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Harmala Alkaloids as Bee Signaling Chemicals

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Harmala alkaloids are pharmaceutically active molecules that can be found in various plants. [1, 7] These alkaloids are fluorescent molecules in the range of 300-700nm.[7] Coincidently, bees have a similar visible range of 300-600nm. [4, 5, 6] This study takes these observations and interweaves them into a hypothesis: since bees use their sight to find flowers to pollinate,[5] then these flowers contain harmala alkaloids that would be visible to bees. It can then be inferred harmala alkaloids attract bees. In other words, harmala alkaloids are functional components of plants. In order to determine harmala alkaloids content, standard solutions of harmine, harmaline, harmane, harmol, and harmalol will be compared with extractions from plant samples using high performance liquid chromatography and fluorescence.

A variety of plants were chosen to represent three categories. The first is plants that are found to be insect pollinated, these include lemon balm *(Melissa officinali*), common rue *(Ruta graveolens*), meadow rue (*Thalictrum aquilegifolium*), hydrangea *(Hydrangea arborescens)*, spirea (*Spirea japonica*), forget-me-not (*Myosotis scorpioides*), blue star grass *(Sisyrinchium augustifolium)*,[6] common rue *(Ruta graveolens*) and meadow rue (*Thalictrum aquilegifolium*). The second category represents wind pollinated plants, including sugar maple (*Acer saccharum)*, white velvet *(Tradescantia sillamontana)*, meadow rue *(Thalictrum ichangense)*, rhoeo *(Rhoeo spathacea)*.[16, 17] Finally, a control was also analyzed. The lady fern *(Athyrium felix-femina*) was chosen because it is not genetically related to the plants in categories one or two and is not insect or wind pollinated.

Following chemical analysis, each of the insect pollinated plants was found to contain harmala alkaloids. The lady fern *(Athyrium felix-femina)* contained no harmala alkaloids, as well as the wind pollinated plants. Due to these results as well as a study of bee behavior, we were able to conclude that harmala alkaloids are present in plants that attract bees. This study both contributes to an understanding of factors involved in pollination and can be used as a guide for further investigation into a natural source of harmala alkaloids.

Keywords: Harmala alkaloids, Bee signaling chemicals

Introduction

Harmala alkaloids are found in a number of plants throughout the world.[1, 7] The most abundant source of these harmala alkaloids is found in the seeds of Syrian rue (*Penganum harmala*).The most abundant harmala alkaloids found in this particular seed are harmaline and harmine. [1, 7] The potential clinical uses of the harmala alkaloids found in these seeds range from a monoamine oxidase inhibitors to cures for Parkinson's disease.[9] Harmala alkaloids can induce tremors in order to study Parkinson's disease; in fact, people diagnosed with Essential Tremor, a mild form of Parkinson's, have harmala alkaloids present in their blood naturally.[9] Essential tremor is a disorder of the nervous system, in which small shaking movements happen during everyday tasks. [19] Using this property of harmala alkaloids in blood, they can be injected into laboratory animals to induce tremors for study on their properties and possible cures.

When an alkaloid is present in a plant, the plant will taste bitter to insects; therefore, the alkaloids will sometimes repel insect pests.[12] However, observation of plants known to contain them, such as passion flowers, demonstrates that not all insects are deterred from the plant.[3] Moths, butterflies, flies, bees can be found on plants that contain harmala alkaloids; in fact, bees and hummingbirds pollinate the passionflower, which is the most concentrated with harmala alkaloids.[20] This leads us to wonder if there is another purpose for the harmala alkaloids that are found in plants, other than deterring pest insects.

Specifically, bees seem be attracted to plants that contain harmala alkaloids. Bees use their sight and smell to detect flowers, although only in ultra violet and visible light spectrum, in which they can detect around 300nm to 600nm.[4, 5, 6] It has been determined that the harmala alkaloids fluoresce in the same range of the spectrum that the bees can see.[3, 7] Harmala alkaloids do not volatize at ambient temperatures, which means that the bees must be attracted only by sight to the alkaloids. Due to this and the correlation between the visible range of bees and the fluorescence of harmala alkaloids, we expected that the bees would be attracted to the plants that require insect pollination and that many of these will contain harmala alkaloids.

From casual observation of these plants, it has been observed that bees are attracted to the lemon balm (*Melissa officinali)*, common rue *(Ruta graveolens)*, hydrangea *(Hydrangea arborescens)*, spirea (*Spirea japonica*), forget-me-not (*Myosotis scorpioides*) and blue star grass *(Sisyrinchium augustifolium)*[6]; whereas, bees are not attracted to the sugar maple *(Acer saccharum)*, white velvet *(Tradescantia sillamontana)*, meadow rue

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(Thalictrum ichangense), rhoeo *(Rhoeo spathacea)* and lady fern *(Athyrium felix-femina)*. [16, 17] Therefore, it was hypothesized plants that do not attract bees will not contain harmala alkaloids or emit light in bees visible range of 300-600nm.

