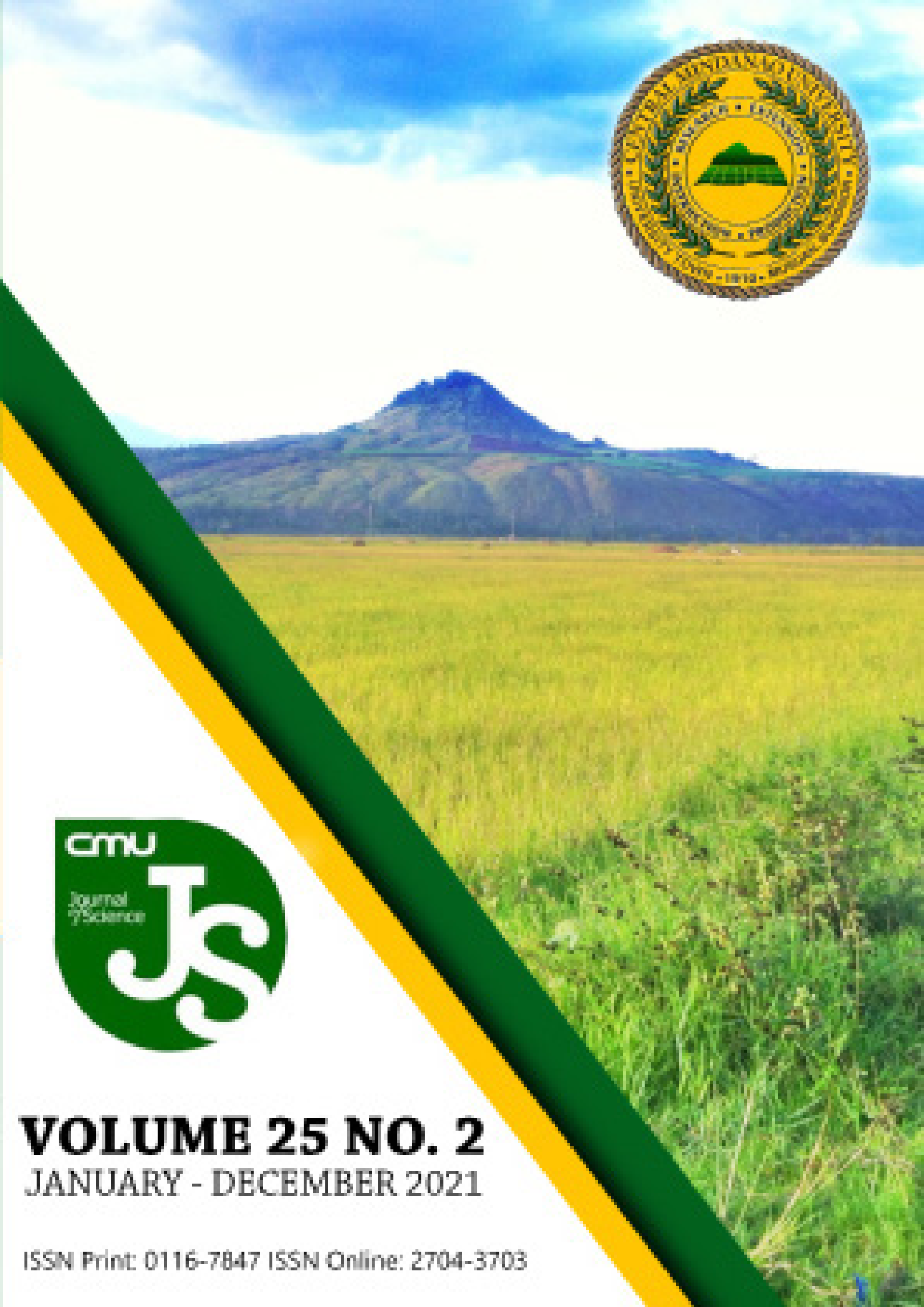


CMU Journal of Science

VOLUME 25 NO. 2

JANUARY - DECEMBER 2021

ISSN Print: 0116-7847 ISSN Online: 2704-3703

Table of CONTENTS

Perspective Article

Research Articles

Central Mindanao University
Journal of Science
ISSN Print: 0116-7847 ISSN Online: 2704-3703

