



Investigation Of The Individual Apoptotic Effects Of Vitamin D3 And Various Species Of Verbena In AML Cells

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Evidence from previous studies has concluded that *Verbena officinalis* has many potential anticancer effects in hematological malignancies such as chronic lymphocytic leukemia and chronic myeloid leukemia. However, this essential oil has not yet been investigated in Acute Myeloid Leukemia (AML). In addition, many other species of *Verbena* such as Lemon Verbena (*Aloysia citriodora*), and Wild Verbena (*Lippia javanica*) have also been said to have many therapeutic properties in terms of cancer due to numerous bioactive compounds being present that could induce selective apoptosis in cancer cells. Vitamin D3 has also been investigated in the field of AML research in which the results have been associated with cancer metabolism. Furthermore, this study will look towards the properties of Vitamin D3 which will strengthen the effects of *Verbena* in AML treatment. This experiment will suggest whether *Verbena officinalis*, *Lippia javanica*, *Aloysia citriodora*, and/or Vitamin D3 exhibit individual therapeutic effects in AML cells.

INTRODUCTION

Acute Myeloid Leukemia (AML) is an extremely aggressive hematological malignancy in which patients only have a 27.4% five-year survival rate. [5] AML particularly affects the geriatric age category in which age is found to be a risk factor in the diagnosis of AML. Hence, this disease leads to substantial morbidity in the elderly generation and these rates are hypothesized to grow within the next few years.

Studies were recently done to analyze the effects of various essential oils in AML and many other hematological malignancies. One study had shown that *Verbena officinalis* and its citral component acted as an apoptotic-inducing agent in chronic lymphocytic leukemia [1] which is also rather the inspiration towards researching the effects of this essential oil in AML. Information in this study also states that *Verbena officinalis* essential oil has apoptotic properties in chronic myeloid leukemia (CLL) which is quite a closely comparable type of leukemia to AML. Furthermore, this research has led this study to compare the effects of various types of *Verbena* since *Lippia javanica*, and *Aloysia citriodora* have similar phytochemicals and bioactive compounds which may be able to stimulate similar apoptotic responses in AML cells.

The purpose of this study is to evaluate the individual anti-cancer effect(s) of each species of *Verbena* on in vitro AML cells to determine whether *Verbena* is a viable treatment option in the progression of AML.

Additionally, the alternative purpose of this study is to discover whether Vitamin D3 can induce apoptosis and/or strengthen the apoptotic effects of *Verbena* in AML cells. Through the analysis of previous studies, it has been hypothesized that the varying species of *Verbena* exhibit many apoptotic abilities through the possession of numerous bioactive compounds and that Vitamin D3 would have positive anti-cancer effects when put in conjunction with alternative therapies.

MATERIALS AND METHODS

{Equipment provided by MaRS centre with the affiliation of Princess Margaret Cancer Centre under the supervision and mentorship of Dr. Steven Chan MD, PhD}

Four 15 ml conical tubes were filled with 5 ml of media [MEM Alpha (1X) Minimum Essential Medium with L-Glutamine, Ribonucleotide, and Deoxyribonucleotide]. A 1:50 dilution of *Verbena officinalis* [Bach Flower Remedies Essence Vervain (Diluted with 27% alcohol)], *Lippia javanica* [Edens Garden Verbena Essential Oil, 100% Pure Therapeutic Grade (Highest Quality Aromatherapy Oils), *Lippia javanica*, 5 ml], and *Aloysia citriodora* [1 OZ PURE ORGANIC LEMON VERBENA ESSENTIAL OIL with glass dropper AROMATHERAPY HIGH QUALITY] were completed in three of the individual conical tubes. Similarly, vitamin D3 was diluted in 2µg/ml due to the concentration presented on the source [Life Brand Vitamin D3 1000 IU Liquid, contains flaxseed, medium-chain triglycerides, and d-alpha-tocopherol, 25mcg] within the fourth conical tube.

Two 96-well microplates were prepared in a manner of which 48 wells were dedicated to a certain essential oil or vitamin D3 (figure 1). One microplate was used in the testing of *Lippia ja-*



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vanica and *Verbena officinalis* and the other microplate was used in the testing of *Aloysia citrodora* and Vitamin D3.

