Valdivia et al. 2008, Yang et al. 2013). Yet, the ecological significance of alkenylphenols and their occurrence patterns in *P. sancti-felicis* were, to our knowledge, previously undescribed.

Most neotropical species of *Piper* are largely dispersed by bats (Fleming 2004), and the primary bat dispersers are in the genus *Carollia* (Phyllostomidae), which depend on the infructescences as a predominant, year-round staple in their diet (Fleming 2004, Maynard et al. 2019). Several other species of bat feed on the infructescences, including species in the genera

Artibeus, Dermanura, and *Glossophaga* (Lopez and Vaughan 2007). Furthermore, several species of birds consume the infructescences of *Piper*, including tanagers (Thraupidae), sparrows (Emberizidae), manakins (Pipridae), toucans (Ramphastidae), cuckoos (Cuculidae), pigeons and doves (Columbidae; Palmeirim et al. 1989, Thies and Kalko 2004). Occasionally, other small mammals (Leiser-Miller et al. 2019) or ants (Thies and Kalko 2004, Clemente and Whitehead 2019) consume the infructescences of *Piper*. After consumption, the seeds passed by bats and birds are viable (Palmeirim et al. 1989, Baldwin and Whitehead 2015). However, the two groups of dispersers handle the infructescences differently. Birds typically consume infructescences at the plant, stripping the pulp and seeds and leaving the rachis (Appendix S1: Fig. S1). Bats use a combination of echolocation and olfaction to locate ripe infructescences, which readily abscise from the plant (Thies et al. 1998). They collect the entire infructescence and carry it to a roost for consumption (Fleming 2004; Appendix S1: Fig. S2). Furthermore, birds tend to defecate while perched, whereas phyllostomid bats defecate more often during flight (Charles-Dominique 1986). Thus, seeds consumed by bats may be moved further where they may not be shaded or outcompeted by the parent plant (Levey 1987, Thies and Kalko 2004). These differences in handling behaviors, as well as infructescence removal rate and dispersal distance, likely all play a role in the relative seed dispersal effectiveness of birds versus bats (Schupp et al. 2010).

Structure elucidation of alkenylphenols in **Piper sancti-felicis**

For structure elucidation of alkenylphenols, unripe and ripe infructescences were collected from approximately 20 individuals of *P. sancti-felicis* during June–August 2011. Samples were collected in and around the lab clearing at La Selva: an area of approximately 1.5 ha that includes buildings and maintained natural areas. Approximately 200 infructescences were oven-dried at 50°C for 48 h, ground to a fine powder in a coffee grinder, and transported to the University of

Nevada, Reno. This method of preparation was chosen because the target compounds in this study were non-volatile secondary metabolites and we did not find quantitative differences between samples that were oven- or silica-dried. An analysis of the crude ^{1}H-NMR extracts of the infructescences was performed first. The major components were further fractionated using flash column chromatography and preparatory TLC on silica gel using mixtures of hexanes and ethyl acetate, followed by detailed 1D and 2D ^{1}H-, ^{13}C-NMR, and EI-MS analysis.

Quantification of alkenylphenols across tissues and developmental stages

To examine variation in alkenylphenols across tissues and developmental stages (Objective 2), one branch with fruits spanning a range of developmental stages (one ripe infructescence, two unripe infructescences, two inflorescences, and one developing inflorescence; Fig. 2) was collected from each of 21 *P. sancti-felicis* individuals (*n*=21), during June–July 2017 and June–July 2018. Similar to other species of *Piper*, infructescences of *P. sancti-felicis* ripen in the afternoon and are typically removed by bats the first night they are ripe (Fleming 2004). Thus, each branch was collected in the afternoon, usually between 1300 h and 1600 h, so that it included a ripe infructescence that had matured on that day. Mature, fully expanded leaves from the branches were also collected. Each sample was dried in a separate envelope in the field with silica, transported to Virginia Tech, and further lyophilized prior to alkenylphenol extractions.

To separate reproductive tissues (i.e. pulp, seeds, and rachis), the dried samples were processed through stainless steel mesh sieves (0.01 mm or 0.0075 mm, depending on sample stage and seed size). Seeds were separated from the pulp for both ripe and unripe infructescences. However, only seeds from ripe infructescences were able to be fully cleaned of pulp; thus, only ripe seeds were analyzed. Dried leaves were ground whole. All samples were extracted and analyzed by GC-MS using a process similar to that in Whitehead et al. (2013) and Aziz et al. (2017). Additional methodological details are provided in Appendix S2: Section S1.

Effects of alkenylphenols on fruit-associated fungi (antagonists)

To assess whether alkenylphenols that occur in *P. sancti-felicis* have a potential defensive role against fruit-associated fungi (Objective 3), we conducted a microdilution assay in September 2018 using methods modified from Zgoda and Porter (2001). To extract large quantities of alkenylphenols, ripe infructescences of *P. sancti-felicis* were locally collected at La Selva, oven dried at 60°C, and ground. A scaled-up version of the extraction procedure described above was used, beginning with a 10 g aliquot of dried plant material.

As there is no prior documentation about the fungal taxa in our study system, three of the most common fungal taxa, which are well-known pathogens in other study systems, were selected from *P. sancti-felicis* seed fungi cultures (Slinn, unpublished data): *Microdochium lycopodinum*, *Fusarium* A, and *Fusarium* B (Appendix S4: Table S4). Seeds were taken from sterile-collected, ripe infructescences and separated from pulp using sterile deionized water. Four seeds from each fruit were plated on malt extract agar (Thermo Fisher Scientific Oxoid Malt Extract) and left at ambient temperature (approximately 26ºC) for 6–7 d. Mycelia were harvested from the plate by adding 1 mL of sterile water and probing the culture with the end of a tip to dislodge fungi. Fungi were stored in a sterile microcentrifuge tube at 4ºC until needed. For details on DNA extraction, PCR and sequencing, see Appendix S2: Section S1.

BLAST was used to align sequences to taxa in the UNITE v8.2 database which features additional quality control checks for fungi deposited in GenBank (Altschul et al. 1990, Kõljalg et al. 2005, accessed 28 February 2020). Taxa were assigned to ecological guilds using the FUNGuild database (Nguyen et al. 2016, accessed 14 May 2019). Two of the three taxa were classified to the genus *Fusarium* (*Fusarium* A and B; guilds: endophyte, plant and animal pathogen, wood saprotroph), and the third taxa was classified as *M. lycopodinum* (guilds: endophyte, plant pathogen; White et al. 1990, Nguyen et al. 2016; Appendix S4: Table S4). *Microdochium lycopodinum* accounted for 43% of the fungal isolates from our seeds, while the two *Fusarium* taxa accounted for 28%. Other fungi that were isolated and not used in this experiment primarily came from the class Sordariomycetes and accounted for 5% or less of fungal isolates. Sequences were deposited at the National Center for Biotechnology Information (NCBI) on GenBank under accession numbers MT093652 - MT093654 (Appendix S4: Table S4).

To determine if the compounds had an effect on fungal growth, we performed a microdilution assay with eight-serial dilutions. Each well received half of the alkenylphenol extract concentration compared to the previous well, fungal inoculum, 2% malt extract to provide nutrients for fungal growth, and sterile DI water. The final volume of each well was 200 µl. The first well received the highest concentration of extract with 5 µl at 73.15 mg/ml of total alkenylphenols in ethanol and an additional 195 µl of water. Next, the remaining seven wells received 100 µl of sterile DI water. The water and extract in well 1 were mixed by pipetting before 100 µl of the 200 µl solution was aliquoted into the second well. This process of mixing newly aliquoted extract into 100 µl of water in subsequent wells was continued across the wells until the eighth well where the 100 µl taken was discarded. Once the wells had the appropriate concentration gradient of alkenylphenols, each well received 80 µl of 2% malt extract and 20 µl of fungal inoculum at a spore concentration of 10^6 cells/ml. Thus, the final concentration of extract in the growth media at the highest concentration was 0.91 mg/mL, approximately equivalent to 0.0003 proportion fresh weight of a ripe infructescence and 6.2% of average alkenylphenol concentration found in one ripe infructescence. We began with these low concentrations due to limited availability of material, but they provide a conservative estimate of the effect of alkenylphenols at concentrations typical of ripe fruits. In addition to the dilution wells, a sterile control with no fungi and no alkenylphenol extract was mixed, including 95 µl of water, 80 µl of 2% malt extract, and 25 µl of 100% ethanol. A negative control was also included with 20 µl of fungal inoculum and no alkenylphenols. These wells then received 95 µl of water, 80 µl of 2% malt extract, 5 µl of 100% ethanol in addition to the inoculum. To assess fungal growth, a spectrophotometer was used at 450 nm with 96 well plates. Hyphal growth was estimated as the difference in optical density (or absorbance) at 72h minus 0h. Additional methodological details are provided in Appendix S2: Section S1.

Effects of alkenylphenols on seed dispersers (mutualists)

To determine if alkenylphenols that occur in *P. sancti-felicis* impact interactions with mutualistic vertebrate seed dispersers (Objective 4), we paired field observations with flight cage feeding experiments. For the field studies, we collected observational data on the removal of infructescences of *P. sancti-felicis* from natural plant populations. We chose 10 individual plants for monitoring. All plants were at least 30 m apart in clearings and along trails within 1 km of the field station. On each plant, we marked and mapped all unripe and ripe infructescences on up to 11 branches. Each plant was then visited twice daily (at dawn and dusk) during 26 May–31 May 2009, and we recorded all nocturnal and diurnal removal events for unripe and ripe infructescences on marked branches. In addition, to further describe the bird species that use plants of *P. sancti-felicis*, we conducted focal observations of six individual plants during 5 July–10 July 2018. Visiting bird species and their behavior (i.e. frugivory, gleaning, calling, etc.) were recorded (Appendix S4: Fig. S2).

To better understand vertebrate responses to the compounds, we conducted feeding trials in a controlled flight cage setting with the dominant consumers of the infructescences of *Piper*.

One representative species was chosen from each group of dispersers: Seba's Short-tailed Bat (*Carollia perspicillata* Linnaeus, 1758) and Passerini's Tanager (*Ramphocelus passerinii* Desmarest, 1805; Appendix S1: Fig. S1 & S2). These species were chosen for feeding experiments because they are dominant consumers of infructescences of *Piper* (Palmeirim et al. 1989, Loiselle 1990, Thies and Kalko 2004, Appendix S4: Fig. S2) and adapt well to captive settings (Denslow et al. 1987, Baldwin and Whitehead 2015, Whitehead et al. 2016). Feeding trials were conducted during January–March 2018. An experimental diet of mashed bananas and agar was used, which allowed us to test the effects of alkenylphenols on animal preference without the confounding effects of natural variation in alkenylphenols found in infructescences of *Piper*. The amount of food provided was equivalent to the fresh weight of one ripe infructescence of *P. sancti-felicis* (approximately 3 g). For the treatment diet, 1 mL of alkenylphenol extract was added at an estimated concentration of 14.6 mg/mL (0.0049 proportion fresh weight). For the control diet, 1 mL of ethanol was applied. All ethanol was evaporated before trials began by allowing diet to air-dry at room temperature (~26℃). For each choice test, one dish each of control and treatment diet were presented simultaneously to each animal (bats N = 16, birds N = 10) for between one and four trials that occurred over consecutive nights (bat trials N = 58) or days (bird trials N = 27). Each dish was pre-weighed and then placed in the flight cage on separate trays to account for any food displaced from the dish but not consumed. Bat trials began at 1800 h and bird trials began at 0600 h. Animals were checked every 30–60 min. After the animal had participated by consuming some portion of either diet, the dishes were removed and weighed to determine the amount eaten from each dish. Any food found in the trays was added to the respective dish. Additional methodological details are provided in Appendix S2: Section S1.

Statistical analyses

All analyses were performed in R v3.6.1 (R Core Team 2020). Linear models, chi-square analyses, and paired t-tests were performed using base R, beta-regressions were performed using the package betareg v3.1-2 (Zeileis et al. 2019), estimated marginal means were performed using package emmeans v1.3.5.1 (Lenth et al. 2019), AIC model comparisons were performed using package AICcmodavg v2.2-2 (Linden 2019), analysis of variance (ANOVA) was performed using package car v3.0-3 (Fox et al. 2019), and plots were created with package ggplot2 (Wickham et al. 2019). All annotated code, data, and metadata are publicly archived at GitHub (https://github.com/ldmaynard/Alkenylphenols_Psf) and will be permanently archived at Dryad Digital Repository upon publication (DOI TBD).

Variation in alkenylphenols across tissue types and stages of development

To determine how alkenylphenol concentration varied across tissue types, we fit the data to a beta-regression. The response variable was the proportion dry weight of total alkenylphenols (summed across all detected compounds). Tissue type was a categorical predictor with five levels: leaves, late flowers (stage 4), late unripe pulp (stage 2), ripe pulp (stage 1), and ripe seeds. Estimated marginal means were computed for tissue type.

To determine how alkenylphenol concentration changed during fruit development and to assess support for linear and non-linear patterns (Fig. 1), we fit the data to two beta-regression models: one linear and one nonlinear (i.e. including a quadratic term). The proportion dry weight of total alkenylphenols was the response and developmental stage was a continuous predictor variable (as described in Fig. 2). AIC model comparison was used to select the model of best fit.