Azadirachta indica A. Juss., *Tinospora rumphii* Boerl. and *Vitex negundo* against *Sarcoptes scabiei in vitro*

Obedencio, Jose Jr M[a], Daguro, Ted Aries A[b], Dargantes, Alan P[a],
Abella, Jose Alexander C[b], Montemayor, Maria Lebeña B[a], Batain, Jesalyne Joy Marie B[a]

[a]Department of Medicine, Surgery and Zootechnics, College of Veterinary Medicine
Central Mindanao University, Musuan, 8714 Maramag, Bukidnon
[b]Department of Microbiology, Parasitology, Pathology and Public Health, College of Veterinary Medicine
Central Mindanao University, Musuan, 8714 Maramag, Bukidnon

ABSTRACT

The study aimed to evaluate the acaricidal efficacy of the ethanol leaf extracts of *Azadirachta indica* A. Juss., *Tinospora rumphii* Boerl., and *Vitex negundo* against *Sarcoptes scabiei var. canis*. It also aimed to determine the concentration level of each leaf extract which has the highest acaricidal activity. Ethanolic leaf extracts from the plants were prepared in 10% and 50% concentrations. Mite mortality was not ed as percentage efficacy and was measured 0.5, 1, 2, and 6 hours post-exposure. The study utilized Completed Randomized Design and One-way Analysis of Variance (ANOVA) for the data. All concentration levels of the plant extracts showed acaricidal potential. Activity for 10% and 50% ethanolic extracts of *T. rumphii*, *V. negundo*, and *A. indica* were 73.33% and 93.33%, 63.33% and 93.33%, and 36.67% and 76.67%, respectively. As indicated, *T. rumphii* showed the best acaricidal activity followed by *V. negundo* and *A. indica*. It also showed that only the 50% concentration level of *T. rumphii* and *V. negundo* have comparable acaricidal effects with a commercial acaricidal solution. It is recommended that further studies will be conducted to determine the lowest effective concentration level for each leaf extract, its toxicity, its bioactive compounds, and *in vivo* trials.

Keywords: acaricide, ethanolic extract, sarcoptic mange

INTRODUCTION

Mange mites are one of the common causes of dermatological problems in a wide range of mammals, causing intense pruritus and unsightly dermal lesions of animals. One of these common skin mites is *S. scabiei*, which causes severe pruritic dermatitis in domestic animals (Ahmed et al., 2012), and is an especially common problem in dogs. Pruritus disturbs the regular activity of the dog and thus, affecting its general health. Due to this problem, anti-mange products are commercially available, but these products are considered expensive.

Natural remedies have been steadily receiving recognition in popular consciousness and pharmaceutical science (Doughari, 2012). Many plants were already ascribed to have considerable positive effects on the body. This effect can be homeostatic, rehabilitative, preventive, or even curative. While a significant number of natural treatments have been described as specific to treating dermal lesions in domestic animals, few of these studies had shown exclusive use for the treatment of mange in domestic animals. Three plants that have been used on scabies are *V. negundo*, *A. indica*, and *T. rumphii*. These three are widely available locally and had been mentioned in anecdotal evidence to have effects on canine mange (Agrawal, 2011; Hamid, 2013; Khan et al. in 2012).

The purpose of this study is to assess the acaricidal activity of the plants mentioned above by using different concentration levels of their ethanolic leaf extracts against *S. scabiei var. canis*. It also aims to compare the efficacy between the plants, as well as with a commercially available acaricide. The study seeks to serve as baseline data for further research and development of specialized products that exhibit acaricidal effect against mange mites in animals and humans.