The plants were dived into three categories to promote a varied data collection. The first was plants that are found to be insect pollinated, these included lemon balm *(Melissa officinali*), common rue *(Ruta graveolens*), meadow rue (*Thalictrum aquilegifolium*), hydrangea *(Hydrangea arborescens)*, spirea (*Spirea japonica*), forget-me-not (*Myosotis scorpioides*), blue star grass *(Sisyrinchium augustifolium)*,[6] common rue *(Ruta graveolens*) and meadow rue (*Thalictrum aquilegifolium*). The second category represents wind pollinated plants, including sugar maple (*Acer saccharum)*, white velvet *(Tradescantia sillamontana)*, meadow rue *(Thalictrum ichangense)*, rhoeo *(Rhoeo spathacea)*.[16, 17] The two meadow rue plants are from the same genus; however, the meadow rue (*Thalictrum aquilegifolium*) is insect pollinated and the meadow rue *(Thalictrum ichangense)* contains spores that make it wind pollinated. The role of these two taxonomically related plants is to determine if harmala alkaloid content is based on genetics. They were also included in the study because Syrian rue (*Penganum harmala*) is a rich source of harmala alkaloids. Finally the third category, a control, was also analyzed. The lady fern *(Athyrium felix-femina*) was chosen because it is not genetically related to the plants in categories one or two and is not insect or wind pollinated.

In order to begin the procedure, the harmala alkaloids were extracted from the plant material. This was done by the low environmental impact method described in previous research by Dr. Haustein and her students.[3] The extraction process took us from plant material to a useable solid containing harmala alkaloids. In order to determine which specific harmala alkaloids were in the sample, the HPLC (high performance liquid chromatography) was conducted using standard procedures established by previous studies at Central College.[3] The molecules traveled through a chromatography column, where each molecule exited the column according to its non polar interactions with the stationary phase C_{18} inside the column (the time it takes the molecule to travel the length of the column is called the retention time). By comparing the retention times of the standards of harmala alkaloids, the content of an each plant species was observed. This determined if there were harmala alkaloids (or related alkaloids) in the plant. From here, fluorescence scan of the plant's alkaloid content determined if these harmala alkaloids are visible to the bees.

A quantitative analysis of harmala alkaloid content for each of the species in the plant was helpful. A quantitative analysis on the meadow rue *(Thalictrum aquilegifolium)* plant, that is known to contain harmala alkaloids, was conducted. This was done by performing several fluorescence scans of standards of harmala alkaloids found in meadow rue (*Thalictrum aquilegifolium*) at various concentrations. These were plotted together to form a calibration curve. A fluorescence scan of the meadow rue (*Thalictrum aquilegifolium*) gave the intensity of the signal, which was plotted as a calibration curve to yield the concentration of each of the harmala alkaloids in the plant material.

Methods

Each of the plant samples were grown organically, without the use of compound altering chemicals. The meadow rue (*Thalictrum aquilegifolium*), lady fern (*Athyrium felix-femina*), hydrangea (*Hydrangea arborescens*), sugar maple (*Acer saccharum*), lemon balm (*Melissa officinali*), blue star grass (*Sisyrinchium augustifolium*) and forget-me-not (*Myosotis scorpioides*) were from the garden of Dr. Haustein. The meadow rue *(Thalictrum ichangense)*, white velvet (*Tradescantia sillamontana*), and rhoeo (*Rhoeo spathacea*) were from Dr. Mary Stark's greenhouse. The spirea (*Spirea japonica*) was from Natalie Harrington's garden, and finally the common rue (*Ruta graveolens)* came from Mountain Rose Herbs.

The standards for the quantitative and qualitative analysis were obtained from Sigma-Aldrich. A HP 1110 High Performance Liquid Chromatography using a 250mm by 4.6mm Phenomenex C-18 Luna column and a 20 µL injection loop was used in accordance with an ultraviolet-visible detection at a wavelength of 340nm. The column had constant temperature of 30 °C. Flow rate was 1 mL/min. Each analyte's retention time varied depending on the strength of its interactions with the stationary phase, the ratio of solvents used, and the flow rate of the mobile phase. For this experiment, the mobile phase was set to a gradient of DI water and methanol to optimize the retention times. This gradient was a 1:3 mixture of DI water and pure methanol respectively for 2 minutes; followed by methanol for the remaining 8 minutes. To confirm the qualitative analysis and provide quantitative analysis, the Cary Eclipse Fluorescence Spectrometer was used. This was conducted at varying excitation and emission wavelengths for each harmala alkaloid.