This test was done using the serial dilution method; hence the first column had received the highest concentration of each compound. For the essential oils and vitamin D3, the highest concentration present in the first column of wells was 1:100 and 1µg/ml, respectively. The remaining 10 wells had then been bifold serial diluted. In the example of the essential oils, the first column was diluted in a 1:100 ratio, the second column in a 1:200 ratio, the third column in a 1:400 ratio, etc. The last column of wells had been left without a drug as the control group.

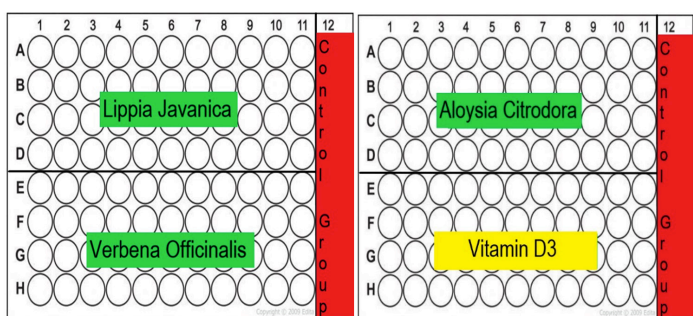


Figure 1: Representation of how substances were prepared in the microplates

OCI-AML2 cells (cell line derived from 65-year-old male diagnosed with AML) were diluted in the media in a 1:100 ratio as well and then 100µl of cells were added into each column of wells. During each dilution, the contents were mixed thoroughly to obtain accurate results.

The microplates were incubated at a temperature of 37°C with 5% carbon dioxide. Following the 4 days of incubation, 5µl of Resazurin (fluorescence medium) was diluted into each well. Four hours after the dilution, an Alamar Blue assay (Fluorescence microplate reader) was performed to formulate the results.

RESULTS

The substance *Lippia javanica* had presented with tremendous apoptotic effects in AML cells. As demonstrated in Figure 2, *Lippia javanica* already had some therapeutic effects within the smallest dilution factors (1:51200). Typically, other substances would present an efficacy similar to the 1:102400 dilution in the 1:51200 dilution factor, however this substance was unique in the way that it had minimal apoptotic properties in the 1:51200 dilution factor. When comparing the 1:25600 dilution to the 1:51200 dilution factor, there was also a substantial decrease in living AML cells (nearly a 73% decrease compared to the 1:51200 dilution and nearly 76% compared to the control group). The half-maximal inhibitory concentration (IC50) of *Lippia javanica* is approximately the 0.00003051 dilution factor. Essentially, the concentration at which *Lippia javanica* induces apoptosis to half of the AML cells is between the 1:6400 and 1:12800 dilution factors, which is a small dosage of *Lippia javanica*.

As demonstrated in the data, *Verbena officinalis* exerts therapeutic effects in AML cells and it only starts to take effect when diluted in a 1:25600 dilution factor of *Verbena officinalis*. There is also a gradually declining trend of the percent of living AML cells as the dilution increases. However, it is not as effective as *Lippia javanica* since the percentages do not enter the negative values. It does have therapeutic value since it induces apoptosis to all of the AML cells when the highest dilution factor (1:200) of *Verbena officinalis* is administered. The IC50 of *Verbena officinalis* is 0.00006811. The IC50 of this substance comes to show that only a dilution factor of 0.00006811 is needed to induce apoptosis in half of the AML cells. The IC50 dilution factor lies between the 1:6400 and 1:12800 dilution factors, which is a fairly small dosage when looked at from a greater scale.

Table 1: Conversions of dilution ratios to decimal values

Ratio 1:x	200	400	800	1600	3200	6400	12800	25600	51200	102400	0
Decimal Value	0.005	0.0025	0.00125	0.000625	0.0003125	0.00015625	0.000078125	0.0000390625	0.0000195313	0.00000976563	0

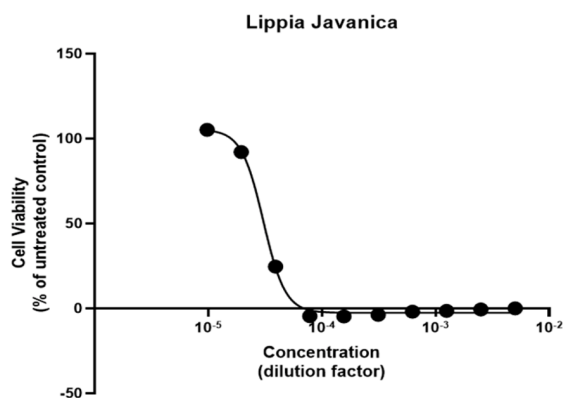


Figure 2: Portrays the effects of *Lippia javanica* on AML cells. The concentrations for this are expressed as a dilution factor since the concentrations were not clearly expressed on the containers.