METHODOLOGY

Study Animals as Source of Mites

The study required ten mange positive local dogs, regardless of age, sex, and breed. Natural infection was confirmed through skin scrapings. The mange positive dogs were housed exclusively in an Experimental Animal Station during the duration of the study. These dogs were fed once a day with a mixture of commercial dog food and table food and provided with adequate water. Accommodation for the dogs was cleaned regularly for feces and urine.

After the study, the dogs were treated with ivermectin (Ivomec, Merial, France), 10 mg/ml, given at 200 mcg/kg BW, two weeks apart for two to three

ARTICLE INFORMATION
Jose M. Obedencio Jr.
Email Address: jmobedenciojr@cmu.edu.ph
Received: June 16, 2020; Accepted: Sept 15, 2021
DOI: https://doi.org/10.52751/cczy2874

treatments before they were returned to their owners or released for adoption. This study was approved by the Institutional Animal Care and Use Committee of Central Mindanao University, Philippines (2016-27B).

Mite collection

Skin scraping (Elsheikha and Wright, 2015) was done once a day. The scraping was done at least an hour, but not more than 12 hours before the application of treatments. A maximum of ten skin scrapes, each within an area of 2 cm2, was done. The collected scabs were placed in an untreated Petri dish and inspected for mites. Using the dark field of a stereo microscope, the Petri dishes were inspected thoroughly to collect the adult mites. A total of 360 adult mites were collected for this study.

Preparation and Extraction of Leaves

The plants for this research include; *A. indica*, *T. rumphii*, and *V. negundo*. The leaves were collected early in the morning, and only the fresh, mature, and insect bite free leaves are selected, approximately one kilogram per plant. The leaves were washed with tap water and were air-dried for seven days. The dried leaves were then finely ground using a grinder before submerging into 1.0 L absolute ethanol (99.8%). This solution was stirred twice a day for 48 hours to obtain the standard extract. The resulting solution was strained using fine-holed cheesecloth, and the strained fluid was the stock solution. The stock solution was subjected to the rotary evaporator to obtain the 100% ethanolic leaf extract. The 100% leaf extracts were reconstituted using sterile water to achieve the desired 10% and 50% concentration.

Treatments

The treatments were the following: T0- – sterile water, T0+ – 0.025% amitraz solution; T1 – 10% ethanolic leaf extracts for *A. indica*, T2- 50% ethanolic leaf extracts for *A. indica*, T3 – 10% ethanolic leaf extracts for *T. rumphii*, T4- 50% ethanolic leaf extracts for *T. rumphii*, and T5- 10% ethanolic leaf extracts for *V. negundo*, and T6- 50% ethanolic leaf extracts for *V. negundo*.

In vitro Application of the Leaf Extract and Controls in Petri Dishes

There were 36 Petri dishes used for this study. For each treatment, three petri dishes were alloted, and for each Petri dish, there were ten mites. At least one hour before the isolation of mites from the collected scabs, the inner base and sides of the Petri dishes with smaller diameter were coated with 0.5 ml reconstituted ethanolic leaf extracts (10% and 50% concentration levels) and the control groups, 0.025% amitraz solution for the positive control and sterile water for the negative control. The Petri dishes were covered and labeled accordingly.

Assessment of Acaricidal Activity of each Leaf extract and Controls

Assessment activity of acaricidal activity was measured by mite mortality, observed at 0.5, 1, 2, and 6 hours after exposure to the control extract. Post-treatment, the mites were inspected in every Petri dish for movement through stimulation with a teasing needle. The mites were touched ten times if they were found immobile. The mites were considered dead if no movements were noted after 5 minutes of visual inspection under a stereo binocular microscope.

The assessment of the efficacy of each leaf extract and control was modified from the formula used by Tabije et al. in 2013 through subtraction of the total remaining live mites after 6 hours per replicate from the total mites used per replicate divided by the total number of mites used per replicate multiplied by 100.

Statistical Analysis

The study utilized a complete randomized design. The significant differences in the acaricidal effects of different leaf extracts and their comparison with the commercially available anti mange bath product and sterile water were determined using the F-test or analysis of variance (ANOVA) and Tukey's honesty significant test. The comparison between the concentration levels was assessed using an independent sample t-test.

