Antifungal activity of three plant-based essential oils against *Colletotrichum gloeosporioides* the causative agent of mango anthracnose: Its implications on inquiry-based learning through Science investigative project

Ronilo V. Aponte[1], Charisse V. Samaniego[2], and Ruth F. Jardeleza[3]
[1]*West Visayas State University and Iloilo National High School*
[2]*Iloilo National High School – Senior High School*
[3]*Bureau of Plant Industry (BPI) – Guimaras National Crop Research Development and Production Support Center (GNCRDPSC), Brgy. San Miguel, Jordan, Guimaras*

Keywords:
Science Inquiry
Science Investigative Project
Antifungal Assay
Essential Oils
Central Philippines

ABSTRACT

The conception, development, and reporting of the science investigative project (SIP) exemplifies science inquiry in the basic education and addresses deficiency in the laboratory facilities. A community-based problem concerning the occurrence of sunken black spots (Anthracnose) on mango fruit caused by fungal pathogen Colletotrichum gloeosporioides which was shown to exhibit resistance to fungicide. Hence, in vitro antifungal activity of essential oils from Ocimum sanctum (Yerba buena), Curcuma longa (Dulaw), and Origanum vulgare (Oregano) against Colletotrichum gloeosporioides was investigated. Initially, essential oils from three plant species were distilled using the MIDAS processor. Pure culture of Colletotrichum gloeosporioides was aseptically seeded by pour plate method on the potato dextrose agar plates. Bioassay following the Disc diffusion method by impregnating 6mm diameter filter paper discs with the specific oils, compared to mancozeb (positive control) and distilled water (negative control) prepared in five replicates and laid out in Completely Randomized Design. Incubation proceeded for five days at ambient temperature and measured the diameter of mycelial inhibition. Results exhibited strong and significantly different ($p<0.05$) fiungicidal activity (Mean±SD) of Origanum vulgare essential oil (40.80 mm±7.01) followed by Ocimum sanctum oil (27.20±5.22) while fungistatic activity of Curcuma longa oil (8.40 mm±2.19) and mancozeb (6.40 mm±0.89), and inactive in distilled water (6.00 mm±0.00) as revealed by One-way ANOVA and LSD. Contrastingly, mancozeb being a short-termed antifungal agent showed fungistatic activity after five-day of incubation. It was demonstrated that essential oils from Origanum vulgare and Ocimum sanctum are potent alternative bio-fungicidal agents while Curcuma longa oils was fungistatic against Colletotrichum gloeosporioides. On that account, Science inquiry through SIP implicated collaboration, critical-thinking and personality development among budding researchers. Additionally, SIPs strengthen linkage to research institutions, decipher solutions on community-related problems, and augment the lack of laboratory facilities in the basic education high schools.

Introduction

A universal Science education integrates varied disciplines (Cobern, 1994) in the construction of personal knowledge through a collaborative and culture relevant process (Taber, 2006). Acquring this viable and pragmatic knowledge is a product of continuous negotiation and consensus building process called science inquiry (Tobin, 1983) which is founded on the scientific method (Michael, 2002).

Keys and Bryan (2001) pinpointed the importance of contextualizing inquiry in science education as the framework of teaching the subject. This places the teachers' knowledge, actions, and meanings in the inquiry-based science to bridge the gap between theory and practice. Minner, Levy, and Century (2009) emphasized inquiry-based instruction utilizes scientific investigation that encourages critical-thinking and making conclusions from data that engages students more on the learning process. Thus, construction of knowledge is culturally contextualized (Cobern, 1991).

Making students as budding researchers more conscious of the pressing problems occurring in their surroundings and providing opportunities to apply the Science concepts learned to unravel sustainable solutions. Resourcesfulness are encouraged by utilizing locally available raw materials for the creation of products. Hence, a culturally related and environmentally relevant problems are identified, resolved, and mitigated. The milieu that employs inquiry-based learning in Research and Science Subjects distinguishes Science Technology and Engineering (STE) curriculum from other special programs.