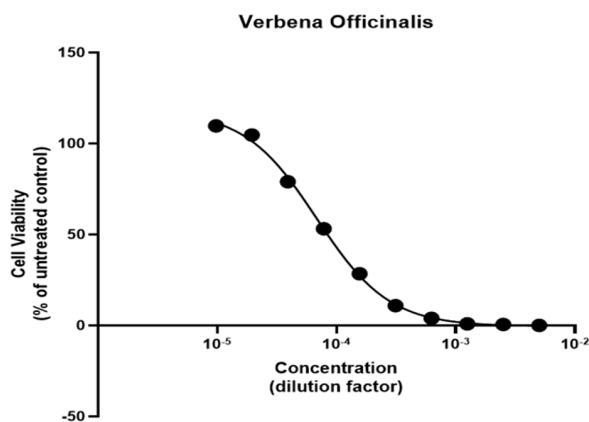


Figure 3: Shows the effects of *Verbena officinalis* on AML cells. The concentrations for this are expressed as a dilution factor since the concentrations were not clearly expressed on the containers.

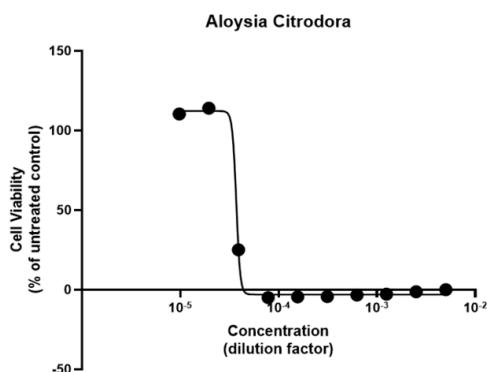


Figure 4: Shows the effects of *Aloysia citriodora* on AML cells. The concentrations for this are expressed as a dilution factor since the concentrations were not clearly expressed on the containers.

As can be observed in the data presented for *Aloysia citriodora*, approximately 75% of the untreated control of AML cells had undergone apoptosis at the 1:25600 dilution factor. This was seen as a significant presence of anticancer activity since the percent of untreated control had slightly increased in the 1:51200 and 1:102400 dilution which then drastically dropped to 25.05. As the concentration (or dilution factor) of the substance increased, it had shown more therapeutic effects in the AML cells which lead to negative percentage values near the higher concentrations. The IC50 of this substance is 0.00003708. It is a fairly small IC50 and it appears to be that this dilution factor is placed between the 1:6400 and 1:12800 dilution factors which are fairly small quantities of the substance.

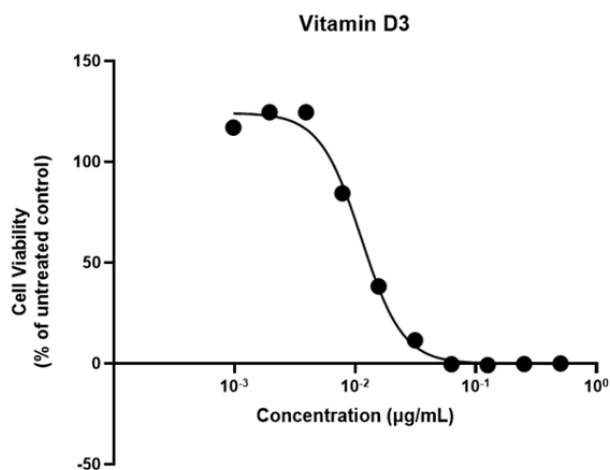


Figure 5: Shows the effects of Vitamin D3 on AML cells.