Effects of alkenylphenols on fruit-associated fungi (antagonists)

To determine the effect of alkenylphenol concentration and species of fungi on fungal growth, we fit the data to a linear model (LM). The response variable was the difference in absorbance values of wells containing fungi, measured in average optical density (OD), at 72 h minus 0 h, averaged across triplicate readings taken from each well. The predictor variables were fungal taxa, alkenylphenol concentration in the growth media, and the interaction between the two. AIC model comparison was used to compare the full model to all possible component models. A two-way ANOVA was performed on the model of best fit (full model), which indicated a significant interaction between the species of fungi and concentration of alkenylphenols. Based on this result, the data were analyzed separately for each fungi, using only concentration of alkenylphenols as a predictor variable.

Effects of alkenylphenols on seed dispersers (mutualists)

To examine the temporal differences in removal of unripe and ripe infructescences in our field observational study, a chi-squared analysis was used to test for independence between ripening stage and removal period in predicting the number of infructescences removed. To determine the effect of alkenylphenol presence on disperser response in our feeding trials, we performed paired t-tests, conducted separately for birds and bats, comparing the total amount of control eaten versus total amount of treatment eaten by each animal in the behavioral trials.

RESULTS

Structure elucidation of alkenylphenols in **Piper sancti-felicis**

Analysis of the crude ^{1}H-NMR extracts of the infructescences revealed the presence of *para*-alkenylphenols due to characteristic AB sets of coupled doublets in aromatic region (6-7ppm, J = 8.5-9.0 Hz), multiplets in the alkene region ($-C\underline{H}=C\underline{H}-$ 5.4-4.5 ppm), and characteristic aliphatic resonances of a long-chain hydrocarbon [$(C\underline{H}_2)_n$ 1.2 ppm and CH_3 0.88 ppm] (Fig. 3). GC-MS analysis revealed the presence of up to 10 distinct chromatographic peaks corresponding to compounds A-J (Appendix S3: Fig. S1). Compound F (R_t=13.6) was the dominant peak in the extract with compounds B and D (Rt=12.4 and 12.9) as the other major components. Analysis of the mass spectra of all components suggested *para*-alkenylphenol in all cases based upon dominant fragmentation to the hydroxytropilium ion (m/z = 107) and compared favorably to literature data for other alkenylphenols that have been isolated from other species of *Piper* (Vieira et al. 1980, Galinis and Wiemer 1993, Jinno and Okita 1998, Li et al. 2008, Valdivia et al. 2008, Yang et al. 2013, Rajeev and Jain 2014, Dung et al. 2015, Yoshida et al. 2018**.** Full structural characterization data for F and tentative assignments of A-E and G-J are reported in the supplementary information (Appendix S3: Section S1).

Variation of alkenylphenols across tissue types and stages of development

Alkenylphenols were abundant in unripe and ripe fruit pulp, present in flowers, and almost undetectable in leaves and seeds (Fig. 4). All 10 compounds were found in flowers and fruit pulp, nine were found in developing flowers, and only two were detected in seeds and leaves (compounds B and F; Appendix S4: Fig. S1 & Table S1). Our statistical analyses showed that pulp had higher concentrations of alkenylphenols compared to other tissues, including leaves, seeds, and late flowers. The average total concentration of alkenylphenols in unripe pulp was 1.5, 2.6, 36.2, and 534.9 times higher than ripe pulp, flowers, seeds, and leaves, respectively (Fig. 4, Appendix S4: Fig. S2).

When examining alkenylphenol concentration across reproductive structure developmental stages, AIC model comparison indicated that the nonlinear model (with the quadratic term) was a better fit compared to the linear model (dAICc = 12.25; Appendix S5: Table S1), and the quadratic term was significant ($P < 0.001$), supporting a non-linear pattern in alkenylphenol concentration during development that peaked just before ripening. Developmental stage was also a significant predictor of alkenylphenol concentration across reproductive structure development (*P* = 0.023; Fig. 5). The average concentration of total alkenylphenols in ripe pulp was 1.8, 3.0, and 5.3 times higher compared to late, early, and developing flowers (stages 4, 5, and 6, respectively; Appendix S4: Table S3. However, the average concentration of total alkenylphenols in unripe pulp (stages 2 and 3) were 1.5 and 1.1 times higher compared to ripe pulp (Appendix S4: Table S3). Concentrations of individual compounds are provided in Supplemental Material (Appendix S4: Table S1).

Effects of alkenylphenols on fruit-associated fungi (antagonists)

The model that best fit the data was the LM that incorporated the interaction between fungal taxa and alkenylphenol concentration (dAICc = 11.4; Appendix S5: Table S2). The effect of alkenylphenol concentration varied based on fungal species (F = 10.30, P = 0.0008; two-way ANOVA of LM interaction). The growth of *M. lycopodinum* (t = -7.03, P = 0.0002, R^2 = 0.88; LM) and *Fusarium* A (t = -5.21, P = 0.001, R^2 = 0.80; LM) experienced clear negative effects when exposed to alkenylphenols; however, *Fusarium* B did not (t = -1.00, P = 0.35, R^2 = 0.13; LM). For every 1 mg/mL increase in alkenylphenol concentration, the average absorbance (a proxy for growth) of *M. lycopodinum* and *Fusarium* A decreased by 0.50 OD and 0.43 OD, respectively (Fig. 6).

Effects of alkenylphenols on seed dispersers (mutualists)

During field observations, most infructescences of *P. sancti-felicis* were removed at night (presumably bats; 67 infructescences, 91.8%). We found that diurnal removal events (presumably birds) were more likely to involve unripe infructescences (five unripe infructescences, 83.3%) whereas nocturnal events were more likely to involve ripe infructescences (59 ripe infructescences, 82.2%) (χ^2 = 14.609, df = 1, P = 0.00013; chi-square test; Appendix S4: Table S5). During flight cage experiments, we found that alkenylphenols had a negative effect on bat feeding response (*t* = 3.90*, df* = 15*, P* = 0.001; paired t-test), but no detectable effect on birds (*t* = 0.24*, df* = 9*, P* = 0.81; paired t-test). Bats consumed an average of 2.4 times more control than treatment (Fig. 7a), whereas birds only consumed an average of 1.1 times more control than treatment (Fig. 7b, Appendix S4: Table S6).

DISCUSSION

The alkenylphenols in fruits of *P. sancti-felicis* occur primarily in fruit pulp, follow a non-linear pattern during development, reduce the growth of seed-associated fungi, and reduce the preferences of seed dispersers. Taken together, these results support the hypothesis that alkenylphenols play an adaptive role in fruits, likely as a defense against pathogens. Furthermore, our results suggest that alkenylphenols are allocated in fruits based on the fitness costs of tissue consumption: increasing as plants invest more resources in fruit pulp and seeds during development, peaking when that investment is at a maximum but seeds are not yet viable, and then decreasing once seeds are viable and fruit consumption begins to have a net benefit for the plant. These results do not support predictions from the non-adaptive physiological constraints hypothesis that fruit secondary metabolites are present due to strong selection for defense of leaves, combined with physiological constraints on their exclusion from fruit tissues (Swain 1977, Cipollini and Levey 1998, Eriksson and Ehrlén 1998, Cipollini et al. 2002). Instead, alkenylphenols were found primarily in pulp (Fig. 4), a pattern also observed in other species (e.g. capsaicin in *Capsicum annuum,* Iwai et al. 1979; amides in *Piper reticulatum,* Whitehead et al. 2013; anthocyanin in *Vaccinium macrocarpon*, Zhou and Singh 2002). In these cases, selection in fruit may be the primary driver of allocation patterns, and the presence of the same compounds in leaves and seeds may be due to physiological constraints on their exclusion from those tissues.

Further evidence for function of alkenylphenols comes from our results showing developmental variation in these compounds. Of the three adaptive hypotheses we posited for secondary metabolite allocation across development (Fig. 1), our results suggest that the fitness consequences of tissue loss may be a better explanation of allocation patterns than relative risk of attack, assuming the early developing tissues are indeed at higher risk of attack, which has not been explicitly shown in this system. We also show a decline in allocation during ripening, which, combined with the deterrent effects on bats, suggest that this may be an adaptive pattern to increase dispersal success. However, we cannot rule out the second non-adaptive hypothesis that the reduction in alkenylphenols during ripening could be due to enzymatic degradation that occurs during fruit softening (Brady 1987).

Our results from the fungal bioassays suggest that alkenylphenols may mediate *P. sancti-felicis* interactions with seed fungi. Alkenylphenols exhibited antifungal activity against *Fusarium* A and *M. lycopodinum*, two of the three dominant fungal taxa associated with seeds in natural forest environments, even at concentrations less than 1/10th those in ripe fruit pulp (Fig. 4). This suggests that anti-fungal defense may be at least one important function of alkenylphenols, similar to the role of other classes of secondary metabolites found in high concentration in fruits (e.g. capsaicinoids in *Capsicum chacoense*, Haak et al. 2012; amides in *Piper reticulatum,* Whitehead and Bowers 2014). While certain fungi could benefit fruits by acting as biocontrol agents against pests or pathogens (Cipollini and Stiles 1993, Busby et al. 2016), or even boosting fruit odors and increasing fruit removal rates (Peris et al. 2017), fungal pathogens are also some of the most important antagonists that reduce plant fitness. Fungal pathogens can destroy seeds, inhibit seed germination, and deter vertebrate seed dispersers (Whittaker and Feeny 1971, Janzen 1977, Gallery et al. 2010). For example, certain species of *Fusarium* that are associated with fruit rot produce mycotoxins that reduce the preferences of seed dispersers (Cipollini and Stiles 1992, 1993). The three fungal taxa used in our study were chosen because they were the dominant isolates in our cultures from *P. sancti-felicis* seeds. We focused on seed-associated fungi because of the potential for these fungi to damage seeds and directly reduce plant fitness. While this study did not directly test the pathogenic relationships between the fungi and *P. sancti-felicis*, both fungal taxa have been documented in other systems as having pathogenic properties (Tedersoo et al. 2014, Blacutt et al. 2018). *Fusarium* contains many well-known pathogens that infect both reproductive and vegetative tissues of many crops in temperate and tropical habitats (Booth 1971, Summerell 2019, Marasas 2001, Goswami and Kistler 2004, Tembo et al. 2013). Some *Fusarium* are known pathogens of *Piper* species, including *P. betle* and *P. nigrum*, and affect the roots and leaves (Shahnazi et al. 2012, Edward et al. 2013). However, to our knowledge*,* *Fusarium* has not been previously documented in *Piper* infructescences. *Microdochium* is a common pathogen of grasses (Hernández-Restrepo et al. 2016); however, it can also act as a dark septate endophyte which colonizes grass roots and, in some cases, can increase plant biomass (Mandyam et al. 2012). Thus, although both fungal genera used in this study contain common plant pathogens, it is possible that the taxa we isolated have no effect or even beneficial effects in *Piper* fruit.

It is also important to note that the effects of alkenylphenols were variable across fungal taxa, as *Fusarium* B was unaffected by alkenylphenols (Fig. 6). One explanation for this pattern is acquired resistance by this strain, and alkenylphenols are ineffective as a defense against this species at the doses we tested. A high tolerance to secondary metabolites can evolve in fungi through mechanisms such as the production of alternative enzymes (Kerscher et al. 1999, Marcet-Houben et al. 2009, O’Donnell et al. 2011, Adams et al. 2019). Alternatively, it is possible that the fungal isolates we tested vary in their effects on host plant fitness, and alkenylphenols represent an adaptation to defend against specialized antagonists. Cipollini and Stiles (1993) suggested that the negative effects of fruit rot fungi on fitness should be highest for pathogens (which can directly destroy seeds), intermediate for toxic opportunists (which are associated with fruit rot and deter dispersers), and lowest for latent opportunists (which are associated with fruit rot but are non-toxic). Past work has shown that fruit secondary metabolites have stronger inhibitory effects against these mycotoxic fungi than fruit rot fungi that are nontoxic (Cipollini and Stiles 1992). Considering this variation in the outcomes of plant-fungal interactions, further work is necessary to understand the ultimate fitness consequences of antifungal alkenylphenols in *Piper* infructescences.

In addition to their anti-fungal effects, our results show that alkenylphenols can also mediate *P. sancti-felicis* interactions with seed dispersers. Our study expanded on the natural history knowledge of seed dispersal in this system by quantifying nocturnal and diurnal fruit removal (Appendix S4: Table S5) and further documented the community of birds that utilize *P. sancti-felicis* as a resource (Appendix S4: Fig. S2). A key unanswered question for determining the fitness consequences of bird interactions with *P. sancti-felicis* is whether the seeds consumed in unripe infructescences contain viable seeds that are dispersed intact following bird consumption.