In this particular scientific investigation, the student-researcher is from the province of Guimaras. It is well known for its sweet and large mangoes of export quality. Over the years, there is a reduction in the exportation of mangoes (Mojica, 2005) due to fungal pathogen (McGrath, 2014) *Colletotrichum gloeosporioides,* the causative agent of mango anthracnose showing symptoms of black lesions on leaves, fruits and panicles (Nelson, 2008). To treat these fungal infections, farmers opted to use short-termed commercially available broad-spectrum antifungal agent mancozeb which requires regular application (Asita & Makhalemele, 2009). However, studies show that undesirable side effects resulted from the indiscriminate use of agricultural fungicides in developing countries out way its benefits. As confirmed by Sokovic *et al.* (2009) ingestion of the fungicide's residue in the fruit cause side effects to the consumers.

In vivo studies on the toxicity effects of mancozeb to laboratory animals is very high causing thyroid gland malfunction (Axelstad *et al.,* 2011), disruption of endocrine glands (Bisson *et al.,* 2002), and genetic damage and congenital malformation of exposed population in a dose-dependent effect (Castro *et al.,* 1999). Joshi *et al.* (2005) investigated the biomagnification of mancozeb from the contaminated tissues, water and soil can also caused indirect threats to humans and non-target species. Moreover, constant application of synthetic fungicide can develop resistance fungicide-resistant pathogens and environmental pollution (Katooli, Maghsodlo, Honari & Razavi, 2012; Abd-Alla & Hagga, 2010)) also has been concerned in *Colletotrichum* spp. (Chung *et al*., 2006) that can lead to costly production of new synthetic chemicals (Katooli, Maghsodlo, Honari & Razavi, 2012) which urged to find more eco-friendly alternative.

In this respect, the following are underlying problems identified when synthetically-produced mancozeb is used: its toxicity effect to non-target species particularly to the vertebrate (Joshi *et al*., 2005), its short-termed antifungal activity necessitated regular applications (Biswas *et al*., 2003) and *Colletotrichum gloeosporioides* develop resistance to regular application. Thereby a need to search for a natural and effective alternative to mancozeb arises. The Department of Agriculture – Bureau of Plant Industries - Guimaras National Crop Research, Development and Production Support Center at Jordan, Guimaras, Philippines is supporting investigation on finding a natural and effective alternatives for mancozeb.

Plant-derived biologically active secondary metabolites have been reported to be safe and without side-effects (Bansod & Rai, 2008). Among secondary plant metabolites, plant essential oils may offer potential alternatives which were previous studied to exhibit fungicidal properties (Macias *et al*., 2007; Soliman & Badeaa, 2002) on humans fungal pathogens (Plooy, Regnier & Combrinck, 2009; Elshafie, Mancini, Camele, Martino, & Feo, 2015) and to certain postharvest phytopathogenic fungi (Plotto *et al.*, 2003). Essential oils are hydrophobic liquid containing volatile aromatic compounds extracted from plants (Isman, 2000) and are not only used as fragrance and flavor source for food and beverages but are also being discovered as reservoirs of bioactive substances (Lawrence, 1984).

Herbal plant essential oil from *Ocimum gratissimum* commonly known as *clove basil* belonging to the holy basil family has shown to exhibit both fungicidal and fungistatic dose-dependent activities on *Colletotrichum gloeosporioides* (Kouame *et al.,* 2015). This study was used as platform to identify locally available herbal plants riched in essential oils as the potential plant samples. These herbal plants include *Ocimum sanctum* commonly known as *Holy basil* which contain eugenol as the active constituent (Prakash & Gupta, 2005), *Origanum vulgare* commonly known as *Oregano* which contain carvacrol, β-fenchyl alcohol, thymol, and γ-terpinene (Teixeira *et al., 2013*) and *Curcuma longa* commonly known as *Dulaw* which exhibits antimicrobial activity (Ferreira *et al.*, 2013). Prior studies revealed that *Ocimum sanctum* exhibited antifungal activity on *Candida albicans* (Agarwal, 2015), *Aspergilus fumigatus*, and *Aspergilus niger* (Bansod & Rai, 2008) as well as *Origanum vulgare* (Adams, Kumar, Clauson, & Sahi, 2011).

Furthermore, unavailability of the laboratory facilities and expert on this field in the school encourages linkage to research institution. The Bureau of Plant Industry (BPI) – Guimaras National Crop Research Development and Production Support Center (GNCRDPSC) located in Brgy. San Miguel, Jordan, Guimaras is conducting parallel studies being one of their priorities. The institution provides assistance to students who wanted to study on areas in line with their expertise. Moreover, microbiological assays of these essentials oils were limited to human pathogens and its possible effect to control anthracnose was not investigated as such, this study was putforward.