The extraction process can be viewed as a flow chart in *Appendix I* . For each sample, the extraction process began by grinding the plant material in a coffee grinder until finely grated. The plant material was massed; then, transferred into a 100 mL beaker. To the beaker, 5 times the mass of the ground plant in mL of a 30% acetic acid was added. For example, if the mass was 2.42g, the amount added was approximately 23 mL. This was stirred for 5 minutes on a stir plate. After the 5 minutes, the solution was vacuum filtered with a Buchner funnel and the plant material was discarded. At this point, the desired harmala alkaloids have now been acidified. This causes the chemical species to be protonated into a positively charged salt. A salt can be dissolved into an aqueous layer which is important for the next step of separation. To the species, a solution of 50 mL of hexanes and 50 mL of ethyl acetate were added to wash the species through separation a total of 3 times. The solutions were mixed in the separatory funnel by way of inversion. The organic layer rose to the top and separate from the aqueous layer. The harmalas

were present in the aqueous layer, so it was separated from the bottom layer into a separate beaker from the organic layer.

The next part of purification was to alter the alkaloids in the aqueous layer to make it dissolve in the organic layer to remove any other impurities. This was done by basification. In order to make the solution neutral again, saturated sodium bicarbonate was added drop wise until the solution showed basic (green, pH≈8) on pH paper. The neutral harmala alkaloids were then dissolved in the organic layer. Another separation was conducted using 100mL of ethyl acetate as an extracting solution. The organic layer was collected and dried using sodium sulfate, until it stops to clump, to remove any extra water. The harmala alkaloids were in the organic layer with all impurities removed. Next, clumps of sodium sulfate in the solution were removed by filtering it into a pre-weighed round bottom flask with a glass funnel with a cotton ball. This was washed with the hexanes to ensure that all of the harmalas make it into the round bottom flask. In order to get the harmalas from the solvent, the solution was placed on a rotary evaporation aspirator. Once the solvent was removed, the flask contained only a small amount of solid. In order to use this material for testing, it was dissolved in 10mL of methanol.

The standards were obtained from Sigma-Aldrich. Solutions were made by diluting each alkaloid (harmine, harmaline, harmane, harmol, and harmalol) with methanol. The solutions for the HPLC were created by dissolving .0125 g of each harmala alkaloid with methanol in a 100mL volumetric flask. This created a total of 5 different solutions that are approximately equal to the concentration previously found in a plant sample. The other concentrations for the fluorescence analysis were using this solution and making further dilutions.

The extraction process took us from plant material to analyzable solid containing harmala alkaloids. In order to determine which specific harmala alkaloids were in the sample, the HPLC was used. By comparing the retention times of the standards of harmala alkaloids, the content of an unknown can be observed. Once the content of the plant sample was predicted, each plant extract was tested with fluorescence to confirm its harmala alkaloid content. In order to do this, fluorescence emission intensities were observed for each extract. Once each sample has been qualitatively analyzed for its harmala alkaloid content, it is important to know how much of the alkaloid is present in a given sample. This quantitative analysis was done for the meadow rue (*Thalictrum aquilegifolium)*. The emission intensity was related to concentrations via a calibration curve. In order to do the analysis, a slit width of 2.5nm and an excitation wavelength were used. The emission wavelengths that were used were 374 nm for harmaline and 420 for harmine. Once the coordinates were plotted, a calibration line was made. By substituting the intensities of the unknown meadow rue sample with the same constraints into the equation, the concentration of harmala alkaloid in meadow rue (Thalictrum aquilegifolium) was determined.

Results and Discussion

For the HPLC analysis, each standard was injected onto the chromatography column. Depending on the polarity of the molecule, it took a specific time for the molecule to travel the column. This time is called the retention time. A sample chromatogram for harmane is shown below in *Figure 1*.

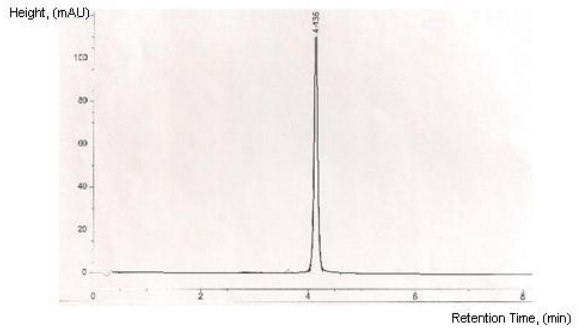

Figure 1: Harmane Sample HPLC Chromatogram

Each of the standard harmala alkaloids has a similar chromatogram; the retention times from these standards are shown in *Table 1*.

Table 1: Harmala Alkaloid Standard Retention Times

Standard	T_R (min)	Standard Deviation (min)
Harmalol	3.2297	.021
Harmane	4.135	.042
Harmine	3.929, 4.48	.033, .026
Harmaline	3.465	.050
Harmol	3.493	.029

Where N=6 for all standards

In order to qualitatively determine the molecules present in our plant samples, we must compare the retention times of our plant chromatograms to these standards shown in *Figure 1*. This is done in the following manner for the meadow rue (*Thalictrum aquilegifolium*) plant. The following is *Figure 2*, showing the HPLC chromatogram and the retention times for the peaks of the unknown meadow rue (*Thalictrum aquilegifolium*) sample.