RESULTS AND DISCUSSION

Tables 1, 2, and 3 summarized the data on the acaricidal efficacy (%) of the ethanolic extracts of *A. indica*, *T. rumphii*, and *V. negundo*, respectively.

As shown in Table 1, the percentage efficacies of *A. indica* are 36.67% for 10% and 76.67% for 50%. The results supported the claims of Prashanth and Krishnaiah in 2014. The acaricidal efficiency of *A. indica* was also reported against *S. scabiei var. cuniculi* (Seddiek et al., 2013). Aside from its acaricidal potential, it had been well known to be a mosquito repellent (Agrawal, 2011). The effects might be slow-acting, but A. indica is relatively a potential source for organic insecticides against *S. scabiei var. canis*.

Tinospora rumphii showed a relatively high efficacy compared to the other plants against S. scabiei var. canis. In Table 2, it revealed the percentage efficacy of 10% and 50% ethanolic leaf extract of *T. rumphii* were 73.33% and 93.33%, respectively. This study utilized leaves, although the most studied part of *T. rumphii* is its stem, which is characterized by fleshy protuberances (Devprakash et al., 2011). Nevertheless, phytochemical analyses showed that the bioactive compounds found in stems could also be found in leaves (Hamid, 2013). There were fewer studies about the pharmacological importance of *T. rumphii* compared to other plants, but past researches and the results of this study showed that the plant could be a potent insecticide against adult *S. scabiei var. canis*.

The percentage efficacies of *V. negundo* for 10% and 50% ethanol extract were 63.33% and 93.33% (Appendix 2), respectively, as shown in Table 3. The results of this study conforms to the results of Khan et al. in 2012 that showed the potential of this plant against *S. scabiei* showing not less than 70% mite mortality in 40% methanol extraction.These acaricidal efficacy of the present study

Table 1

Mean percentage (%) efficacy and the total number of remaining live mites for 10% and 50% A. indica ethanolic leaf extracts against adult S. scabiei var. canis after a given time of exposure

Treatment	N	Total number of remaining live mites/time of exposure					Mean percentage efficacy
		30 min	1 hr	2 hr	4 hr	6 hr	
10% *A. indica*	30	30	28	28	23	19	36.67
50% *A. indica*	30	27	26	23	19	7	76.67
Amitraz**	30	0	0	0	0	0	100.00
Sterile water*	30	30	30	30	30	30	0.00

Table 2

Mean percentage (%) efficacy and the number of remaining live mites for 10% and 50% T. rumphii ethanolic leaf extracts and control groups against adult mites (S. scabiei var. canis) after a given time of exposure.

Treatment	N	Total number of remaining live mites/time of exposure					Mean percentage efficacy
		30 min	1 hr	2 hr	4 hr	6 hr	
10% *T. rumphii*	30	30	27	27	19	8	73.33
50% *T. rumphii*	30	30	27	22	7	2	93.33
Amitraz**	30	0	0	0	0	0	100.00
Sterile water*	30	30	30	30	30	30	0.00

*- negative control, **- positive control, N- total number of mites per treatment, min-minutes, hr- hour.

conforms to the results of Nandini and Sriniwasa (2018).

In terms of percent efficacy, results show that at 10% concentration level, *T. rumphii* has the highest percentage efficacy followed by *V. negundo* and *A. indica* with the following percentage efficacies; 73.33%, 63.33%, and 36.67%, respectively. For the 50% concentration level, *T. rumphii* and *V. negundo* have the same percentage efficacies of 93.33% and *A. indica* has 76.67%.

In Table 4, it showed that 10% *A. indica* leaf ethanol extract (T1), *T. rumphii* (T3), and *V. negundo* (T5) are significantly different from amitraz solution, but *A. indica* is not significantly different with sterile water (T0-). Thus, none of these leaf extracts have comparable acaricidal effects with the amitraz solution.