In the present investigation, the antifungal activity of essentials oils from *Ocimum sanctum*, *Curcuma longa,* and *Origanum vulgare* against *Colletotrichum gloeosporioides* was evaluated *in vitro*. This study is directed to the utilization of the essentials oils as natural and effective fungicide in the mycelial inhibition of *Colletotrichum gloeosporioides* a causative agent of anthracnose in mangoes. Inquiry learning through scientific investigation of community-based problems directed to provide avenue for the 21st Century learners to engage in creative and collaborative learning experience, developed critical-thinking and problem solving skills, and imbibe a personal construction of new knowledge among budding researchers.

Research Objectives

Generally, this study determined the antifungal activity of *Ocimum sanctum*, *Curcuma longa*, *Origanum vulgare* essential oils against *Colletotrichum gloeosporioides*.

Specifically, this study aimed to :

1. compare and characterize the diameter of mycelial inhibition of *Ocimum sanctum*, *Curcuma longa*, *Origanum vulgare* essential oils against *Colletotrichum gloeosporioides* in reference to mancozeb (+) and distilled water (-);
2. determine the significant difference in the diameter of mycelial inhibition of *Ocimum sanctum*, *Curcuma longa, Origanum vulgare* essential oils against *Colletotrichum gloeosporioides* in reference to mancozeb (+) and distilled water (-); and
3. elucidate the implications of inquiry-based learning through Science Investigative Project in Research of the Science, Technology and Engineering (STE) curriculum of the Junior High School (JHS).

Materials and Methods

A. Scientific Investigation Formulation

Conceptualization

In Research 3 of the Special Science Class, the students are encouraged to develop a researchable topic anchored on addressing environmental issues that predominate in the locality. Certain distinct criteria are set to guide the students to come up with the expectation. Once a problem is identified the review of related studies from published science articles are gathered to supplement the students with the knowledge on the variables that influence the study, scientific concepts, and procedure on how to go about with the experimentation.

The students then present the title for approval wherein; the feasibility of the proposed title is scrutinized. Afterwards, a Research Proposal is developed and subjected to a proposal defense with a panel. Once the study is approved, preparation of communications toward the research institutions were prepared, signed and submitted for approval.

Development

The experiment commences with the collection and preparation of samples used in the study. Plant samples were submitted for authentication to the recognized institution. The research adviser monitors the whole research process. A brief internship occurred prior to the experimentation to equipped researchers with the knowledge and skills for the assay. After the data were collected and analyzed, the manuscript wass written based on the guidelines of the Intel International and Engineering Fair (ISEF).

B. Scientific Investigation Procedure

Regulated Research Institution

The essential oils from *Ocimum sanctum, Curcuma longa, Origanum vulgare,* were freshly collected and distilled from Herbanex Laboratories, Bago City. The microbiological assay was conducted at the Bureau of Plant Industry (BPI) – Guimaras National Crop Research Development and Production Support Center (GNCRDPSC) located in Brgy. San Miguel, Jordan, Guimaras.

Research Design

In this experimental research the independent variables includes the different essential oils from *Ocimum sanctum*, *Curcuma longa*, *Origanum vulgare* compared to distilled water (negative control) and mancozeb (positive control) while the dependent variable is the diameter of fungal growth inhibition. The petri dishes were assigned and arranged in Completely Randomized Design (CRD) with five replications.

Distillation

Approximately, 2 kgs of each cleaned fresh leaves of *Ocimum sanctum*, *Origanum vulgare* and rhizome of *Curcuma longa* were availed from the Herbanex Laboratory farm. The first batch of plant sample - *Ocimum sanctum* was loaded in a Midas processor, a

distillation device that eventually separate the oil from the water, added with 4 liters of water and distilled for 3 hours yielding the essential oil. Similar process was followed for the two remaining plant samples namely

Origanum vulgare and *Curcuma longa* in availing the essential oils.

Figure 1. Distillation of essential oils using the Midas processor

Sterilization and PDA agar Preparation

Materials such as gauze, filter paper, forceps, and beakers were sterilized for an hour in an autoclave after it reached 15 ppm. Exactly, 0.18 kg of PDA (Potato Dextrose Agar) was diluted in 1 liter of distilled water and dispensed in five 200 mL volumetric flasks for sterilization close 25 minutes.