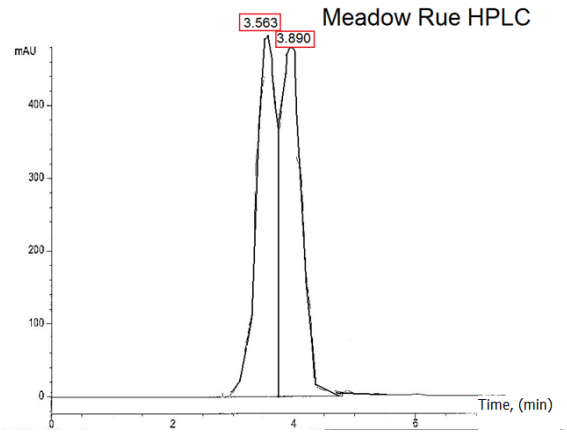

Figure 2: HPLC of Meadow Rue (Thalictrum aquilegifolium)

From the chromatogram, it can be determined that the two peaks result from two species with a small difference in polarity. In order to determine exactly what these peaks come from, they are compared to a standard solution. The image in *Figure 3* shows how the HPLC from meadow rue (*Thalictrum aquilegifolium*) matches with the harmine standard. The HPLC for the meadow rue (*Thalictrum aquilegifolium*) (blue) is pasted over the HPLC for the standard in order to see the correlation better.

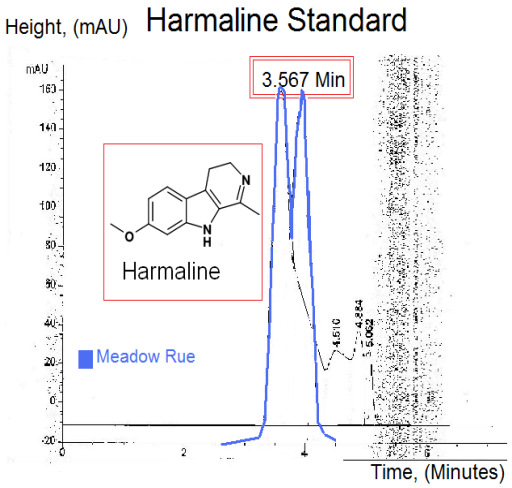

Figure 3: Harmaline compared to Meadow rue (Thalictrum aquilegifolium)

This shows us that the first peak of the meadow rue (*Thalictrum aquilegifolium*) must be from the harmaline present in the molecule. This method gives good qualitative results that are reproducible. It allows for relatively rapid results which makes it suitable for undergraduate research. The standard deviation of the retention time values range from .015 min to .162 minutes. This is reproducible enough for the time allotted for this undergraduate research; therefore, further method development would improve the quantization. The same process was used to determine the second peak in *Figure 4.*

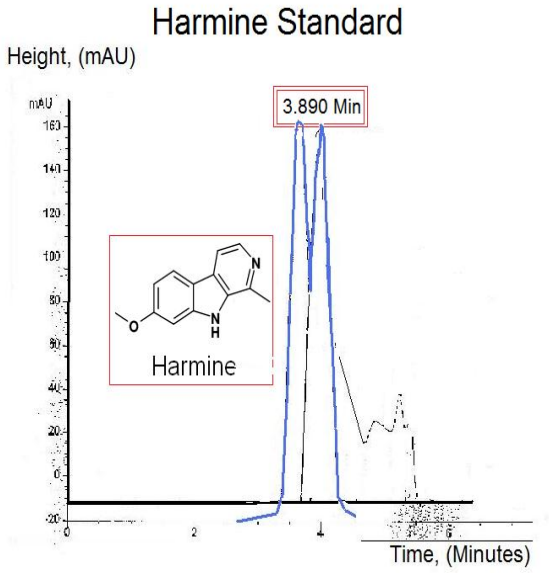

Figure 4: Harmine compared to Meadow rue (Thalictrum aquilegifolium)

Figure 4 shows that the second peak on the HPLC chromatogram comes from harmine. This is the quantitative analysis that was conducted to determine that harmaline and harmine were present in our species. It also makes sense that they appear in the order that they do based on polarity. The molecules are similar and only differ in conjugation. So we should expect them to have about the same retention times. The order results from the reverse phase chromatography that was done here meaning that with the non polar mobile phase and semi polar mobile phase, the more polar molecules elute more rapidly. In this case we would expect the harmaline to come out first since it is a little less conjugated, therefore a little more polar than harmine. This is exactly what was observed.

The same process is done for each of the samples that were analyzed. The retention times corresponding to each plant sample are recorded below in *Table 2.* These times are an average of 2-3 trials HPLC chromatograms from different extractions from the same plant.