Our disperser preference trials indicated that alkenylphenol compounds decrease palatability, but only for bats (Fig. 7). A similar scenario has been shown in other systems, where birds seem to have a higher threshold for secondary metabolites in fruits compared to small mammals (Tewksbury and Nabhan 2001, but see Karasov et al. 2012). However, in this case the deterrent effect is against the most frequent (and likely most effective) seed disperser. Quantifying the fitness consequences of this deterrent effect would require extensive field studies to track seeds and seedlings, but there are likely important costs associated with reduced bat preference. Even if most infructescences are removed, infructescences containing deterrent metabolites could be rejected once bats begin to feed and dropped partially intact below a feeding roost (as is the case with amides, Whitehead et al. 2016), where competition and pathogen loads are likely high. In addition, less-preferred fruits may experience shorter dispersal distances if they are removed later in the evening once the peak hours of bat activity have passed (Baldwin et al. 2020). Thus, a deterrent effect of alkenylphenols on bats likely carries a fitness cost in terms of dispersal success. This scenario also provides a parsimonious explanation for our results showing nonlinearity of alkenylphenol concentration across development (Fig. 5)—the decrease upon ripening could be a product of selective pressure exerted on fruit chemistry by bat feeding preference.

Taken together, our results are consistent with the hypothesis that alkenylphenols are an adaptation in fruits to defend against pathogenic fungi, but also lead to trade-offs by deterring mutualist seed dispersers (i.e. the defense trade-off hypothesis, Cipollini and Levey 1997). Additional work is necessary to understand the ultimate fitness consequences of alkenylphenols, exploring, for example, the fitness outcomes of specific plant-fungal interactions or the extent to which birds removing unripe fruits are destroying seeds. Future work may also work to isolate and screen individual compounds for bioactivity or explore the metabolic fate of alkenylphenols during ripening. This study demonstrates that alkenylphenols have important ecological consequences in fruits and can serve as a roadmap for using intraplant allocation patterns to better understand the evolutionary ecology of plant chemical traits.

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FIGURE LEGENDS

Figure 1. Hypothesized patterns of secondary metabolite concentrations during fruit development.

Figure 2. *Piper sancti-felicis* reproductive structures developing successively along a branch. From left to right: developing inflorescence, two inflorescences, two unripe infructescences, and one ripe infructescence. Illustration by Sherri Maynard.

Figure 3. Secondary metabolites (alkenylphenols) isolated from the infructescences of *Piper sancti-felicis* (A-J).

Figure 4. Alkenylphenol concentration (proportion dry weight) differed across plant tissues. Pulp, including ripe and late unripe, had higher concentrations of alkenylphenols compared to all other tissue types. Late flowers had higher concentrations than leaves and seeds. Box margins indicate the 25th and 75th percentiles, whiskers the 5th and 95th percentiles, solid lines within the boxes the median, and points individual data observations of total alkenylphenol concentrations from $N = 21$ plants. Letters indicate significant differences from post-hoc pairwise comparisons among tissue types. Ripe pulp (N=20), late unripe pulp (N=21), late flowers (N=21), mature leaves (N=4), and seeds (N=6).

Figure 5. The concentration of alkenylphenols follows a nonlinear trend over reproductive structure stage of development that peaks just before ripening. Stage of development ($z = 2.27$, $P = 0.023$; beta regression) was significant in predicting alkenylphenol concentration. Colored

lines are individual plants, and the bold black line is the nonlinear fit of the data with the gray band indicating 95% confidence intervals. Ripe pulp (*N*=20), late unripe pulp (*N*=21), early unripe pulp (*N*=19), late flowers (*N*=21), early flowers (*N*=15), and developing flowers (*N*=11). Lower sample sizes for some stages was due to small tissue sizes, thus a lack of adequate starting material. Concentrations of alkenylphenols calculated as internal standard equivalents (mg/ml).

Figure 6. The effect of alkenylphenols on hyphal growth of three types of fungi harvested from unsterilized seeds of *Piper sancti-felicis*. Hyphal growth was measured as the difference in optical density (or absorbance) at 72h minus 0h. Points are individual observations, lines are linear fits of the data with gray bands indicating 95% confidence intervals. Alkenylphenols had antifungal effects for two of the three naturally occurring fungi (*Fusarium* A and *Microdochium lycopodinum*) but not *Fusarium* B. Concentrations of alkenylphenols are estimated as internal standard equivalents (mg/ml).

Figure 7. Overall effect of alkenylphenols on bat (*Carollia perspicillata*; a) and bird (*Ramphocelus passerinii*; b) feeding responses. Treatment diets contained approximately 14.6 mg infructescence-extracted alkenylphenols in 3 g of diet, a concentration that mimicked the average concentration found in a ripe infructescence of *Piper sancti-felicis* (0.0049 proportion wet weight). Amount of control and treatment diets eaten were averaged for each individual (bats *N* = 16, birds *N* = 10) for all trials (bat trials *N* = 58, bird trials *N* = 27). Alkenylphenols had a negative effect on bat feeding response but no detectable effect on birds. Box and whisker plots show the median, 25th and 75th percentile, and range of average amount of each dish consumed, and points are the average amount consumed across trials for each individual.

Figure 1

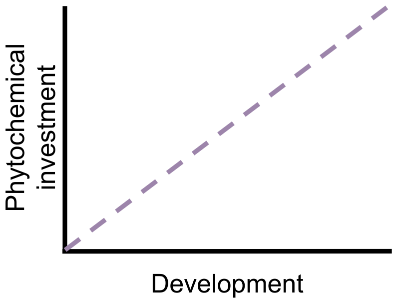
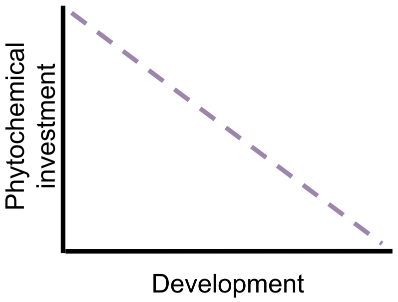
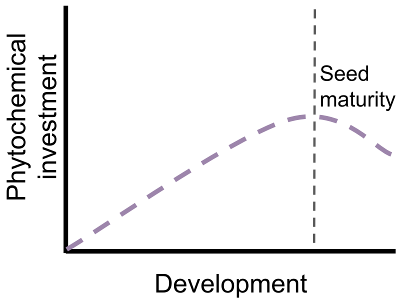
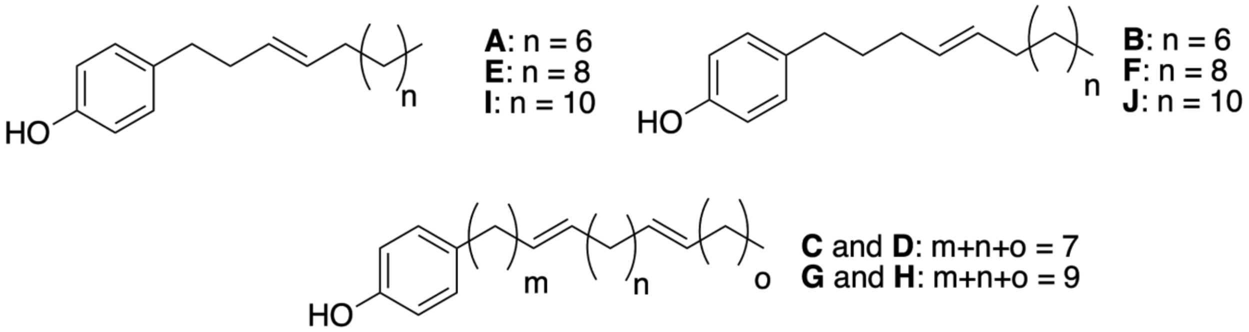
Manipulation of mutualists *Increasing linear*	**Defense allocated by risk** *Decreasing linear*	**Defense allocated by fitness** *Nonlinear*
		
Hypothesized function: Suites of secondary metabolites function in ripe fruits to manipulate mutualists and increase the effectiveness of dispersal services.	**Hypothesized function:** Suites of secondary metabolites function in unripe fruits to defend against antagonists and are allocated according to risk of attack.	**Hypothesized function:** Suites of secondary metabolites function in unripe fruits to defend against antagonists and are allocated according to fitness consequences of removal.
Predicted trend: Phytochemical investment increases linearly with fruit development and is highest when the fruit is ready to be dispersed from the parent plant.	**Predicted trend:** Phytochemical investment decreases linearly with fruit development and is highest in young tissues that are not yet physically defended.	**Predicted trend:** Phytochemical investment increases initially with development then decreases as fruits ripen. It is highest just prior to seed maturity when the fitness costs of loss would be greatest.