When comparing the effects of Vitamin D3 to the effects of the essential oils, it is less effective. Rather than exhibiting apoptotic effects in the smaller concentrations, Vitamin D3 had only started to exhibit effects in the 1:12800 concentration. Although it started to only have effects in the 1:12800 concentration, the number of living AML cells had started to drop drastically. The IC50 of Vitamin D3 is 0.01121 which is only between the 1:200 and 1:400 concentration values (0.005µg/ml and 0.0025µg/ml). Hence, Vitamin D3 has effective properties in treating AML cells, however, is not as effective as the other substances tested in this study.

With regards to the essential oils, each respective oil has substantial apoptotic effects on the AML cells at even the smallest dilution factors. Both *Aloysia citriodora* and *Verbena officinalis* had started to induce apoptosis in the 1:25600 dilution factor which is only the third smallest dilution factor (omitting the control group). Despite the apoptotic effects presented by these two substances, *Lippia javanica* had the most substantial apoptotic effects regarding that it was effective in the second smallest dilution factor and that it had neared the negative percentages at a smaller dosage (dilution factor).

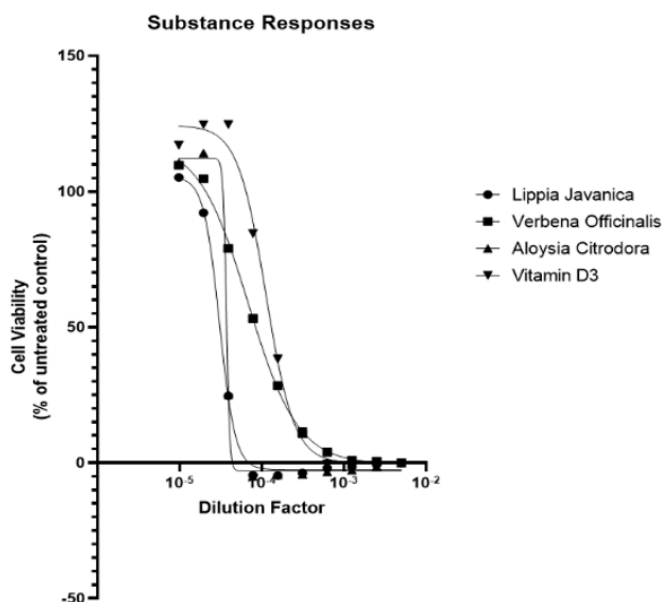


Figure 6: Compares the efficacies of all substances tested through measuring cell viability

When regarding the trends present in the graphs, the trend seen in *Verbena officinalis* mimics the trend present in Vitamin D3 since the effects of each substance respectively gradually progress as the dose increases. In contrast, *Aloysia citriodora* and *Lippia javanica* present sudden changes in its efficacy in which the percent of AML cells substantially drops at a certain concentration (dilution factor) in which these trends are similar.

In terms of the IC₅₀s presented by each substance, the IC₅₀ of *Aloysia citriodora* matched that of *Lippia javanica* (0.00003708 and 0.00003051). *Verbena officinalis* also had a close IC₅₀ to the other two essential oils since all of their IC₅₀s fell between the 1:6400 and 1:12800 threshold, but the IC₅₀ was still slightly greater in terms of the dilution factor (0.00006811). When comparing the essential oils with Vitamin D3, there is an extensive difference between the IC₅₀s because Vitamin D3's IC₅₀ was in between the higher concentrations.

DISCUSSION

Lippia javanica, *Aloysia citriodora*, *Verbena officinalis*, and Vitamin D3 have been shown to exhibit apoptotic effects in AML cells. This study suggests that any of these substances can be used to treat AML cells, however *Lippia javanica* had exhibited the highest apoptotic effect in AML cells. In addition, *Aloysia citriodora* had exhibited very similar effects to *Lippia javanica* as well, hence could be used as an alternative to *Lippia javanica*. It can be suggested that both of these substances possess similar quantitative measures of bioactive compounds of similar efficacies. When examining the other compounds' performance in exhibiting apoptosis, *Verbena officinalis* and Vitamin D3 were quite ineffective compared to the two aforementioned compounds, Vitamin D3 having the worst efficacy. Higher dosages of Vitamin D3 and *Verbena officinalis* would be needed to

meet the equilibrium of the effects of the other essential oils to induce a similar level of apoptosis in AML cells. Therefore, as hypothesized, all of these substances exert apoptotic effects on AML cells while each substance has varying effectiveness.