Table 2: HPLC Retention Times of Plant Samples (For each plant, N=3)

	Plant Sample	T_R (min)		StDev (min)	
1	Lemon balm (*Melissa officinali)*	3.131	3.938	.020	.033
	Hydrangea (*Hydrangea arborescens*)	3.233	3.476	.015	.067
	Spirea (*Spirea japonica*)	3.074	3.311	.102	.082
	Blue star grass (*Sisyrinchium augustifolium*)	3.132	3.230	.125	.056
	Forget-me-not (*Myosotis scorpioides*)	3.291	3.752	.232	.325
	Common Rue (*Ruta graveolens*)	3.474		.085	
	Meadow Rue (*Thalictrum aquilegifolium)*	3.563	3.890	.105	.126
2	Meadow Rue (*Thalictrum ichangense)*	3.121	3.604	.153	. 129
	Sugar maple (*Acer saccharum*)	3.089	3.227	.086	.111
	White velvet (*Tradescantia sillamontana*)	3.260	3.668	.096	.099
	Rhoeo (*Rhoeo spathacea)*	3.115	3.331	.102	.103
3	Lady fern (*Athyrium felix-femina*)	4.105		.162	

Key: 1-Insect Pollinated, 2-Wind pollinated, 3-Control

Now that we have all of the sample retention times, these times can be matched to the standards with very similar retention times from *Table 1*. They may all seem to match the standards, but they are confirmed using fluorescence. Each of the standards will emit light at a specific wavelength. This is found using the fluorescence emission spectra. The average emission wavelengths are shown below in *Table 3*, based on the excitation wavelength of 340nm.

Table 3: Standard Emission Wavelengths of Harmala Alkaloids (Where N=6, for each standard)

Standard	$\lambda_{emission}$ (nm)	StDev (nm)
Harmine	375	1.04
Harmaline	490	1.22
Harmalol	475	1.32
Harmane	380	1.41
Harmol	410	.99

The emission of each plant sample was recorded and compared to the standards above. In some cases, the plant was found to have no fluorescent molecules in it; if this is the case, then none is recorded in the table. The results can be seen in *Table 4.*

Table 4: Emission Wavelengths of Plant Samples (Where N=3 for each plant sample)

	Plant Sample	λ_{em} (nm)	StDev (nm)	Insect Pollinated
1	Lemon balm (*Melissa officinali)*	381.84	2.22	Yes
	Hydrangea (*Hydrangea arborescens*)	426.96	2.12	Yes
	Spirea (*Spirea japonica*)	410.00	1.41	Yes
	Blue star grass (*Sisyrinchium augustifolium*)	670.00	.500	Yes
	Forget-me-not *(Myosotis scorpioides)*	418.93	1.98	Yes
	Common Rue (*Ruta graveolens*)	458.93	1.12	Yes
	Meadow Rue (*Thalictrum aquilegifolium)*	365, 483	1.00, .98	Yes
2	Meadow Rue (*Thalictrum ichangense)*	None	None	No
	Sugar maple (*Acer saccharum*)	None	None	No
	White velvet (*Tradescantia sillamontana*)	None	None	No
	Rhoeo (*Rhoeo spathacea)*	None	None	No
3	Lady fern (*Athyrium felix-femina*)	None	None	No

Key: 1-Insect Pollinated, 2-Wind pollinated, 3-Control

The plant samples that are shown to fluoresce were expected to have harmala alkaloids in them according to the hypothesis that these plants attract bees. This means that the plants in the first category that are insect pollinated all contained harmala alkaloids. In the opposite manner, the wind pollinated did not fluoresce. Similarly, the lady fern contained no fluorescent molecules. Each of the plants that contained fluorescent components was compared with the standards in the areas of retention time and emission wavelength in order to make a qualitative analysis. By comparing the retention time closest to the standard and the fluorescence, the standard that is present in the sample can be noted. This comparison and determination is shown in *Table 5.*

Table 5: Qualitative Analysis of Plants Containing Harmala Alkaloids

#	Plant Sample	T_R (min)	λ_{em} (nm)	Standard	Std T_R (min)	Std λ_{em} (nm)
1	Lemon balm (*Melissa officinali)*	3.938	381.84	**Harmine**	3.929	375
	Hydrangea (*Hydrangea arborescens*)	3.476	426.96	**Harmol**	3.493	410
	Spirea (*Spirea japonica*)	3.311	410	**Harmol**	3.493	410
	Blue star grass (*Sisyrinchium augustifolium*)	3.23	670	**Outside range**		
	Forget-me-not (*Myosotis scorpioides*)	3.291	418.93	**Harmol**	3.493	410
	Common Rue (*Ruta graveolens*)	3.474	458.93	**Harmaline**	3.465	490
	Meadow Rue (*Thalictrum*	3.89	365	**Harmine**	3.929	375
	aquilegifolium)	3.563	483	**Harmaline**	3.465	490
2	Meadow Rue (*Thalictrum ichangense)*	3.604	None	**None**	None	None
	Sugar maple (*Acer saccharum*)	3.227	None	**None**	None	None
	White velvet (*Tradescantia sillamontana*)	3.668	None	**None**	None	None
	Rhoeo (*Rhoeo spathacea)*	3.331	None	**None**	None	None
3	Lady fern (*Athyrium felix-femina*)	4.105	None	**None**	None	None