Pour Plate Method

Aseptically, two plates of *C. gloeosporioides* previously prepared by the experts from the research institution were gradually homogenized in 60 mL sterilized distilled water and added to 200 mL of sterilized PDA. Exactly 25 petri dishes were randomly laid out and seeded with 8 mL of the inoculum combined to PDA.

Disc Diffusion Method

Previously sterilized filter papers with 6 mm diameter were impregnated with 10 µL of the the essential oils. On the other hand, a 6.5 g/10 mL concentration of the positive control mancozeb diluted in distilled water was prepared and 10 µL was also impregnated the designated filter discs for the positive control. Similarly, 10 µL of distilled water was also used to impregnate the filter discs designated for the negative control. The impregnated filter discs of the designated treatments were dispensed on the PDA agar plate in a clockwise manner and the negative control was placed in the middle of plate. The plates were then inverted and incubated at 28-30°C (Ambient Temperature) for 5 days (Al-Reza *et al*., 2010).

Data Gathering Procedure

The antifungal activity was evaluated by measuring the diameter of the mycelial inhibition formed around the disc. The diameter of mycelial inhibition was measured using a ruler expressed in mm on the undersurface of the plate preventing the opening of the lid and compared to mancozeb as the positive control (Asita & Makhalemele, 2009) and distilled water as the negative control. Wider diameter of mycelial inhibition indicated fungicidal activity of the essential oils while minimal diameter of mycelial inhibition indicated fungistatic activity of the essential oils.Moreover, absence of clearing zone closely around the disc indicate inactivity of the sample/treatment being tested (Katooli, Maghsodlo, Honari, & Razavi, 2012).

Disposal

After the experiment was conducted and the results were gathered, the plates containing the *C. gloeosporioides* that were examined were disinfected using Lysol solution and set aside in the laboratory for 24 hours and finally the petri dishes were cleaned and autoclaved.

Statistical Data Analysis

The diameter of fungal inhibition per treatment were utilized to determine the mean and standard deviation using the PASW. The variance as implicated in the significant difference among and between treatment means were determined by One-ANOVA and LSD at 5% level of significance.

Results and Discussion

Antifungal assay of essential oils from plant sources against *Colletotrichum gloeosporioides* offers baseline information on the natural method of preventing postharvest disease in mango fruit. The antifungal activity of the essential oils was determined through the measurement of the diameter of mycelial inhibition after 5 days of incubation (Al-Reza *et al*., 2010) and compared to mancozeb and the negative control. The appearance of the clear zone around the filter disc impregnated with the essential oils indicated an area of no fungal growth occur and measured along the diameter of mycelial inhibition. The mean values of the diameter of mycelial inhibitions and its respective Standard Deviation were presented in the proceeding paragraph accordingly.

Results of the antifungal assay revealed that essential oil from *Origanum vulgare* exhibited strong antifungal activity of 40.80 mm ± 7.07 followed by *Ocimum sanctum* essential oil of 27.20 mm ± 5.22. On the other hand, essential oil from *Curcuma longa* showed antifunga ; activity of 8.40 mm ± 2.19 and the positive control - mancozeb of 6.40 mm ± 0.89. While distilled water used as the negative control exhibited 6.00 mm ± 0.00 indicating the diameter of the filter disc used.

Antifungal activity of the essential oils of *Origanum vulgare* and *Ocimum sanctum* are characterized as fungicidal due to a wider diameter of mycelial inhibition demonstrated (Figure 2) while *Curcuma longa* essential oil and mancozeb characterized as fungistatic due to minimal inhibitory activity by blocking fungal growth closely around the impregnated filter discs after the 5-day incubation.

Table 1. *Antifungal activity of three essential oils against Colletotrichum gloeosporioides*.

Essential oils	Diameter of mycelial inhibition (mm) *Mean±SD
Ocimum sanctum essential oil	27.20 ±5.22 b
Curcuma longa essential oil	8.40 ±2.19 c
Origanum vulgare essential oil	40.80±7.01 a
mancozeb (+)	6.40 ±0.89 c
distilled water (-)	6.00 ±0.00 c
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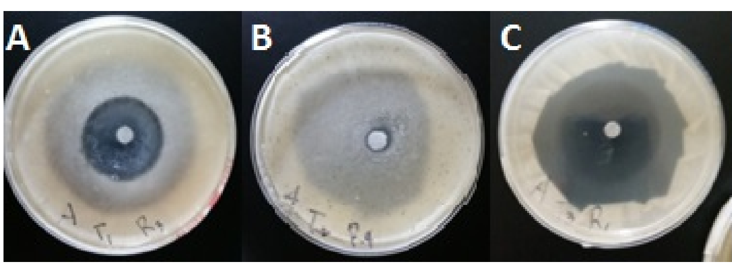
Note: 6mm – disc diameter
*Mean values with similar letters are not significantly different at p<0.05.