Figure 2

Figure 3

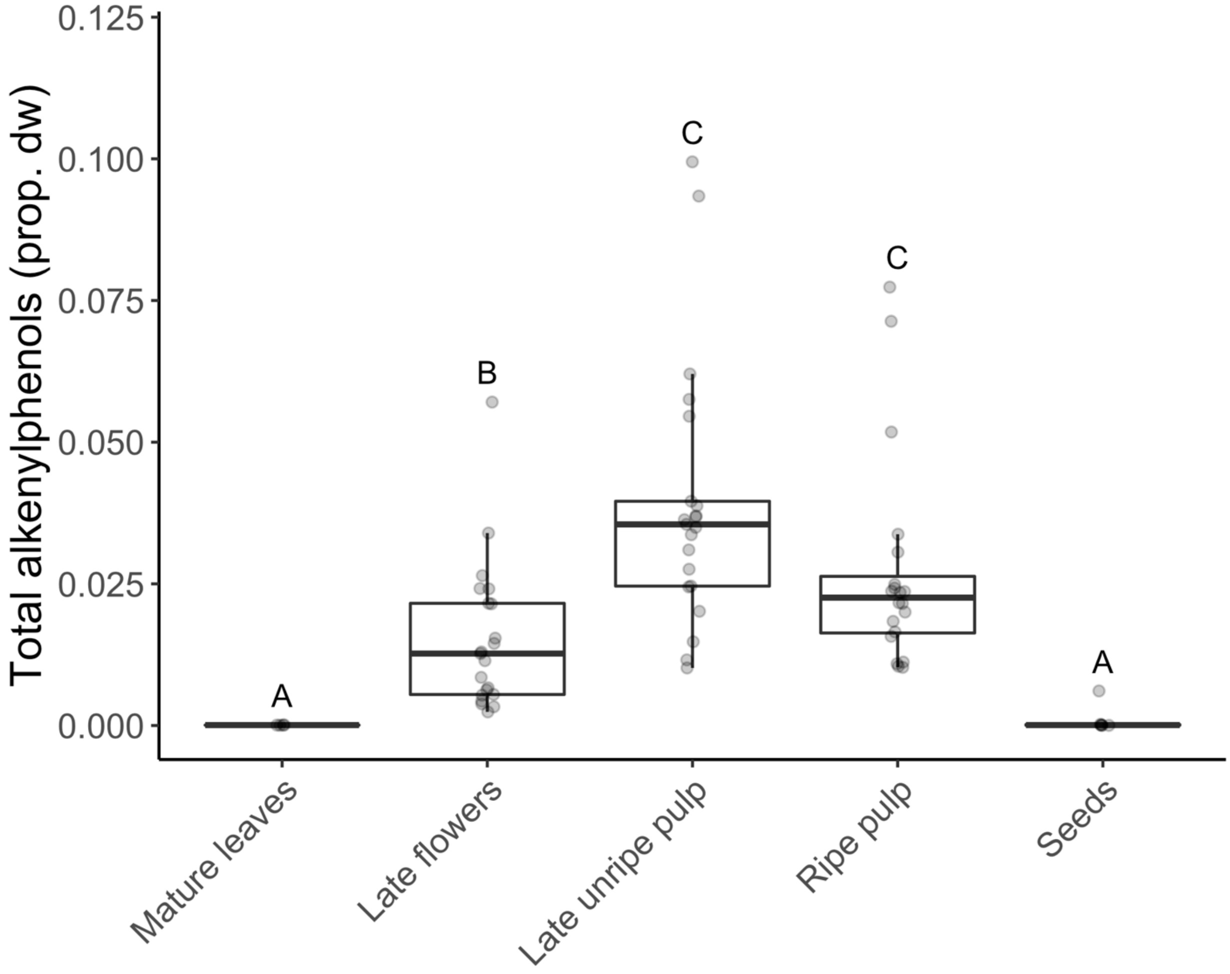


A: n = 6
E: n = 8
I: n = 10

B: n = 6
F: n = 8
J: n = 10

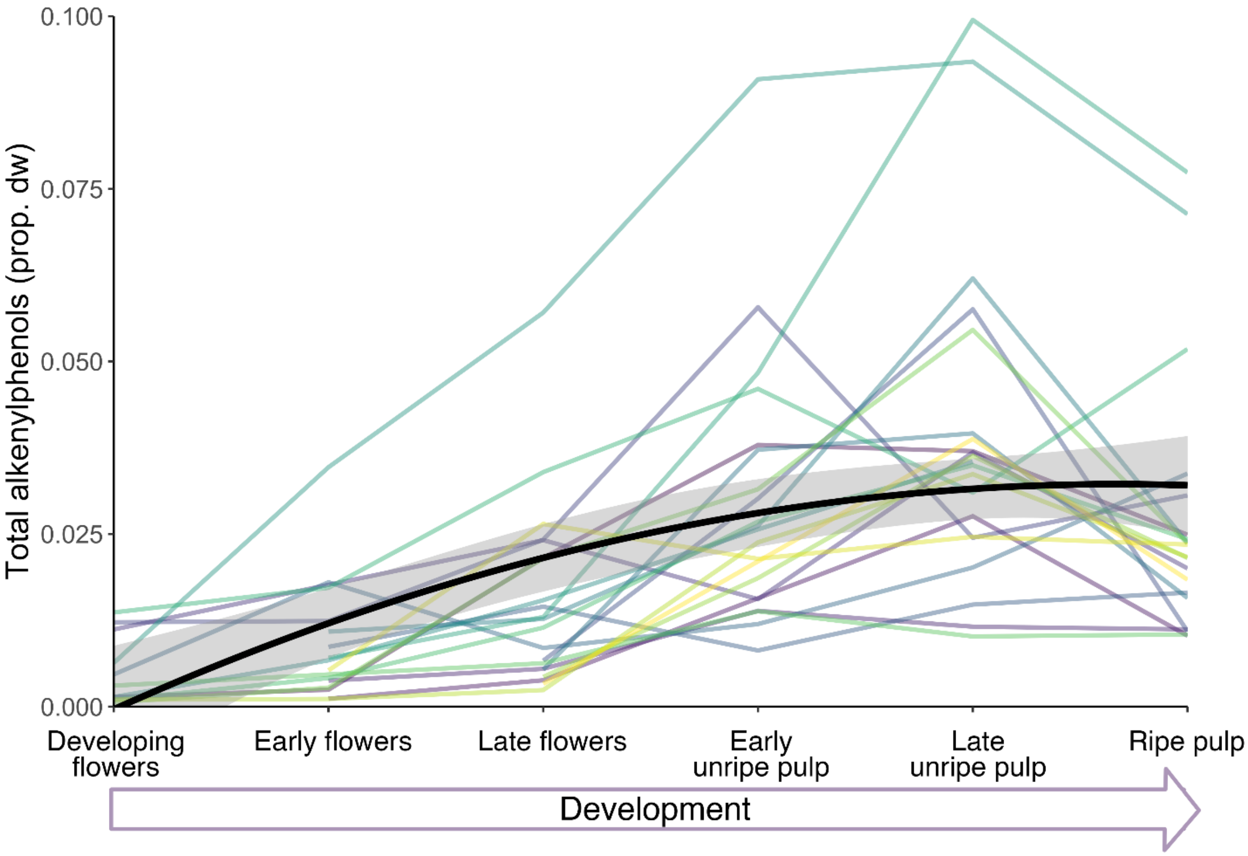
C and **D**: m+n+o = 7
G and **H**: m+n+o = 9

Figure 4



Total alkenylphenols (prop. dw)
0.125
0.100
0.075
0.050
0.025
0.000
A
B
C
C
A
Mature leaves
Late flowers
Late unripe pulp
Ripe pulp
Seeds

Figure 5



0.100
0.075
0.050
0.025
0.000
Total alkenylphenols (prop. dw)
Developing flowers
Early flowers
Late flowers
Early unripe pulp
Late unripe pulp
Ripe pulp
Development

Figure 6

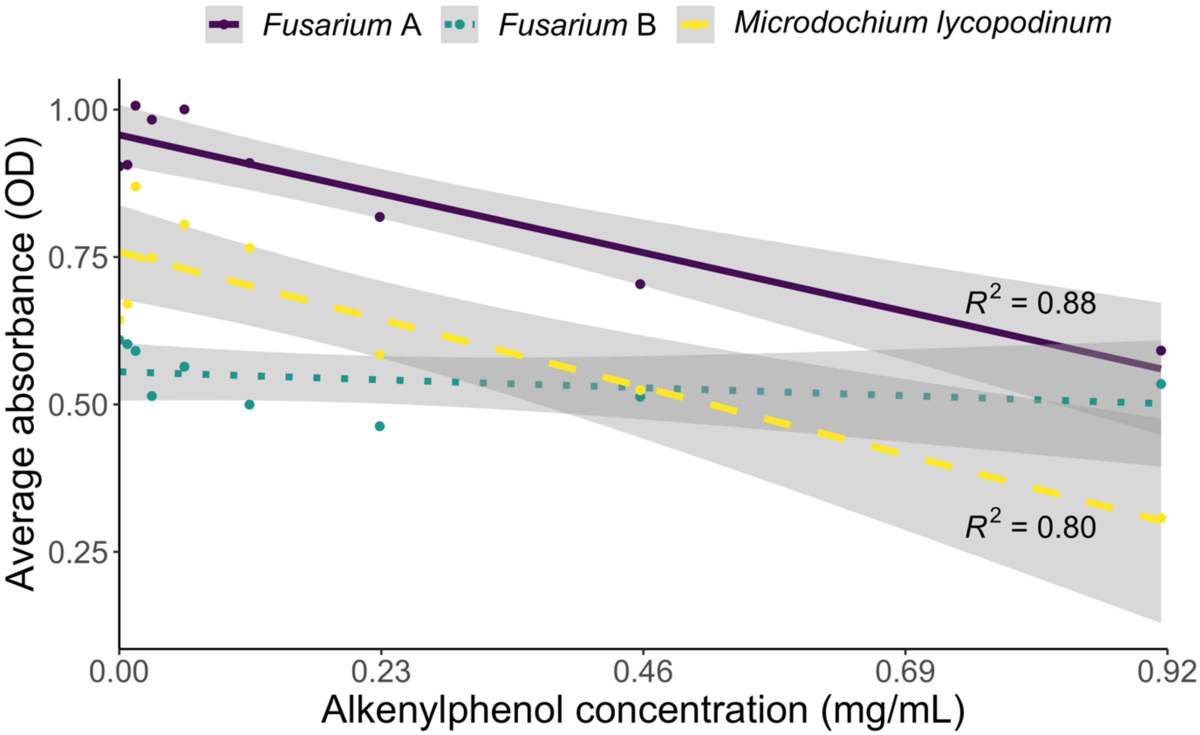
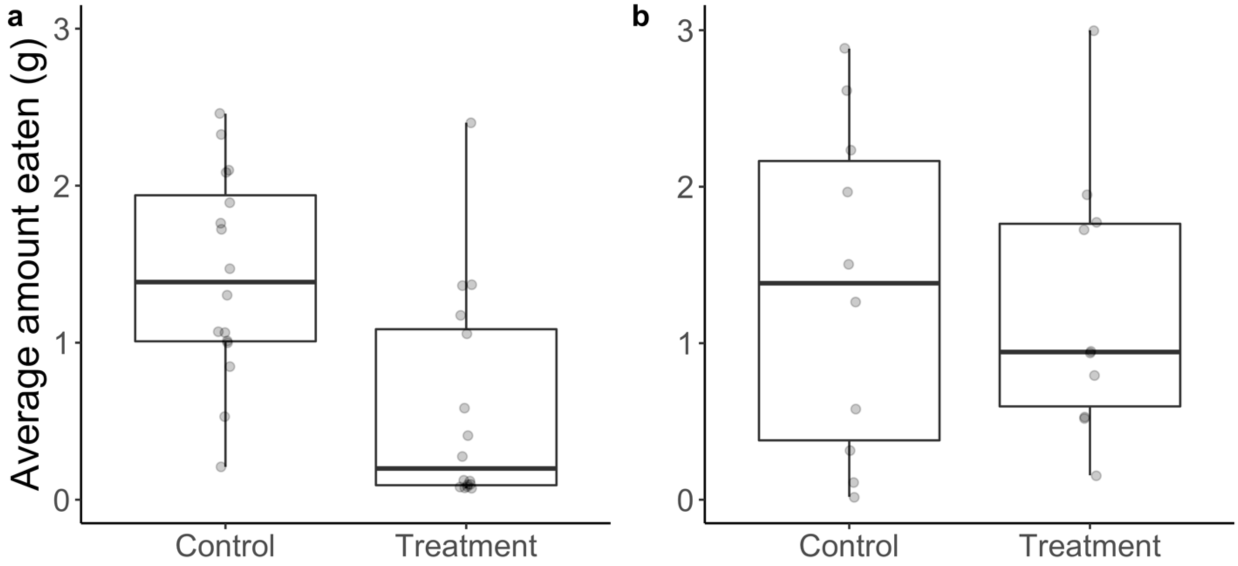

Fusarium A
Fusarium B
Microdochium lycopodinum
Average absorbance (OD)
1.00
0.75
0.50
0.25
0.00
0.23
0.46
0.69
0.92
Alkenylphenol concentration (mg/mL)
$R^2 = 0.88$
$R^2 = 0.80$

Figure 7



SUPPLEMENTAL MATERIAL

Appendix S1: Supplemental information on disperser species

Figure S1. A male (top) and female (bottom) Passerini's Tanager, *Ramphocelus passerinii,* consuming infructescences of *Piper sancti-felicis*, photos by Bernadette Rigley

Figure S2. *Carollia perspicillata* collecting ripe infructescences of *Piper sancti-felicis*, photos by Susan Whitehead.

Appendix S2: Supplemental information on experimental methods

Section S1.

Quantification of alkenylphenols across tissues and developmental stages

Aliquots of homogenized ground material (10 mg) were prepared in 2 mL vials and 4-butylresorcinol (200 µg) was added as an internal standard. Two successive extractions were performed on each aliquot with 95% ethanol by processing for 30 sec at 4 m/s using a FastPrep-24™ (MP Biomedicals, Santa Ana, California, USA) instrument. The two extractions were combined and evaporated under nitrogen to dryness. A chloroform partition was performed by adding 3:1 water:chloroform to each sample. The water partition was discarded, and the chloroform layer (containing the alkenylphenol compounds) was evaporated to dryness. The final extract was resuspended in dichloromethane (100 µL) for GC-MS analysis. All samples were analyzed in the Whitehead Lab using an HP Agilent 7820A GC System coupled with an Agilent 5977B MSD (Agilent Technologies, Santa Clara, CA, USA). The instrument was equipped with a HP-5ms Ultra Inert capillary column (30 m x 250 µm x 0.25 µm; Agilent Technologies, Santa Clara, CA, USA). The carrier gas used was ultra-pure He set at a flow rate of 1.5 mL/min with a pressure of 12.6 psi. The oven conditions were as follows: initial temperature 50°C, initial hold time 1 min; ramp 1, 20°C/min to 275°C, hold time 5 min. GC-MS output data were recorded and processed using MassHunter Workstation Quantitative Analysis software (Version B.08.00). Quantities of individual compounds were estimated as internal standard equivalents based on peak areas in total ion current chromatograms. The internal standard showed a linear response across the range of concentrations present in our samples ($R^2 = 0.96$).

Effects of alkenylphenols on fruit-associated fungi (antagonists)

Fungi were used to make a liquid media culture, which was necessary to promote the production of blastopores (asexual spores) which enable the quantification of fungal concentration in liquid medium. A 2% malt extract broth was made using 100 mL dH_2O and 2 g of malt extract (BD Bacto malt extract). The inoculum was mixed for a few minutes and then autoclaved at 120°C for 20 min. Once the malt extract broth cooled, 100 µl of the inoculum was added to 75 mL of the broth in a laminar flow hood and incubated on a shaker at 200 rpm for 4 d at room temperature. The inoculum was filtered with sterile cheesecloth to remove hyphae, blastospores were counted using a hemocytometer, and the inoculum was diluted to 1 million spores/ml.

In August of 2018, DNA extractions (QIAGEN: Plant mini kit) and the first round of PCR were performed at LSBS. Negative controls were used with every PCR run. Round 1 of PCR was conducted to generate forward and reverse reads of the internal transcribed spacer (ITS) region between the ribosomal RNA genes using primers ITS1F (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) (University of Guelph: Laboratory Services – Oligo Synthesis and Sequencing; White et al. 1990, Gardes and Bruns 1993). A master mix was made for 50 µL reactions with 3 µL of genomic DNA using 5 µL of 10x DreamTaq Buffer (Thermo Fisher Scientific), 1 µL of 10mM dNTPs (University of Guelph: Laboratory Services – Oligo Synthesis and Sequencing), 2.5 µL at 10µM each of forward and reverse primers, 0.25 µL of 5 U/µL DreamTaq Polymerase (Thermo Fisher Scientific) and 35.75 µL of PCR water. Stage 1 of PCR included 94°C for 1 minute; Stage 2 was 94°C for 1 minute, 51°C for 1 min, then 72°C for 1 min. This stage was repeated 35 times. Stage 3 consisted of 72°C for 8 minutes and then a hold at 4°C. Amplicons were stored at -20°C until they were transported to the University of Guelph in March of 2019.