Key: 1-Insect Pollinated, 2-Wind pollinated, 3-Control

As previously discussed, columbine meadow rue (*Thalictrum aquilegifolium)* contains both harmaline and harmine. As shown in *Table 5*, harmol is present in the hydrangea (*Hydrangea arborescens*), spirea (*Spirea japonica*), and forget-me-not (*Myosotis scorpioides*). Lemon balm (*Melissa officinali*) contains harmine and the common rue (*Ruta graveolens*) has harmaline. The fluorescence that was observed for the blue star grass (*Sisyrinchium augustifolium*) was outside of the range that harmala alkaloids are present. This simply means that something else is contained in the plant species that is fluorescent. When something emits a wavelength that is in the visible range, a color with corresponding intensity is displayed. In the case of the blue star grass (*Sisyrinchium augustifolium*), its emission does not lie where bees can see, but it is bright blue to the eye. These emissions in this study lie in the visible light spectrum, so each of these emissions corresponds with a color as shown in *Table 6*.

Table 6: Visible Light Spectrum

Color	Wavelength (nm)
Red	635-700
Orange	590-635
Yellow	560-590
Green	490-560
Blue	450-490
Violet	380-450

Table 7: Corresponding Color Based on Emission Wavelength

Plant	Standard	λ_{em} (nm)	Color of Emission
Lemon balm (*Melissa officinali)*	Harmine	381.84	Violet
Hydrangea (*Hydrangea arborescens)*	Harmol	426.96	Violet
Spirea (*Spirea japonica)*	Harmol	410	Violet
Common Rue (*Ruta graveolens*)	Harmaline	458.93	Blue
Blue star grass (*Sisyrinchium augustifolium)*	None	670	Blue
Forget-me-not (*Myosotis scorpioides)*	Harmol	418.93	Violet
Meadow Rue (*Thalictrum aquilegifolium)*	Harmaline	483	Blue

In order to understand how close these observations are to determining the harmala alkaloid present in the plant, the percent errors have been calculated below in *Table 8.* This is the percentage that the actual value that was achieved in the lab deviates from the theoretical value of the standard.

Table 8: Percent Error Calculation

%error=((Actual-Theoretical)/Theoretical)*100%		
	Percent Error	**Percent Error**
Plant Sample	**T_R (%)**	**λ_{em} (%)**
Lemon balm (*Melissa officinali*)	0.23%	6.07%
Hydrangea (*Hydrangea arborescens*)	0.49%	4.14%
Spirea (*Spirea japonica*)	5.21%	0
Common rue (*Ruta graveolens*)	0.26%	6.34%
Blue star grass (*Sisyrinchium augustifolium*)	N/A	N/A
Forget-me-not (*Myosotis scorpioides*)	5.78%	2.18%
Meadow Rue (*Thalictrum aquilegifolium)*	2.83%	1.43%
	.99%	1.39%

The calculations above show that all results are within 6.5% or less of the accepted value. In the case of the spirea (*Spirea japonica*), the emission wavelengths observed from the plant sample were the exact same as the standard. This shows a 0% error. This means that if spirea (*Spirea japonica*) contains a harmala alkaloid, it is 100% certain, that it is harmol.

In each of the plants, there may also be other unknown chemical components that have a similar polarity to harmala alkaloids. This is why the HPLC of some species show peaks on the HPLC, but the plants did not contain harmala alkaloids. This is why the fluorescence is done to confirm that the resultant peaks on the HPLC are actually harmala alkaloids that are present in the plant sample. In fact, there are over 110 chemicals in the meadow rue (*Thalictrum aquilegifolium)* alone. [11]

In order to derive an idea if pollinating insects are attracted to harmala alkaloids, other external factors, such as competing fragrances and other unknown alkaloids, should be eliminated. In a brief study of bee behavior, two test tubes containing apricot nectar (to attract bees by sense of smell) were placed in a test tube rack. These tubes were fitted with two circles of paper the same size on their necks. These were the exact same size and pattern to eliminate other external factors that would cause the bees to favor one over the other. On one circle, methanol was painted in an asterisk pattern. The other test tube, containing the second circle, was painted in the same pattern; however, this time the methanol had harmaline and harmine dissolved in it.

The one painted with only methanol was the control, whereas the one with harmala alkaloids was to attract pollinating insects. Two parties that did not know which test tube contained harmala alkaloids were asked to observe what happened, as to avoid prejudices. A total of 6 bees came to the area of this trial within two hours. Of the 6, 5 landed on the one with the harmala alkaloids. The other bee seemed only to circle around the one with the alkaloids, but did not land on either test tube. In this two hour time frame, no bee landed on the one with only methanol. This study was conducted in the sun on a day where the temperature was approximately 20°C. This demonstrates, in a control case, with all outside factors eliminated, bees seem to prefer harmala alkaloids.