Table 5 shows that the acaricidal activity of 50% A. indica (T3) is significantly different with amitraz solution and sterile water while the 50% of *T. rumphii* (T4) and *V. negundo* (T6) leaf ethanol extract is not significantly different with the acaricidal activity of amitraz (T0+) but are significantly different with sterile water (T0-). Therefore, 50% *T. rumphii* and *V. negundo* ethanolic leaf extracts are comparable with the acaricidal property of amitraz solution, but all the three-leaf extracts have more superior efficacy compared with sterile water.

CONCLUSION

All of the leaf extracts showed acaricidal property but varied in efficacy. Among the three mentioned plants, *T. rumphii* showed the promising potential in killing adult *S. scabiei var canis* with percentage efficacies of 73.33% in 10% concentration level and 93.33% in 50% concentration level, followed by *V. negundo* with percentage efficacies of 63.33% and 93.33% in 10% and 50% concentration levels, respectively. The least among the three extracts were *A. indica* with 36.67% for 10% concentration level and 73.33% for 50% concentration level. The control groups had the following percentage efficacies; the positive control (Amitraz) exhibited 100%, and the negative control (sterile water) had 0% in all treatments. In comparison between the concentration levels of each leaf extract, it was shown that a 50% concentration level has more significant acaricidal effects than a 10% concentration level. However, only the 50% concentration level of *V. negundo* and *T. rumphii* have comparable acaricidal effects to the amitraz solution.

Therefore, only the acaricidal activity of the 50% concentration levels of *T. rumphii* and *V. negundo* are comparable with a commercial anti-mange solution. However, the determination and quantification of the the bioactive components responsible for the acaricidal activity of the plant extracts is necessary to validate the potential use of the studied plants.

Table 3

Percentage (%) efficacy and the total number of remaining live mites for 10% and 50% V. negundo ethanolic leaf extracts against adult mites (S. scabiei var. canis) after a given time of exposure

Treatment	N	Total number of remaining live mites/time of exposure					Mean percentage efficacy
		30 min	1 hr	2 hr	4 hr	6 hr	
10% *V. negundo*	30	28	26	25	18	11	63.33
50% *V. negundo*	30	26	25	20	12	2	93.33
Amitraz**	30	0	0	0	0	0	100.00
Sterile water*	30	30	30	30	30	30	0.00

*- negative control, **- positive control, N- total number of mites per treatment, min-minutes, hr- hour.

Table 4

The Comparison between 10% concentration level of each Ethanolic leaf extracts with amitraz solution and sterile water.

Treatment	N	Total number of remaining live mites/time of exposure					Mean percentage efficacy
		30 min	1 hr	2 hr	4 hr	6 hr	
10% *A. indica*	30	30	28	28	23	19	36.67^{c}
10% *T. rumphii*	30	30	27	27	19	8	73.33^{b}
10% *V. negundo*	30	28	26	25	18	11	63.33^{b}
Amitraz**	30	0	0	0	0	0	100.00^{a}
Sterile water*	30	30	30	30	30	30	0.00^{c}

*- negative control, **- positive control, N- total number of replicates per treatment, min- minutes, hr- hour. Treatments with the same letters (superscript) are not significantly different $p>0.05$

Table 5

The Comparison between 50% concentration level of each Ethanolic leaf extracts with amitraz solution and sterile water

Treatment	N	Total number of remaining live mites/time of exposure					Mean percentage efficacy
		30 min	1 hr	2 hr	4 hr	6 hr	
10% *A. indica*	30	27	26	23	19	7	76.67^{b}
10% *T. rumphii*	30	30	27	22	7	2	93.33^{ab}
10% *V. negundo*	30	26	25	20	12	2	93.33^{ab}
Amitraz**	30	0	0	0	0	0	100.00^{a}
Sterile water*	30	30	30	30	30	30	0.00^{c}

*- negative control, **- positive control, R- number of replicates, min- minutes, hr- hour. Treatment with same letters (superscript) are not significantly different $p>0.05$

ACKNOWLEDGEMENT

The authors would like to thank Central Mindanao University for funding the study.