In preparation for Sanger sequencing, amplicons were purified (Thermo Fisher Scientific: Invitrogen PureLink PCR Purification Kit) and DNA was quantitated with a spectrophotometer (Thermo Fisher Scientific: NanoDrop 2000). The next round of PCR and Sanger sequencing was conducted at the University of Guelph's Advanced Analysis Centre Genomics Facility with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on the Applied Biosystems 3730 DNA analyzer (Thermo Fisher Scientific). Primers were used with 1 μL at 10 pmol/μL generating both forward and reverse reads. A master mix was created for approximately 12 μL reactions with 1 μL of BigDye, 2 μL of 5X SeqBuffer and 9 μL of PCR water. Volumes of amplicons were calculated and standardized to be 28ng/1kb. PCR cycling conditions included: Stage 1 at 96ºC for 2 minutes, Stage 2 at 96ºC for 30 seconds, 45ºC for 15 seconds and 60ºC for 4 minutes. Stage 2 was repeated 29 times before moving to stage 3 which was held 10ºC. Sequences were purified with Sephadex columns (Sigma Aldrich) and passed to electrophoresis. Basecalls were performed with the v5.2 KB Basecaller (Thermo Fisher Scientific) with ambiguous bases assigned to any bases with a QV (phred score) of 12 or less, yielding a minimum confidence of 95% for base calls. Primers were trimmed and consensus sequences were generated by merging forward and reverse reads, using CodonCode Aligner v8.0.2 (CodonCode Aligner Company).

Sequences were identified to taxa using UNITE (v8.2) (Kõljalg et al. 2005), rather than directly through other international sequence databases, such as GenBank, because of the high percentage of errors found in them (Nilsson et al. 2006). UNITE adds additional layers of quality control, where it checks for low quality reads and is curated by fungal taxonomists, bioinformaticians and ecologists (Nilsson et al. 2019). UNITE is based on the GenBank sequence database, where it is updated regularly with new sequences (Nilsson et al. 2019). Our *M. lycopodinum* sequence aligned with sequences from type materials based on a curated fungal collection, and therefore we are reasonably confident that our sequence can be classified to species, and highly confident about genus classification despite a 98% identity match. We refrained from classifying our taxa to species using UNITE's species hypothesis algorithm because it uses the ITS2 region to approximate species classification. Moreover, even the full ITS region does not always distinguish equally well across different species (Schoch et al. 2012). However, we have included the species hypotheses and their digital object identifiers in Table S4 as they match genus level identification and are linked to ecological data on the taxa and other probable sequence alignments.

To measure absorbance of fungi, a spectrophotometer (BIO-RAD: iMark Microplate Absorbance Reader) was used at 450 nm with 96 well plates. A preliminary run with the different wavelengths available for the microplate reader was used to select the optimal wavelength for the experiment by generating a series of absorbance curves (415 nm, 450 nm, 490 nm, 520 nm, 595 nm, 655 nm, 750 nm). A wavelength of 450 nm was selected as it was the best at detecting changes in inoculum concentration for our experiment. Due to common variation in the microplate reader's measurements, absorbance duplicate measurements were taken sequentially and averaged to control this source of variance. Measurements were taken at 0 h, 24 h and 72 h. In between measurements, the 96 well plates were stored in sterile Ziploc® bags with damp sterile filter paper to maintain humidity.

Effects of alkenylphenols on seed dispersers (mutualists)

Both birds and bats were captured in the field using mist nets (Keyes and Grue 1982, Kunz et al. 2009); adult males and adult, non-reproductive females were retained for experiments. All animals were housed in individual cages of 1 m x 1 m x 2 m. Each cage had ledges for perching/roosting, adequate cover, and at least one water source. In between trials, animals were fed a maintenance diet *ad libitum* developed by Denslow et al. (1987) for tropical fruit-eating birds. All animal care was in accordance with institutional guidelines. Prior to release, all animals were marked to avoid repeated trials with the same individuals. Bats were marked by clipping a section of fur from the dorsum (Kunz and Weise 2009), and birds were marked by clipping a small section of the tail feathers (Trainor et al. 2014). Animals were only captured over a 3-month period, so permanent markers were not necessary.

Appendix S3: Supplemental information on compound structure determination

Section S1.

Further analysis of the molecular ion of these peaks suggested that the compounds A, B, E, F, I and J were phenols with C_{12} (A, B), C_{14} (E, F), C_{16} (I, J) *para*-alkenyl substituents containing one unit of unsaturation (C_nH_{2n-2}). Analysis of the molecular ion of compounds C, D, G and H demonstrated that the *para*-substituent was a doubly unsaturated dienyl chain of the C_{12} (C and D) C_{14} (G and H) series. Isomeric relationships between compound A and B, E and F, and I and J suggested that these compounds only differed by the double bond position in the mono-unsaturated *para*-substituent. The major components were further fractionated using flash column chromatography and preparatory TLC on silica gel using mixtures of hexanes and ethyl acetate. Detailed 1D and 2D ^{1}H-, ^{13}C-NMR, and EI-MS analysis of the major component were strongly consistent with the structure of a *p*-alkenylphenol. Double bond position was tentatively assigned in the mono-unsaturated series (A, B, E, F, I and J) based upon EI-MS fragmentation and comparisons to the literature. EI-MS analysis was consistent with the $\Delta^{3',4'}$position of the double bond for compounds A, E, and I and the $\Delta^{4',5'}$ position for compounds B, F, and J. Comparison of all spectral data to known alkenylphenols that have been previously isolated from other *Piper* species strongly supported the structures of E and I, and supported the proposed structure of the major component F by comparison (Vieira et al. 1980, Yoshida et al. 2018). Double bond position and stereochemistry of the doubly unsaturated components (C, D, G, and H) in the series could not be unequivocally established based upon NMR and EI-MS analysis.

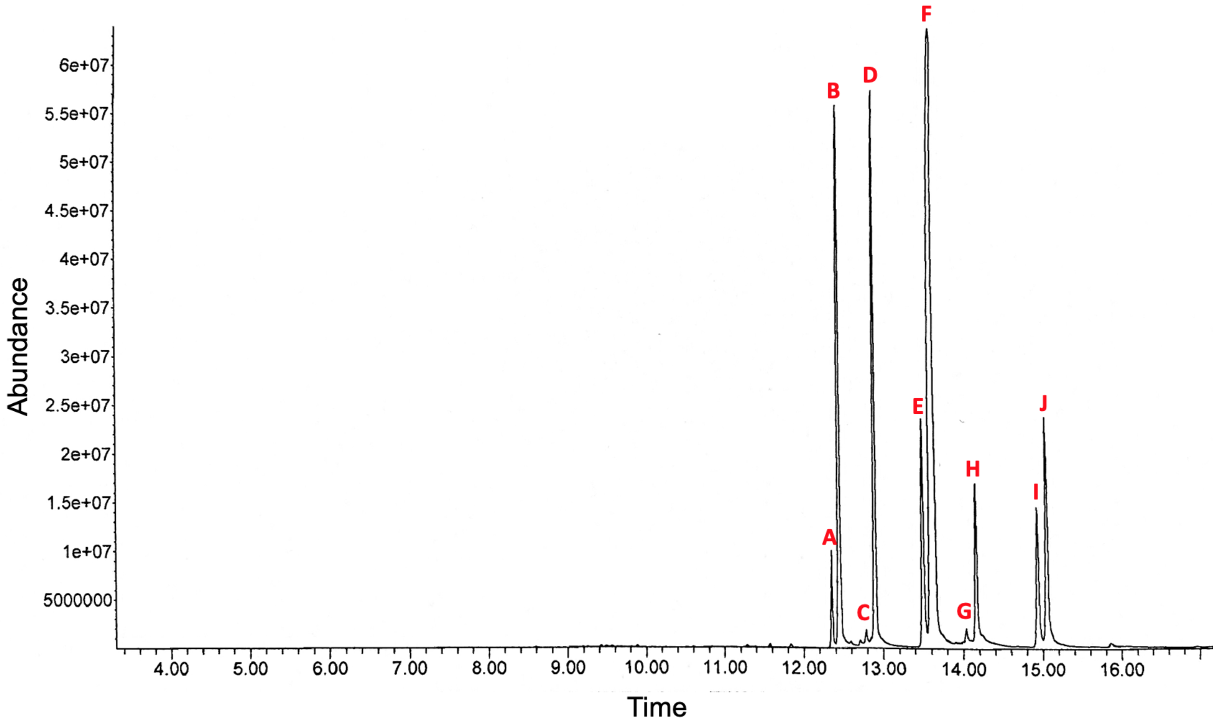
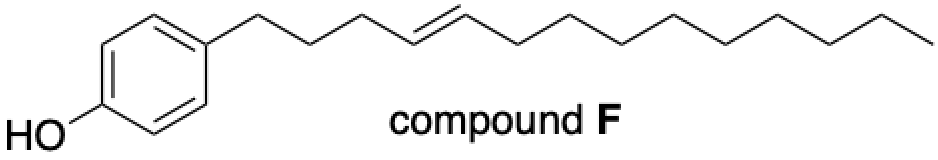


Figure S1. Representative GC-MS chromatogram of ripe infructescences showing ten alkenylphenols (A-J) found in *Piper sancti-felicis.*

Full characterization data for 4-[(4'E)-Tetradec-4'-en-1-yl]phenol (F):



compound **F**

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.04 (d, *J* = 8.5 Hz, 2H), 6.74 (d, *J* = 8.5 Hz, 2H), 5.47 – 5.33 (m, 2H), 4.59 (s, 1H), 2.61 – 2.47 (m, 2H), 2.08 – 1.93 (m, 4H), 1.63 (tt, *J* = 8.0, 6.6 Hz, 2H), 1.39 – 1.16 (m, 26H), 0.92 – 0.79 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (100 MHz, CDCl$_3$) δ 153.6, 135.1, 131.1, 129.9, 129.6, 115.2, 34.6, 32.8, 32.2, 32.1, 32.1, 31.7, 29.9, 29.8, 29.8, 29.8, 29.7, 29.7, 29.5, 29.5, 29.3, 29.3, 29.3, 29.3, 22.8, and 14.3.

FT-IR: 3369 (br), 3024 (w), 2921(s), 2851(s), 1519(m) cm^{-1}

HRMS (ESI-MS) Calcd' for $C_{20}H_{33}O$ $(M+H)^+$ = 288.2453, found 288.2418

LRMS m/z (rel. intensity) 288(54), 133(84), 134(17), 120(100), 121(48),107(100), 108(31)

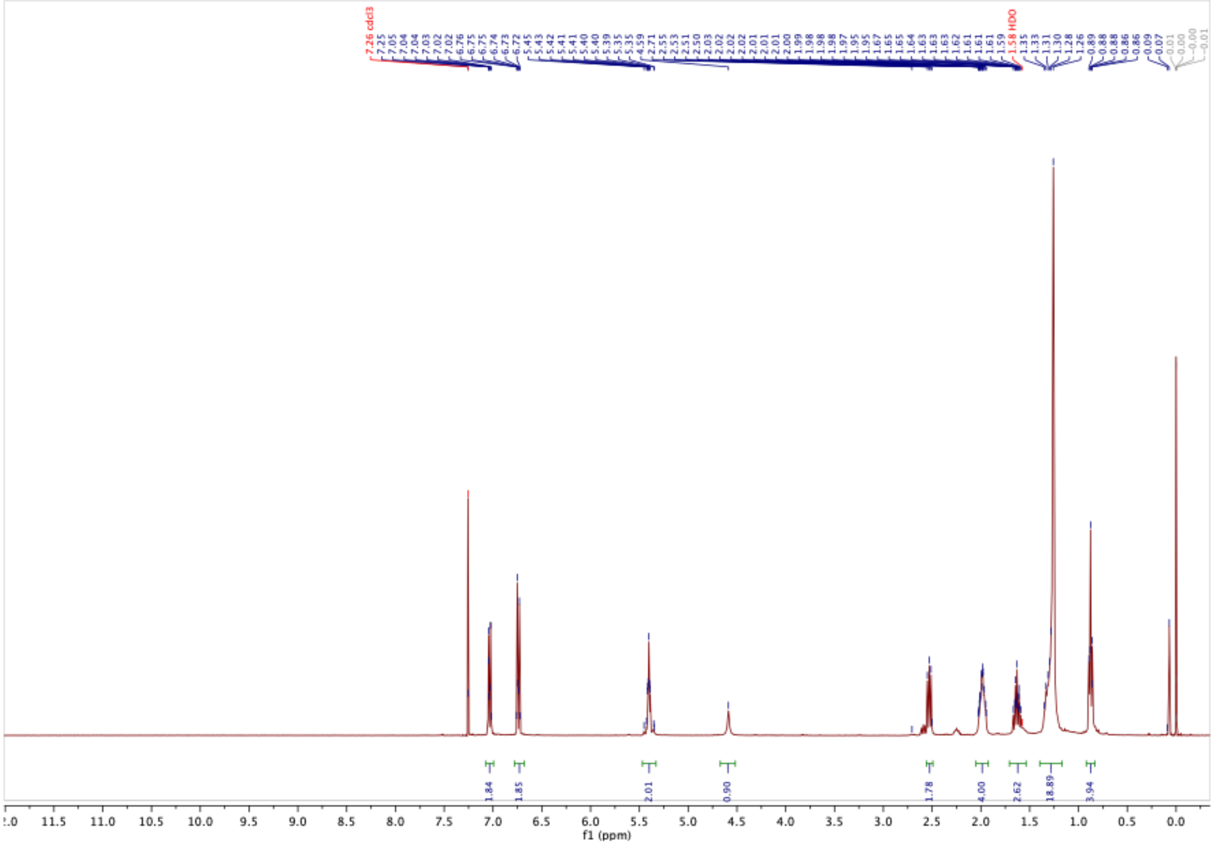


Figure S2. ^{1}H-NMR spectrum of compound F in $CDCl_3$ (400 MHz)