Another study under similar conditions repeated. A set of two test tubes in a rack were done with the same size of paper and asterisk pattern painted on them. Instead of using apricot nectar, this trial used sugar water. The sugar water will not draw the insects based on sight, and has no fluorescence to eliminate the chance that this would affect their vision. Like the first trial, tube 1's collar was marked with only methanol as the control. The second was painted with harmala alkaloids dissolved in methanol. The study was conducted on a warm, end of summer day; therefore, bees were easily found in a nearby garden.

In twenty minutes of observation, the observer counted 11 bees to land on the paper containing harmala alkaloids. Three landed on the one with only methanol. The methanol was just a control to ensure that the same pattern was painted on the paper, as well as to know that only the

harmala alkaloids were the factor differing from one tube to the next. Since bees still seemed to land on the methanol, it shows that methanol is not a deterrent. When the positions of the tubes were exchanged, the bees seemed to visit each one about 50% of the time. Since in this case, there was no preference, this study must be revisited. For the purpose of this preliminary experiment, it seems as though there is a connection between harmala alkaloids and pollinating insects. This does not necessarily prove that bees are attracted to harmala alkaloids; it shows that this is a pattern that should be further explored.

Now qualitatively, the contents of the plant have been determined within a reasonable amount of error. Next, the columbine meadow rue (*Thalictrum aquilegifolium*), was analyzed quantitatively. This way the exact amount of harmine and harmaline can be determined for the common rue. Meadow rue was chosen because it was readily available and contained two harmala alkaloids with small deviations. This quantitative analysis is done by observing the intensity for a specific concentration of a standard. An example of the spectrum used in the fluorescence analysis is shown below in *Figure 5*.

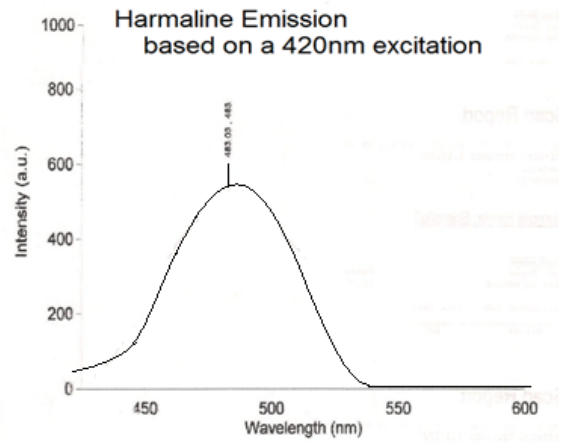

Figure 5: Sample Fluorescence Spectrum

From this spectrum, it can be determined that for this concentration of 125ppm, an intensity of 483 is observed. This means that in the calibration curve, a data point of (125ppm concentration, 483 a.u. intensity) can be added. The data for the calibration curve for both harmine and harmaline is shown below in *Table 9*.

Table 9: Building the Calibration Curve

Emission Spectra Calibration Curve Conc vs Intensity of Standards					
HARMINE			HARMALINE		
Standard	Conc., (ppm)	Intensity, (a,u)	Standard	Conc., (ppm)	Intensity, (a,u)
1	1.27	30.74	1	1.25	92
2	5.08	141	2	2.5	129
3	6.35	270	3	3.75	256
4	7.62	305.2	4	5	342
5	12.7	589	5	6.25	409.5
			6	12.5	721

This data shows how the calibration curves below in *Figure 6* for harmaline and *Figure 7* for harmine were modeled.