REFERENCES

AGRAWAL, A. D. (2011). Pharmacological activities of flavonoids: A Review. *International Journal of Pharmaceutical Science and Nanotechnology. 4*(2), 1394-1398.

AHMED, M. A. M., EL-MAWGOOD, S.Z.A., AWAD, S.W.A. (2012). Studies on mite species cause demodectic mange in dogs. 1-20.

DEVPRAKASH. SRINIVASAN, K. K., SUBBURAJU, T., SUHAS-GURAV, SACHIN-SINGH. (2011). Tinospora Cordifolia: A Review on Its Ethnobotany, Phytochemical & Pharmacological Profile. *Asian Journal of Biochemical and Pharmaceutical Research, 4*(1), 291-302.

DOUGHARI, J. M. (2012). Phytochemicals: extraction methods, basic structures, and mode of action as potential chemotherapeutic agents phytochemicals - A global perspective of their role in nutrition and health. In D. V. Rao (Series Ed.), InTech.

ELSHEIKHA, H. AND WRIGHT, I. (2015). Biology, diagnosis and management of sarcoptic mange. T*he Veterinary Nurse, 6* (5). https://doi.org/10.12968/vetn.2015.6.5.260

HAMID, H. B. A. (2013). Characterization and Biological Activities of *Tinospora crispa (menispermaceae)* Extract with Emphasis on Alkaloids. (Doctor of Philosophy of Science in Industrial Chemistry), Universiti Malaysia Pahang, Malaysia.

KHAN, M. A., SHAH, A.H., MAQBOL, A., KHAN, N., RAHMAN, Z.U. (2012). Miticidal Activity of Methanolic Extract of *Vitex negundo-lam* against *Sarcoptes scabiei* in Animals and Man. *The Journal of Animal and Plant Sciences, 22*(2), 102-107.

NANDINI, K. N. AND SRINIVASA, N. (2018). Vitex species, a potential store house for acaricidal principles. Journal of Pharmacognosy and Phytochemistry. 7(4), 2103-2107

PRASHANTH G.K, AND KRISHNAIAH, G.M. (2014). Chemical composition of the leaves of Azadirachta indica Linn (Neem). *International Journal of Advancement in Engineering Technology, Management, and Applied Science, 1*(5), 21-31.

SEDDIEK S. A., KHATER H. F., EL-SHORBAGY M. M., ALI A. M. (2013) The acaricidal efficacy of aqueous neem extract and ivermectin against *Sarcoptes scabiei var. cuniculi* in experimentally infested rabbits. Parasitology Research. doi: 10.1007/s00436-013-3395-2.

TABIJE, N., VISTE, G., CAMALIG, F., MONTERO, G. (2013). Efficacy trial of Lima bean (*Phaseolus lunatus*) Ointment against Dog Mange. *International Scientific Research Journal, 5*(3), 10.

Central Mindanao University
Journal of Science
ISSN Print: 0116-7847 ISSN Online: 2704-3703

FAUNAL COMPOSITION OF MACROMOTHS OF MT. MUSUAN, MARAMAG, BUKIDNON

Kerk Rekkem R. Rafil and Joliesa Mae S. Toledo

Department of Biology, College of Arts and Sciences
Central Mindanao University, Musuan, Bukidnon, Philippines, 8710

ABSTRACT

The present study was conducted to compile information on species composition of moths found from Mt. Musuan, Maramag, Bukidnon. The moths were collected using a light trapping method from November to December 2014 on three vegetation types namely grassland, pine trees and mixed dipterocarp vegetations of Mt. Musuan.