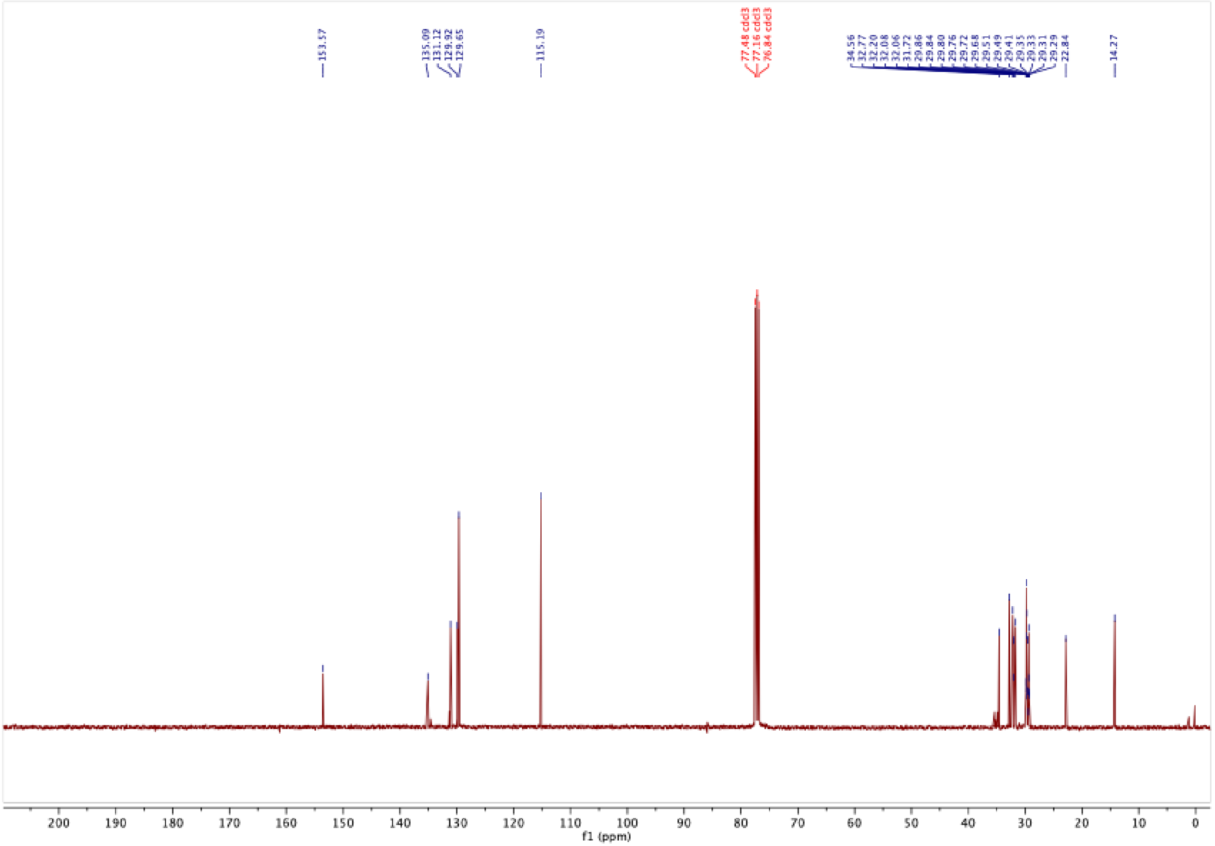


Figure S3. ^{13}C-NMR spectrum of compound F in $CDCl_3$ (100 MHz)

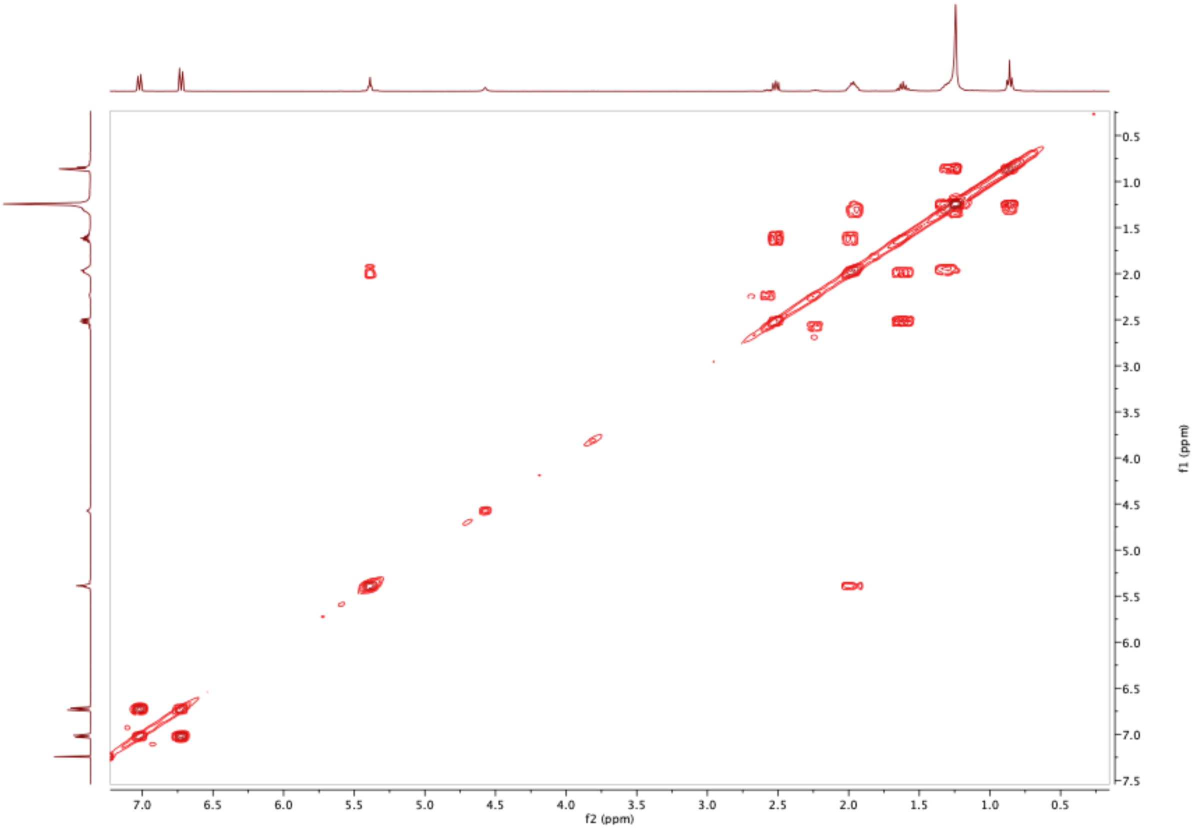


Figure S4. ^{1}H,^{1}H-NMR spectrum of compound F in $CDCl_3$ (400 MHz)

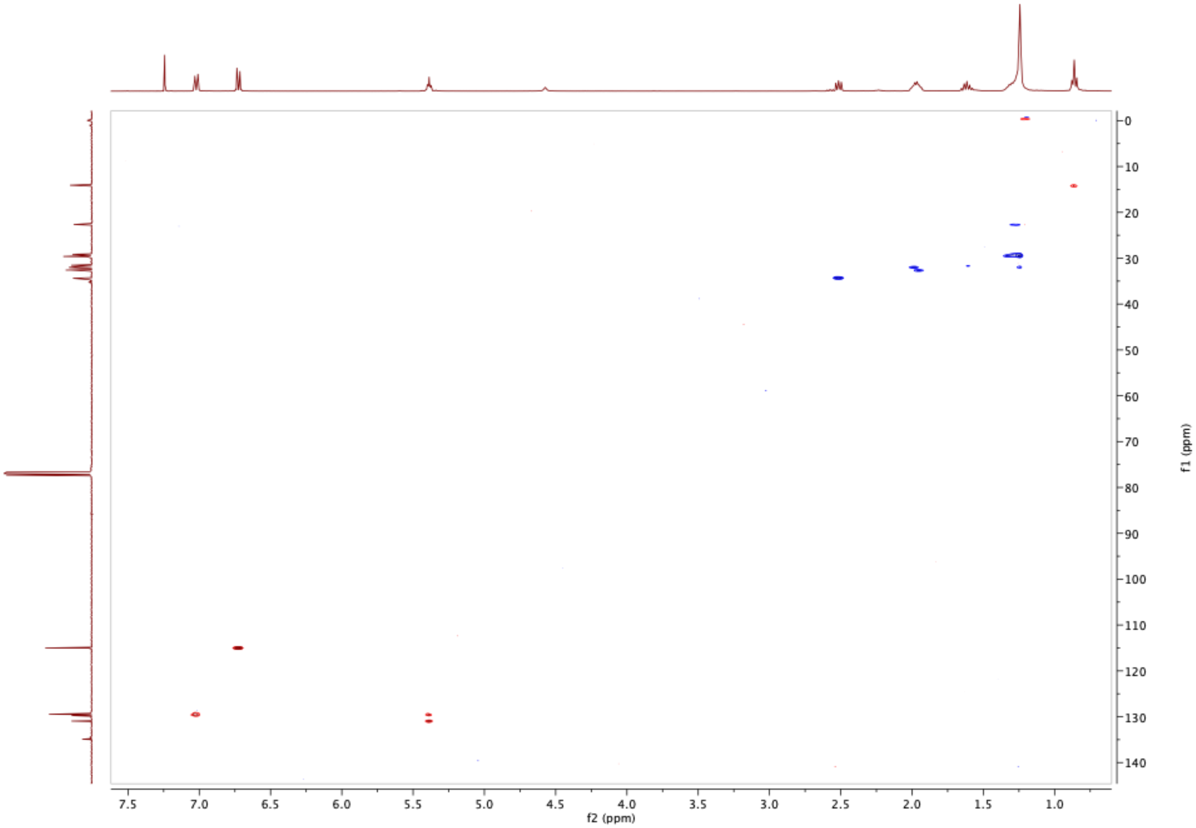


Figure S5. ^{1}H,^{13}C-HSQC NMR spectrum of compound F in $CDCl_3$ (400 MHz)

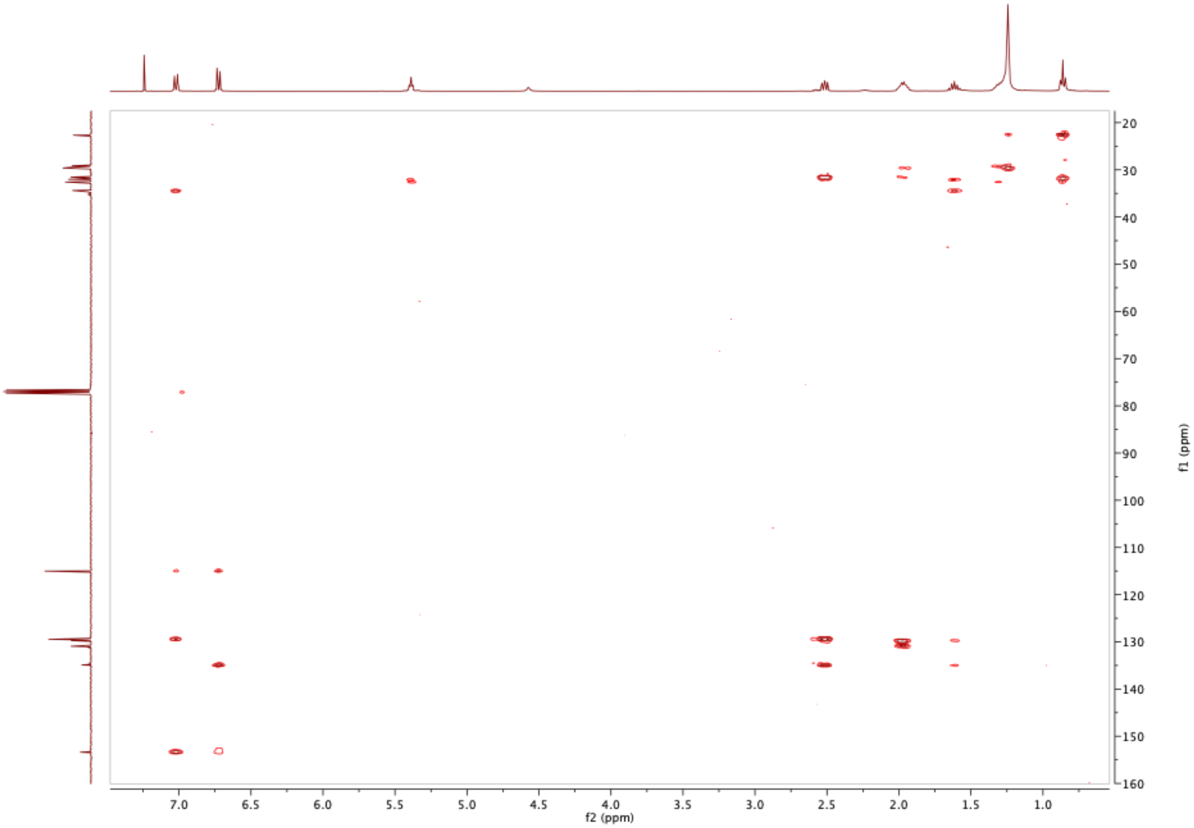


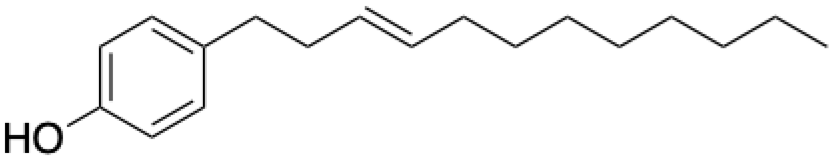
Figure S6. ^{1}H,^{13}C-HMBC NMR spectrum of compound F in $CDCl_3$ (400 MHz)

GC-MS data for compounds A, B, C, D, E, G, H, I and J

<u>Compound A ($C_{18}H_{28}O$):</u>

LRMS m/z (rel. intensity) 260(3, $M^{+\bullet}$), 107(100), 108(10), 77(6), 78(2)

MS fragmentation is consistent with the tentative structure 4-[(3′E)-dodec-3′-en-1-yl]phenol.