This line of investigation is important because AML is still a condition that is rarely cured and patients would still need to continuously undergo therapies such as BTK inhibitors (Ibrutinib) which still have side effects like diarrhea, nausea, musculoskeletal pain, and etc. Some studies are also showing that Ibrutinib can cause long term cardiovascular complications in which the drug which is helping to treat AML is causing more problems to the body. [5] On the other hand, another topic that could be investigated in this topic is combining these substances to create a combination drug. It would be more sensible to look towards combining Vitamin D3 with *Verbena officinalis* since both of these substances have similar trends in terms of therapeutic value as mentioned earlier in the results section of this study. The idea of combining these substances with some mild chemotherapy drugs may also be considered since these substances may have the ability to enhance the effects of certain chemotherapy drugs. This combination product would most likely be aimed to provide patients with a better quality of life consisting of less short and long term side effects. Also, a variety of species of *Verbena* are abundant in many countries like North Africa, China, and Japan in which this treatment option might be optimal for those living in countries limited to conventional pharmaceutical resources.

In terms of future work, many steps could be taken to further advance this study. The next step would be to test these substances on healthy cells to help discover their therapeutic indices to conclude whether it selectively induces apoptosis in AML cells or not. This should be the next step since the target of this treatment is to selectively annihilate cancer cells without affecting healthy cells. If the healthy cells are affected in this treatment, the purpose of creating this novel treatment plan is essentially defeated since this treatment is meant to not cause any adverse effects to the patient undergoing it.

The specific compounds which make each substance proprietary in terms of its efficacy are yet to be determined. Thus, uncovering the bioactive compounds which activate certain biological mechanisms within the cells to trigger apoptosis is probably the next step to look towards. Concerning this topic, it may also be useful to extract certain compounds that exhibit these effects in these substances to create a drug that can help treat the disease without causing any adverse effects.

Typically, in the topic of AML, drugs are administered via Intravenous (IV) orally or spinal injections (stem cells). As future goals, an ambitious idea would be to administer drugs via an oral spray in which the substance(s) (or drugs) would be dissolved into a lipid-soluble compound. Then, the lipid-soluble solution would be able to permeate through lymphoid tissue and enter a lymph node and then circulate throughout



the lymphatic system. However, the expertise required for this field of research was not found during the execution of this study, hence it is a future goal to be attained. This research will help administer drugs for this disease as the typical intravenous and spinal drug administration methods induce substantial pain to patients who are already undergoing treatments that cause both physical and psychological pain. In addition, as AML affects primarily the lymphatic system, utilizing this form of drug delivery can perhaps increase the efficacy of the drug reaching target sites of administration whereas typical intravenous administration will make the drug circulate throughout the body. Hence, lymphoid axis absorption can reduce the amount of adverse effects a patient suffers compared to that of intravenous absorption since the drug will mainly be absorbed by targeted tissue than having to circulate throughout the body and create adverse effects to healthy tissue.

In conclusion, this study is only part of the preliminary research used to conclude whether *Lippia javanica*, *Aloysia citriodora*, and *Verbena officinalis* can be used to treat AML. As mentioned throughout the discussion, there are many future tests yet to be done to discover the efficacy of these substances and to discover their cell viability as well. Future clinical trials evaluating the effects of these substances on human participants will provide us with the conclusion as to whether these substances are feasible substances to treat AML patients with. In conclusion, as hypothesized, species of the *Verbena* genus along with Vitamin D3 does provide hope for the future of AML treatment.

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Haarini Suntharalingam is a grade 10 student attending the gifted program at Markham District High School. She is interested in studying mechanisms in cancer biology in which she enjoys investigating cancer diagnostics and cancer treatment. She has been participating in regional science fairs since seventh grade in the field of health sciences. She is very passionate about STEM and continues to challenge herself by investigating different forms of cancer for her science fair projects to develop solutions that bridge the field of health sciences to engineering. In terms of hobbies, she enjoys reading, playing badminton and singing.