The study revealed the presence of 29 species belonging to 11 families and 24 genera. The species abundance was recorded highest in mixed dipterocarp vegetation with 6.681 mean individuals, followed by in pine tree vegetation with 3.085 mean individuals and 1.723 mean individuals in grasslands. Sixteen (16) species are randomly distributed which includes Asota Hubner, Amerila Walker, Carriola ecnomoda Swinhoe, Creatonotos gangis Linneaus, Nyctemera baulus Boisduval, Oenistis altica Linnaeus, Gastrina cristaria Guenée, Pingasa chlora Stoll, Taxeotis sp. 1 Guest, Hypomecis Hubner, Pingasa ruginaria Guenée, Pinara sp. 1 Walker, Persectania dyscrita Common, Noctuidae sp. 1 Latrielle, Dudusa Walker and Ourapteryx Leach.

Therefore, Mt. Musuan is a home of 29 species of moth belonging to twenty-four (24) genera and eleven (11) families. There are 16 species species of moths that are randomly distributed.

Keywords: Mt. Musuan, moths, species composition, habitat preferences

INTRODUCTION

Lepidoptera has 160,000 described extant species (Kawahara et al., 2019). Moths constitute one of the most prevalent terrestrial insect groups because they play an important role in ecosystems as these organisms are herbivorous and prey for many animals and other insect groups (Lara-Perez et al., 2017). The caterpillars of some species are a vital source of proteins and fats in rural communi. Almost all Lepidoptera are phytophagous species feeding on specific vegetation and exhibiting strong associations with vegetation structure and composition (Schmidt and Roland, 2006).

Amidst the diversity of moths in the group of Lepidoptera and its contribution to the conducive ecosystem, there are studies and articles that account for the species found in the Philippines.However, there is still a need to document more species in the country (Badon et al., 2019). The discussions of the moths found in the Philippines are cited only in few publications, and the knowledge about moths that can be found in Mindanao is minimal.

Therefore, there is a need to determine the species of moths in Mt. Musuan of Central Mindanao University, Musuan, Bukidnon to address further knowledge of moths found in the Philippines.

The objectives of the study was to determine the species composition of moth species and determine its distribution in Mt. Musuan, Maramag, Bukidnon. The study provides information about the species of moths in Mt. Musuan, which could be used by lawmakers in formulating strategies for the conservation of the species.

METHODOLOGY

Study Site and Establishment of Study Station

According to the RA 9147 and following with DAO No. 2004-55, a gratuitous wildlife permit was granted with the permit number R10 2014-35. The research was conducted in Mt. Musuan, Maramag, Bukidnon on the different vegetation types. The study was done from November to December 2014.

Mt. Musuan was located along Sayre Highway and its boundary between Valencia City, Bukidnon, and Musuan, Maramag, Bukidnon. It has an elevation of 646 masl and coordinates of 7.88° north and 125.07° east. There were three stations established in the study area. Station 1 was the mixed dipterocarp an elevation of 380-415 masl / 07° 52.30N 125° 02.30E. Station 2 was grassland with an elevation of 550-595 masl / 07° 50.10N

ARTICLE INFORMATION
Joliesa Mae S. Toledo
Email Address: : joliesamaetoledo@gmail.com
Received: Aug 6, 2020; Accepted: Sept 7, 2021
DOI: https://doi.org/10.52751/ilzb3992

125° 10.05E and station 3 was pine tree forest with an elevation of 615-625 masl / 07° 51.40N 125° 02.25E.

Moth Collection and Preservation

Light trapping was used in collecting moth species. Only one light trap was established per night of sampling. It was done using two hundred fifty (250) watts of mercury bulb set against a 2 x 4 meters white cloth sheet as modified from Sutrisno (2008). Moths were collected by handpicking and directly injected with 90% ethanol in the thorax to paralyze them as modified from Sutrisno (2008). The sampling period was done at 6:00 pm to 9:00 pm. Each station allotted four nights of sampling.

The moth collected was placed in a triangular wax paper to avoid damage of their wings and body parts. Information like the name of the collector, study station, and date of collection was written in the wax paper. The labeled moth collected was placed in a container containing naphthalene balls to preserve them.