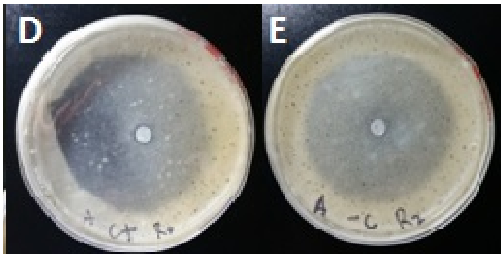
Statistical analysis using One-way ANOVA revealed a probability value of 0.000 < 0.05 level of significance leads to the decision of rejecting the null hypothesis and acceptance of the alternative hypothesis stating that there is a significant difference in the antifungal activity of the essential oils. To determine which essential oils show significant antifungal activity based on the mean values comparison of the diameter of the mycelial inhibition, a post hoc analysis using the Least Significant Difference (LSD) was employed. Analysis revealed that *Origanum vulgare* essential oil's antifungal activity differ significantly with *Ocimum sanctum* essential oil and *Curcuma longa* essential oil, mancozeb and distilled water designated as letter a. This was followed by *Ocimum sanctum* essential oil designated by letter b and statistically comparable antifungal activity of *Curcuma longa* essential oil, mancozeb and the negative control designated wilh letter c (Table 1).

In support to the numerical data, the clear zones surrounding the impregnated filter discs exhibiting antifungal activity (Figure 2) showed widest diameter of mycelial inhibition were observed in *Origanum vulgare* and *Ocimum sanctum* essential oils while *Curcuma longa* and mancozeb showed a narrow clear zone closely around the filter discs. Both results are distinct from the negative control – distilled water where absence of clearing zone around the disc was observed supporting its inactivity against *Colletotrichum gloeosporioides.*



A

Figure 2. Fungal inhibition of the following essential oils: **A**) *Ocimum sanctum,* **B**) *Curcuma longa,* **C)** *Origanum vulgare* **D**) mancozeb (+), and **E**) distilled water (-) against *Colletotrichum gloeosporioides* after 5 days of



incubation.

Figure 2. Fungal inhibition of the following essential oils: **A**) *Ocimum sanctum,* **B**) *Curcuma longa,* **C)** *Origanum vulgare* **D**) mancozeb (+), and **E**) distilled water (-) against *Colletotrichum gloeosporioides* after 5 days of incubation

Comparison of the diameter of mycelial inhibitions of the essentials from *Origanum vulgare*, *Ocimum sanctum*, and *Curcuma longa* revealed a varying antifungal activity. *Origanum vulgare* and *Ocimum sanctum* essential oils are distinctively fungicidal while *Curcuma longa* and the positive control – mancozeb are fungistatic against *Colletotrichum gloeosporioides.* On the contrary, distilled water impregnated filter disc as the negative control showed no clearing zone close around the filter disc implying absence of fungal growth inihibition.

Findings are supported by previous studies revealing that essential oils exhibit antifungal activity. Teixeira *et al*. (2013) investigated that *Origanum vulgare* is riched in essential oils carvacrol, *β*-fenchyl alcohol, thymol, and *γ*-terpinene (Adams, Kumar, Clauson, & Sahi, 2011) that exhibited antifungal activity against *Candida albicans, Aspergilus fumigatus*, and *Aspergilus niger*. Similarly, *Ocimum sanctum* which contain eugenol the active constituent (Prakash & Gupta, 2005) showed antifungal activity to *Candida albicans* (Agarwal, 2015), *Aspergilus fumigatus*, and *Aspergilus niger* (Bansod & Rai, 2008). *Curcuma longa* on other hand, although claimed in previous studies to exhibit antimicrobial activity (Ferreira *et al.*, 2013) displayed fungistatic activity comparable to the positive control - mancozeb. Possible explanation to this findings was confirmed in a study by Asita & Makhalemele (2009) revealing that mancozeb is a short-termed broad-spectrum antifungal agent that requires regular application. More importantly the extensive used of mancozeb triggers the development of fungicide – resistant fungal species (Katooli, Maghsodlo, Honari & Razavi, 2012) particularly *Colletotrichum spp*. (Chung *et al*., 2006) that explained the fungistatic activity after 5 days of incubation.