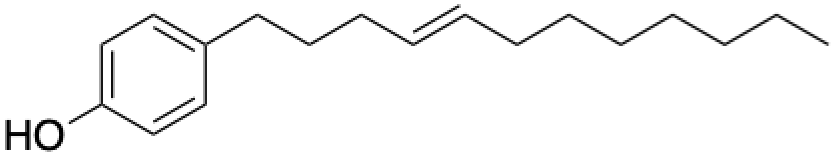


Compound B ($C_{18}H_{28}O$):

LRMS m/z (rel. intensity) 260(51, $M^{+\bullet}$), 133(82), 134(16), 120(100), 121(45),122(4), 107(100), 108(29), 109(4), 105(4), 103(6), 91(12), 78(5), 79(6), 77(23)

Tentative structure:

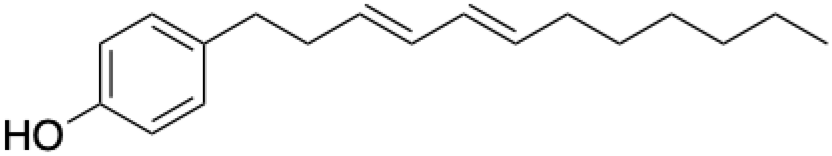
MS fragmentation is consistent with the tentative structure 4-[(4′E)-dodec-4′-en-1-yl]phenol.



Compound C ($C_{18}H_{26}O$):

LRMS m/z (rel. intensity) 258(7, $M^{+\bullet}$), 120(19), 121(3), 107(100), 108(8), 109(2), 77(9), 78(2), 79(6).

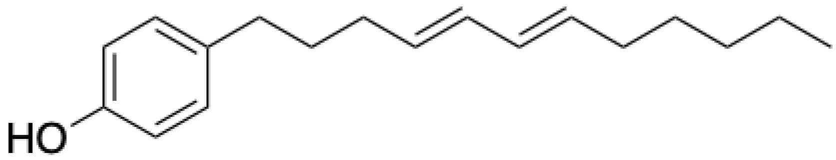
Tentative structure consistent with the data reported for the structure 4-[(3’,5’,E, E)-dodec-3′,5’-dienyl-1-yl]phenol (Villiaramullin B; Galinis and Wiemer 1993)·



Compound D ($C_{18}H_{26}O$):

LRMS m/z (rel. intensity) 258(45, $M^{+\bullet}$), 160(25), 159(100), 158(5), 157(5), 147(3), 146(16), 145(43), 144(15), 141(10), 134(4), 133(21), 132(7), 131(23), 130(2), 129(6), 128(8), 121(11), 120(86), 108(12), 107(100), 91(11), 71(11), 77(16).

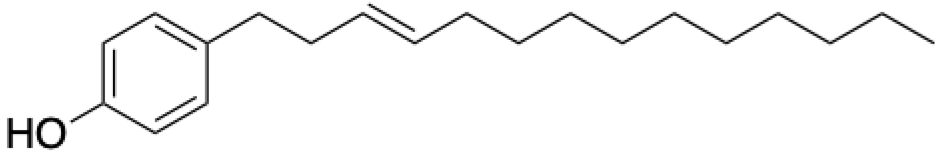
MS fragmentation is consistent with the tentative structure 4-[(4’,6’,E, E)-dodec-4′,6’-dienyl-1-yl]phenol



Compound E ($C_{20}H_{30}O$):

LRMS m/z (rel. intensity) 288(14, $M^{+\bullet}$), 289(3), 108(30), 107(100), 78(4), 77(12).

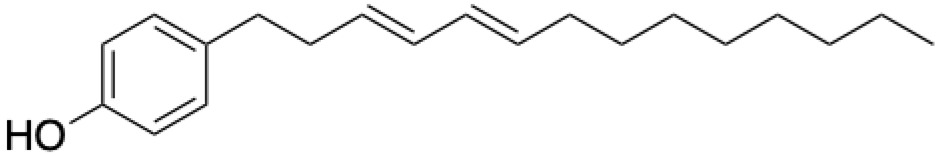
Tentative structure is consistent with data reported for 4-[(3′E)-tetradec-3′-en-1-yl]phenol (Yoshida et al. 2018).



Compound G ($C_{20}H_{28}O$):

LRMS m/z (rel. intensity) 286(3, $M^{+\bullet}$), 121(4), 120(26), 109(2), 108(9) 107(100)

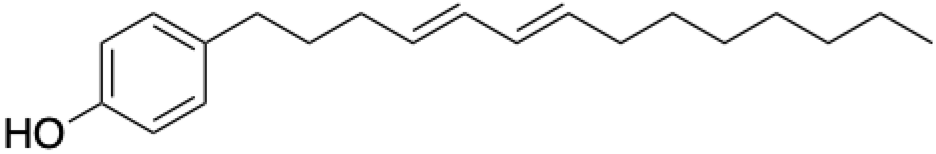
MS fragmentation is consistent with the tentative structure 4-[(4’,6’,E, E)-dodec-4′,6’-dienyl-1-yl]phenol 4-[(3’,5’,E, E)-tetradec-3′,5’-dienyl-1-yl]phenol



Compound H ($C_{20}H_{28}O$):

LRMS m/z (rel. intensity) 286(18, $M^{+\bullet}$), 287(4), 174(2), 173(5), 160(13), 159(63), 158(3), 157(3), 147(3) 146(9), 145(22), 144(7), 134(3), 133(17), 132(3), 131(11), 121(9), 120(74).

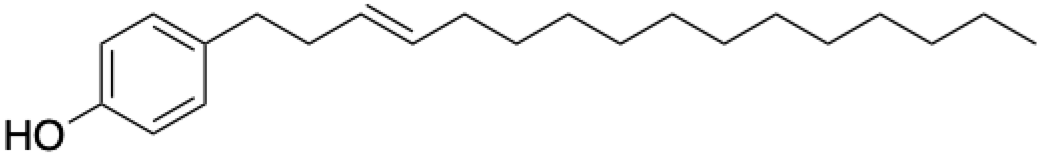
MS fragmentation is consistent with the tentative structure 4-[(4’,6’,E, E)-tetradec-4′,6’-dienyl-1-yl]phenol.



Compound I ($C_{22}H_{32}O$):

LRMS m/z (rel. intensity) 316(4, $M^{+\bullet}$), 159(2), 145(1), 121(1), 120(7), 108(14), 107(100)

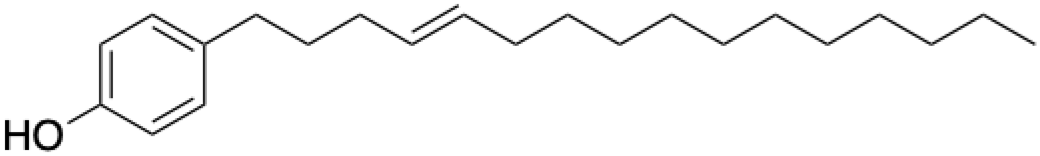
Tentative structure is consistent with data reported for 4-[(3′E)-hexadec-3′-en-1-yl]phenol (Yoshida et al. 2018)



Compound J ($C_{22}H_{32}O$):

LRMS m/z (rel. intensity) 316(14, $M^{+\bullet}$), 317(3), 134(5), 133(32), 121(16), 120(100), 108(12), 107(88)

MS fragmentation is consistent with the tentative structure 4-[(4′E)-hexadec-4′-en-1-yl]phenol



Appendix S4: Supplemental figures and tables of results

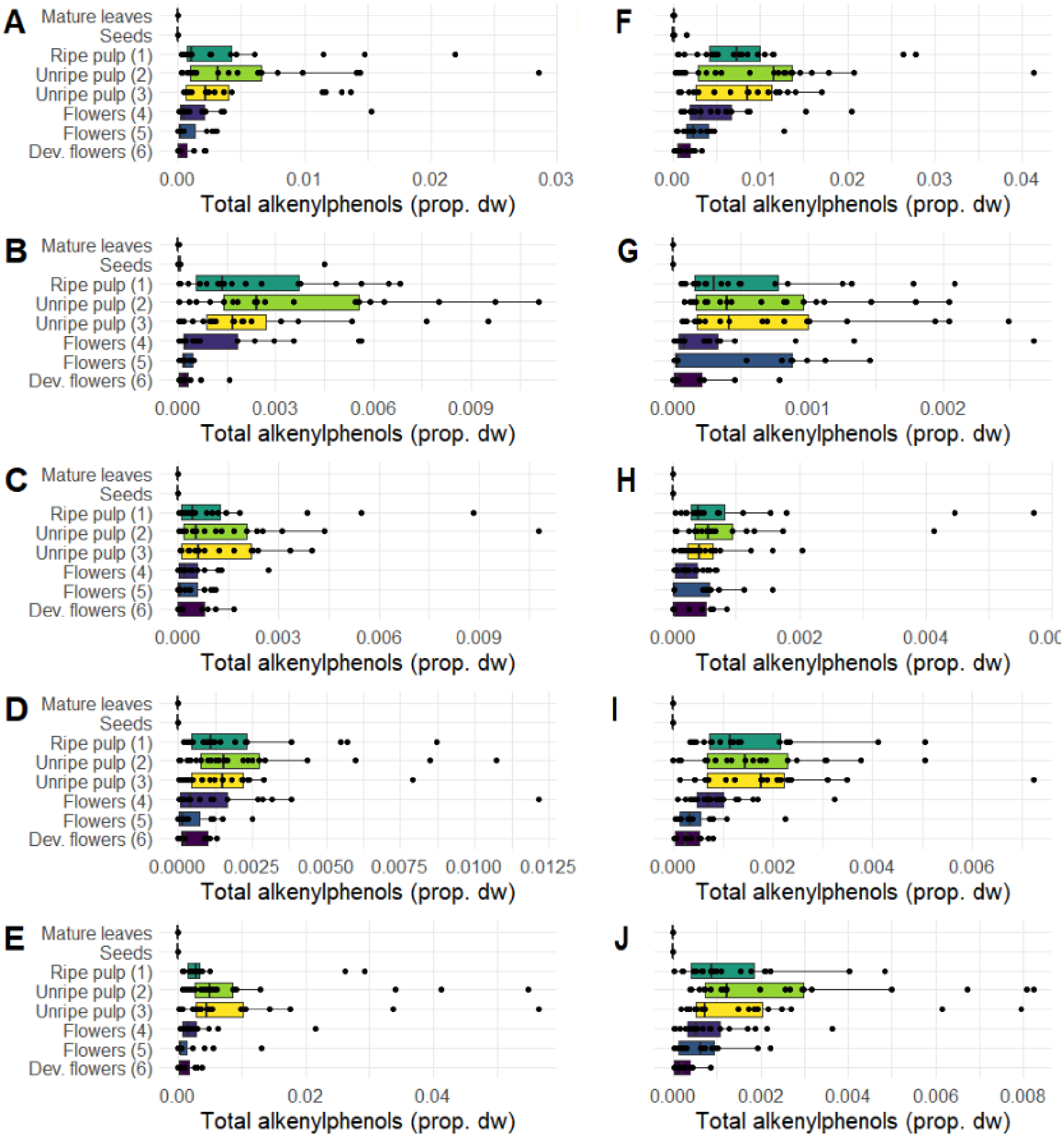


Figure S1. Average estimated concentrations (proportion dry weight) of individual alkenylphenol compounds in different plant parts from 21 individuals of *Piper sancti-felicis*, including ripe pulp (*N*=20), late unripe pulp (*N*=21), early unripe pulp (*N*=19), late flowers (*N*=21), early flowers (*N*=15), and developing flowers (*N*=11). Box margins indicate the 25th and 75th percentiles, whiskers the 5th and 95th percentiles, solid lines within the boxes the median, and points individual data observations of total alkenylphenol concentrations.