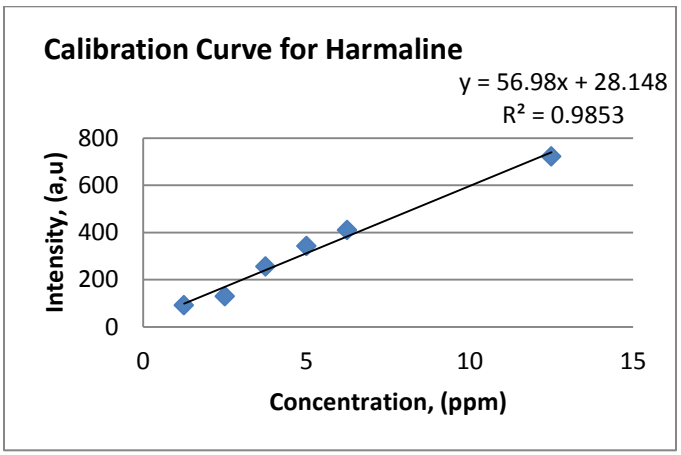

Figure 6: Calibration Curve for Harmaline

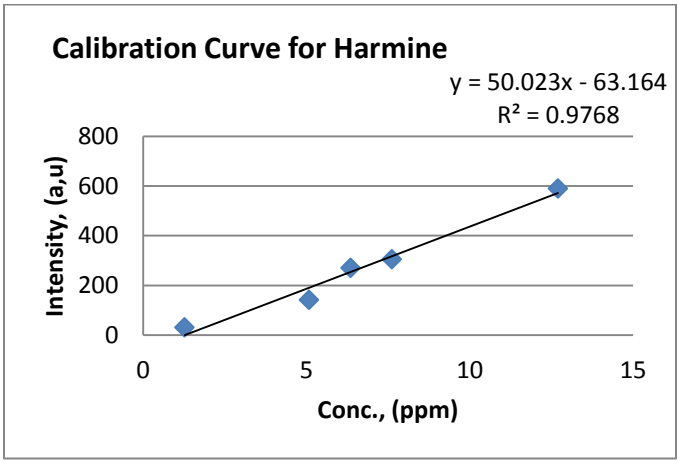

Figure 7: Calibration Curve for Harmine

These calibration curves have fairly high correlation for raw data. This means that the data is stable enough to use the line equations to determine a concentration of the harmine and harmaline in the sample. This calculation is done in *Table 10*.

Table 10: Concentration Calculation

Harmaline	***Harmine***
Intensity=56.98 (Conc) +28.148 Data for Meadow rue Intensity, (a,u) = 654 a.u. 654=57.0 (Conc) +28.1 Concentration= 11.0 ppm	Intensity=50.023 (Conc)-63.164 Data for Meadow rue Intensity, (a,u) = 483 a.u. 483=50.0 (Conc)-63.2 Concentration= 10.9 ppm

From these calculations, the meadow rue (*Thalictrum aquilegifolium*), contained a concentration of 11ppm harmaline and 11 ppm harmine. This was possible through the calibration curve. These numbers have an error of .1ppm associated with them due to this calculation.

Another extraction of meadow rue (*Thalictrum aquilegifolium*) was done to confirm this data; however, the sample size was only .05g so it was hard to get the sample to achieve the same results. The harmine was found in the second sample in similar concentrations of 8ppm; however, the second trial did not contain harmaline in any concentration. All of the other plant samples containing harmala alkaloids were highly reproducible. In fact, for each of the plants, the average of 3 plant samples is what is shown in the data tables.

Conclusions

Harmala alkaloid standards harmine, harmaline, harmane, harmol, and harmalol were compared with the plant sample using high performance liquid chromatography and fluorescence. The following plants contained harmala alkaloids: lemon balm (*Melissa officinali*), hydrangea (*Hydrangea arborescens*), spirea (*Spirea japonica*), common rue (*Ruta graveolens*), forget-me-not (*Myosotis scorpioides*), and meadow rue (*Thalictrum aquilegifolium).* Of these, hydrangea (*Hydrangea arborescens*), spirea (*Spirea japonica*), and forget-me-not (*Myosotis scorpioides*) contained harmol. Lemon balm (*Melissa officinali*) contains harmine and the common rue (*Ruta graveolens*) has harmaline.

The meadow rue (*Thalictrum aquilegifolium)*contained both harmaline and harmine. Calibration curves were constructed to find the concentration of the each of the harmala alkaloids in this plant species. This showed that about 11ppm of harmaline and 11ppm of harmine are present in a sample of meadow rue (*Thalictrum aquilegifolium).* This is helpful to know if there was ever an increased need for harmala alkaloids in pharmaceuticals or other areas.

The second category containing the wind pollinated sugar maple (*Acer saccharum),* white velvet *(Tradescantia sillamontana),* meadow rue *(Thalictrum ichangense),* and rhoeo *(Rhoeo spathacea)* did not contain harmala alkaloids. The genetic relationship between the wind pollinated meadow rue and the columbine insect pollinated rue was also analyzed. This study showed that genetics do not play a role in harmala alkaloid content. The pattern observed was strictly based on insect pollinated and wind pollinated. The insect pollinated contained harmala alkaloids and wind pollinated did not contain them.

Bees use sight in the range of 300-600nm and smell to find flowers. The plants in this study that are insect pollinated were found to fluoresce in the region of 380-480nm. This falls directly into the sight range of the bees. The insect pollinated plants lemon balm (*Melissa officinali*), hydrangea (*Hydrangea arborescens*), spirea (*Spirea japonica*), common rue (*Ruta graveolens*), forget-me-not (*Myosotis scorpioides*), and meadow rue (*Thalictrum aquilegifolium)* all contain harmala alkaloids in the visual region of the bees. Finally, a control that is not insect pollinated is the lady fern *(Athyrium felix-femina)* was analyzed. No harmala alkaloids were found in this plant.

Bees are not attracted to the lady fern, so it fits in with our hypothesis. In the bee observation experiment, it appeared that bees went to the test tube with the paper containing harmala alkaloids; they did not visit the control methanol as frequently. Given the evidence that we have proposed, it seems that bees are attracted to plants that contain harmala alkaloids. Further investigation should be conducted, but at this point there is no evidence to support the opposite. This study has implications in the understanding of pollination. It is also important in identifying which plants contain the pharmaceutically interesting harmala alkaloids.

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