Colletotrichum gloeosporioides that causes the postharvest disease anthracnose, a disease that affects many tropical fruits aside from mangoes (Nelson, 2008). The discovery of effective and natural antifungal agent from the essential oils of *Origanum vulgare* and *Ocimum sanctum* is timely because of the development of resistant *Colletotrichum gloeosporioides* to mancozeb. Uitlizing the essential oils from the herbal plants *Origanum vulgare* and *Ocimum sanctum* are (Katooli, Maghsodlo, Honari, & Razavi, 2012) environment-friendly substitute to synthetic fungicides for the management of fungal diseases in mangoes.

The identification of the exact essential oils that exhibit antifungal activity was not included and subject for further investigation. On the other hand, the outcome of this study can serve as basis for *in vivo* investigation of the bio-fungicidal activity of essential oils from *Origanum vulgare* and *Ocimum sanctum* by coating the mango fruits and challenged by the fungus.

Findings of the study corroborated the importance of Science inquiry through Science Investigative Project (SIP) in Research of the STE program in four fold: it engages researchers to collaborate with experts in the field and the community, it stimulates creativity, innovativeness and critical-thinking in addressing environment-related problems through a natural and sustainable process, it also provides a venue for budding researchers to participate in oral presentations like Science Fairs and Conferences to hone communication skills and self-confidence, and it engages construction of new knowledge owned by the learner. In a larger scale, the lack of laboratory facilities in the basic education high schools is mitigated by strengthening linkage with research institutions.

Conclusion and Recommendation

It was demonstrated that essential oils from *Origanum vulgare* exhibited strong and significantly different fungicidal activity followed by *Ocimum sanctum* and fungistatic activity by *Curcuma longa* and mancozeb (+) against the causative agent of mango anthracnose, *Colletotrichum gloeosporioides.*

Science inquiry through Science Investigative Project implicated collaboration, critical-thinking and personality development among budding researchers. Additionally, SIPs strengthen linkage to research institutions, decipher solutions to community-related problems, and augment the lack of laboratory facilities in the basic education high schools.

Hence, it's highly recommended that *in vivo* screening of the antifungal efficacy of *Origanum vulgare* and *Ocimum sanctum* essential oils by applying on mango peel and challenged by exposure to *Colletotrichum gloeosporioides* spores to determine the extent of its protective capacity, curative capacity, and spore germination inhibition.

In science education, the application of inquiry approach through Science Investigative Project is highly advocated because learning goes beyond the classroom setting, socio-scientific issues are addressed, and developed critical-thinking and problem-solving skills of learners.

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Acknowledgment

First and foremost, the researchers would like to extend gratitude to the following for the outmost support to the accomplishment of this study :

To the Almighty Father, for His blessings of wisdom to the researchers to surmount the challenges.

To the Faculty and Staff of CoEd – Graduate School, WVSU for the guidance and encouragement for the realization of this action research.

To the Accelerated S & T Human Resource Development Program (ASTHRDP), DOST-SEI for the financial support and encouragement to pursue a research driven postgraduate education.

To the Dr. Nordy D. Siason Jr, Officer-in-Charge of Iloilo National High School ; Ms. Rose R. Umadhay and Mrs. Ma. Aries A. Pastolero for the approval and insightful suggestions.

To the Head and Personnel of the Bureau of Plant Industry (BPI) – Guimaras National Crop Research Development and Production Support Center (GNCRDPSC) located in Brgy. San Miguel, Jordan, Guimaras, for the immeasurable and selfless approval and guidance as venue of the experiment.

To Mr. Philip Cruz – Manager, Herbanex Laboratories, Bago City, Negros Occidental, for the source of the plant samples and site of distillation of the essential oils.

To the Mr. and Mrs. Benigno Alan D. Samaniego parents of Charisse, for the relentless support extended to the student researcher.

To the family of Mrs. Ruth Jardeleza, for their unconditional love and support.

To the family of Mr. Ronilo V. Aponte, for the understanding and support.

To persons and institution the researchers failed to mention, this gratitude and thanksgiving is extended.