Table S1. Average estimated concentrations (proportion dry weight) of individual alkenylphenol compounds in different plant parts of *Piper sancti-felicis.* (nd = not detected)

Tissue	**Compound**	**N**	**Mean**	**SD**	**SE**
Ripe pulp (1)	A	20	0.003928643	0.005728091	0.001280840
Ripe pulp (1)	B	20	0.002206696	0.002224326	0.000497374
Ripe pulp (1)	C	20	0.001346650	0.002249166	0.000502929
Ripe pulp (1)	D	20	0.001987187	0.002267601	0.000507051
Ripe pulp (1)	E	20	0.004977501	0.007883599	0.001762826
Ripe pulp (1)	F	20	0.008298529	0.007276669	0.001627113
Ripe pulp (1)	G	20	0.000563378	0.000599846	0.000134130
Ripe pulp (1)	H	20	0.000993436	0.001484151	0.000331866
Ripe pulp (1)	I	20	0.001490589	0.001250917	0.000279713
Ripe pulp (1)	J	20	0.001281158	0.001280079	0.000286234
Unripe pulp (2)	A	21	0.005347396	0.006858371	0.001496619
Unripe pulp (2)	B	21	0.003703359	0.003204574	0.000699295
Unripe pulp (2)	C	21	0.001533471	0.002437913	0.000531996
Unripe pulp (2)	D	21	0.002492772	0.002788817	0.000608570
Unripe pulp (2)	E	21	0.010264210	0.014556068	0.003176395
Unripe pulp (2)	F	21	0.010312668	0.009523466	0.002078191
Unripe pulp (2)	G	21	0.000640000	0.000584609	0.000127572
Unripe pulp (2)	H	21	0.000777375	0.000883225	0.000192735
Unripe pulp (2)	I	21	0.001644595	0.001288308	0.000281132
Unripe pulp (2)	J	21	0.002522828	0.002469268	0.000538838
Unripe pulp (3)	A	19	0.003857605	0.004710262	0.001080608
Unripe pulp (3)	B	19	0.002360804	0.002577936	0.000591419
Unripe pulp (3)	C	19	0.001171454	0.001228178	0.000281763
Unripe pulp (3)	D	19	0.001678165	0.001757198	0.000403129
Unripe pulp (3)	E	19	0.009627121	0.013848875	0.003177150
Unripe pulp (3)	F	19	0.007268165	0.005107066	0.001171641
Unripe pulp (3)	G	19	0.000732980	0.000736568	0.000168980
Unripe pulp (3)	H	19	0.000569358	0.000524666	0.000120367
Unripe pulp (3)	I	19	0.001807324	0.001613549	0.000370173
Unripe pulp (3)	J	19	0.001752484	0.002046959	0.000469605
Flowers (4)	A	21	0.001728104	0.003333087	0.000727339
Flowers (4)	B	21	0.001227505	0.001774678	0.000387267
Flowers (4)	C	21	0.000467772	0.000663872	0.000144869

Flowers (4)	D	21	0.001514603	0.002713615	0.000592159
Flowers (4)	E	21	0.002781402	0.004563550	0.000995848
Flowers (4)	F	21	0.005260610	0.004979063	0.001086521
Flowers (4)	G	21	0.000357638	0.000625334	0.000136459
Flowers (4)	H	21	0.000243740	0.000231352	5.05E-05
Flowers (4)	I	21	0.000896649	0.000685543	0.000149598
Flowers (4)	J	21	0.000852942	0.000868424	0.000189506
Flowers (5)	A	15	0.000836362	0.001171480	0.000302475
Flowers (5)	B	15	0.000272123	0.000176487	4.56E-05
Flowers (5)	C	15	0.000330804	0.000428749	0.000110702
Flowers (5)	D	15	0.000509252	0.000734797	0.000189724
Flowers (5)	E	15	0.001877274	0.003512052	0.000906808
Flowers (5)	F	15	0.003261303	0.002953237	0.000762523
Flowers (5)	G	15	0.000455393	0.000519046	0.000134017
Flowers (5)	H	15	0.000378451	0.000484046	0.00012498
Flowers (5)	I	15	0.000493519	0.000562162	0.00014515
Flowers (5)	J	15	0.000664167	0.000673735	0.000173958
Dev. flrs (6)	A	11	nd	nd	nd
Dev. flrs (6)	B	11	0.000309486	0.000473139	0.000142657
Dev. flrs (6)	C	11	0.000416794	0.00057982	0.000174822
Dev. flrs (6)	D	11	0.000534046	0.000506891	0.000152833
Dev. flrs (6)	E	11	0.001094351	0.001360299	0.000410146
Dev. flrs (6)	F	11	0.001235828	0.001033494	0.00031161
Dev. flrs (6)	G	11	0.000175547	0.000249703	7.53E-05
Dev. flrs (6)	H	11	0.000255986	0.000318171	9.59E-05
Dev. flrs (6)	I	11	0.000304378	0.000296426	8.94E-05
Dev. flrs (6)	J	11	0.000252601	0.000256842	7.74E-05
Seeds	A	6	nd	nd	nd
Seeds	B	6	0.000767416	0.00181971	0.000742893
Seeds	C	6	nd	nd	nd
Seeds	D	6	nd	nd	nd
Seeds	E	6	nd	nd	nd
Seeds	F	6	0.000317936	0.000639511	0.000261079
Seeds	G	6	nd	nd	nd
Seeds	H	6	nd	nd	nd
Seeds	I	6	nd	nd	nd
Seeds	J	6	nd	nd	nd
Mature leaves	A	4	nd	nd	nd
Mature leaves	B	4	1.21E-05	5.77E-06	2.89E-06
Mature leaves	C	4	nd	nd	nd
Mature leaves	D	4	nd	nd	nd
Mature leaves	E	4	nd	nd	nd
Mature leaves	F	4	6.12E-05	2.38E-05	1.19E-05
Mature leaves	G	4	nd	nd	nd
Mature leaves	H	4	nd	nd	nd
Mature leaves	I	4	nd	nd	nd

Mature leaves	J	4	nd	nd	nd

Table S2. Average estimated concentrations (proportion dry weight) in different tissue types of *Piper sancti-felicis.*

Tissue	**N**	**Mean**	**SD**	**SE**
Unripe pulp (2)	21	3.923867e-02	2.339570e-02	5.105360e-03
Ripe pulp (1)	20	2.707377e-02	1.878158e-02	4.199689e-03
Flowers (4)	21	1.533097e-02	1.311570e-02	2.862080e-03
Seeds	6	1.085352e-03	2.457638e-03	1.003327e-03
Mature leaves	4	7.335458e-05	2.890953e-05	1.445477e-05

Table S3. Average estimated concentrations (proportion dry weight) in different tissue developmental stages of *Piper sancti-felicis.*

Stage	**N**	**Mean**	**SD**	**SE**
Ripe pulp (1)	20	0.027073768	0.018781580	0.004199689
Late unripe pulp (2)	21	0.039238673	0.023395701	0.005105360
Early unripe pulp (3)	19	0.030825459	0.019718249	0.004523677
Late flower (4)	21	0.015330966	0.013115700	0.002862080
Early flower (5)	15	0.009078647	0.008876482	0.002291898
Developing flower (6)	11	0.005129660	0.004979878	0.001501490

Table S4. Fungal taxa identification of the full ITS region (ITS1, 5.8S, ITS2), using the UNITE database (v8.2). Lab ID refers to the ID the taxa are given in our data. Our two *Fusarium* taxa matched in both query cover and % identity to other accessions in UNITE that had not been classified to species. UNITE generates estimates of species hypotheses (SH) based on the ITS2 region and SH codes below the species name are digital object identifiers. *Gibberella* is the sexual stage, or teleomorph, of *Fusarium*.

Taxa	**Query cover (%)**	**% Identity**	**Closest UNITE SH**	**GenBank Accession**
Fusarium A	100	100	*Fusarium proliferatum* | SH2456081.08FU	MT093652
Fusarium B	100	100	*Gibberella circinate* | SH2456044.08FU and *Nectriaceae* | SH2456050.08FU	MT093653
Microdochium lycopodinum	100	100	*Microdochium lycopodinum* | SH2261955.08FU	MT093654

Table S5. Temporal and ripeness differences of the removal of infructescences of *Piper sancti-felicis.*

	Diurnal	Nocturnal
Ripe	1	59
Unripe	5	8

Table S6. Overall effect of alkenylphenols on bat (*Carollia perspicillata*) and bird (*Ramphocelus passerinii*) feeding responses measured by the average amount of diet eaten. Treatment diets contained approximately 14.6 mg infructescence-extracted alkenylphenols in 3 g of diet, a concentration that mimicked the average concentration found in a ripe infructescence of *P. sancti-felicis* (0.0049 proportion wet weight).

Animal	**Diet**	**N**	**Mean**	**SD**	**SE**
Bat	Control	16	1.4278438	0.6546030	0.1636508
Bat	Treatment	16	0.5859531	0.6905014	0.1726254
Bird	Control	10	1.347717	1.0596576	0.3350932
Bird	Treatment	10	1.233967	0.8624022	0.2727155

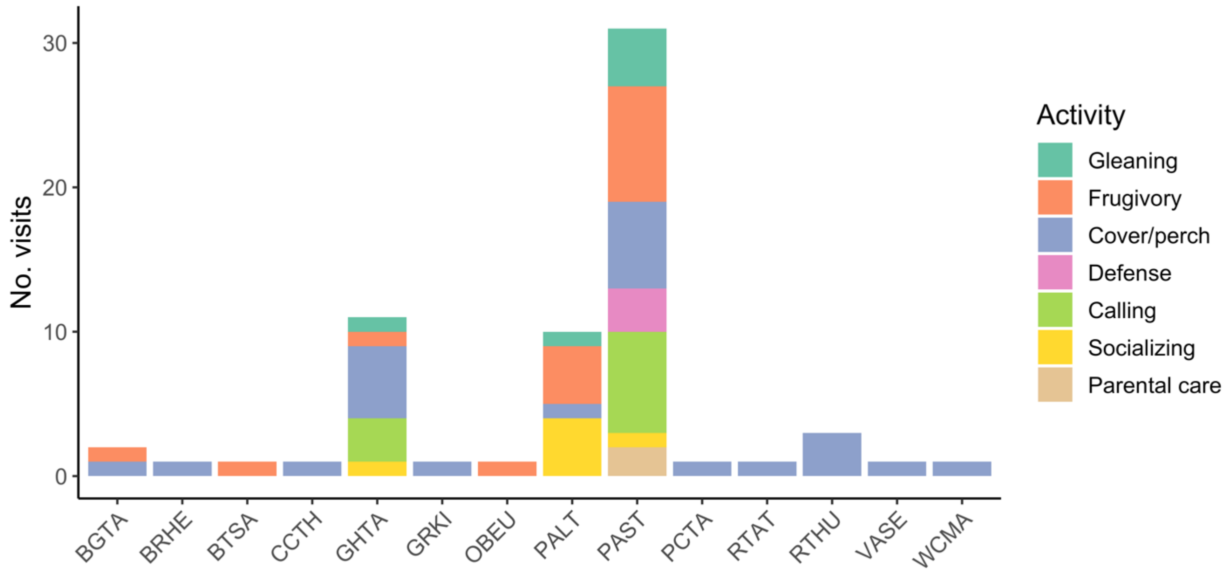


Figure S2. Summary of bird species and their activities on *Piper sancti-felicis* plants 5–10 July 2018 at La Selva Biological Station, Costa Rica. BGTA=Blue-grey tanager (*Thraupis episcopus*), BRHE=Bronzy hermit (*Glaucis aeneus*), BTSA=Buff-throated saltator (*Saltator maximus*), CCTH=Clay-colored thrush (*Turdus grayi*), GHTA=Golden-hooded tanager (*Tangara larvata*), GRKI=Great kiskadee (*Pitangus sulphuratus*), OBEU=Olive-backed euphonia (*Euphonia gouldi*), PALT=Palm tanager (*Thraupis palmarum*), PAST=Passerini tanager (*Ramphocelus passerinii*), PCTA=Plain-colored tanager (*Tangara inornata*), RTAT=Red-throated ant tanager (*Habia fuscicauda*), RTHU=Rufous-tailed hummingbird (*Amazilia tzacatl*), VASE=Variable seedeater (*Sporophila corvina*), WCMA=White-collared manakin (*Manacus candei*)

Appendix S5: Supplemental information on model selection results

Table S1. AIC model comparison for alkenylphenol concentration across fruit ripening.

Model	K	AICc	ΔAICc	ω	LL
Nonlinear	4	-646.78	0.00	1	327.58
Linear	3	-634.52	12.25	0	320.38
Null	2	-591.89	54.88	0	298.00

Table S2. AIC model comparison for average absorbance (fungal growth) across alkenylphenol concentrations and fungal species.

Model	K	AICc	ΔAICc	ω	LL
Species x concentration	7	-58.79	0.00	1	39.34
Species + concentration	5	-47.38	11.41	0	30.12
Species	4	-26.35	32.44	0	18.08
Concentration	3	-16.48	42.31	0	11.76
Null	2	-10.54	48.26	0	7.52

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