Preserved moths were pinned in a spreader board (Styrofoam). Powder mothballs (naphthalene balls) were scattered around the body of the preserved moths. The preserved moths were deposited in the University Museum of Central Mindanao University.

Ecological Parameters Identification of Moth

Ecological parameters were measured and assessed during the sampling period. These include, air temperature, humidity, and elevation. The air temperature was measured using thermometer hung 1 meter above the ground at the start of the sampling period and it ranges from 25 to 28oC. Humidity was measured using a sling psychrometer and result ranges from 94-96%. Elevation was determined by using the Global Positioning System (GPS).

Identification was done using books and journals like Zilli and Hogenes (2002) and Townsend and Paul Waring (2007). Together with Mr. Dave Mohagan, moths were initially identified. Moths collected were photographed, and images were sent through e-mail into different experts like Mr. Greg Watson for confirmation.

Data Analysis

Analysis of the data was done using BioPro software version 2 (McAlleece et al., 1993). BioPro Software calculated the diversity indices and distribution of species.

RESULTS AND DISCUSSION

Species Composition

A total of twenty nine (29) species were collected in three vegetation types of Mt. Musuan, Maramag, Bukidnon. These species belong to twenty-four (24) genera and eleven (11) families. The families are Aganinae, Erebidae, Geometridae, Lassiocampidae, Notodontidae, Sesiidae, Saturniidae, Sphingidae, Uraniidae, Xyloryctidae and Noctuidae.

The species abundance was highest in mixed dipterocarp with 6.681 mean individuals, followed by pine tree forest with 3.085 mean individuals and 1.723 mean individuals in grassland.

The mixed dipterocarp has the most significant number of species collected, 46 moth species with 314 individuals and it has the highest species number and individuals since that Mixed dipterocarp are abundant in grown-up trees greatly covered by underground grasses and sedges and has the present of water source that serves as microhabitats of moths.

According to Ellis (2003), species habitat preferences, just like the larva stage of Noctuidae that are known to be stem borers, prefer to live in mixed dipterocarp and pine tree vegetation. However, as mentioned in the study of Singh et al., 2017, that abundance of flowering plants is the preferred habitat of lepidopteran species. In addition, moth larvae preferred also abundant trees and shrubs which enable them to survive. The availability of critical resources (food and water sources) in different vegetation types makes differences between the Lepidopteran species composition in each vegetation type.

The canopy cover of the mixed dipterocarp indicates the less penetration of sunlight and less interference from other light sources such lunar cycle in time of sampling, which is competing to light trap light during the collection of moth species, which gains the highest moth species documented during the sampling period. Besides, higher canopy cover provides indirect sunlight penetration on moth species since most of the moths were nocturnal species, and a few of them are diurnal species that can adapt to direct sunlight penetration (Sutrisno, 2007). In addition as cited by Horvath et al. (2016) canopy layer per se plays a decisive role for herbivore communities.

There was low moth species composition and individuals in grassland with 32 moth species and 81 individuals. This probably due to the limited availability of food resources since grassland was dominated by cogon grasses and few trees, and there was no water source present. It is consistent with Sutrisno (2007). Hence, larvae of moths often show high specificity to host plants. Also, there were certain moth families associated with grasses, but most moths are forest inhabitants.

As cited by Horvath et al. (2016), habitat suitability among moth species is also critical in species composition, and distribution in different habitats. Most of the insect herbivores respond rapidly to changes in their habitat. Moth species specifically live on the area where they are capable of surviving and a habitat that can give them their resources to survive.

Grassland moth species also preferred a low temperature; their numbers decreased with the increasing temperature that resulted in increasing water precipitation (Kobori & Amano, 2003). Increase of precipitation may lead to less air humidity that causes Lepidopteran immature stages to migrate to other habitats which are suitable for metamorphism (Wallisdevries & Van Swaay, 2006). This is consistent to the study of Lara-Perez et al., (2016) that

Many ectothermic insects depend on external thermal conditions such as evapotranspiration and temperature to maintain viable populations and these variables are the best predictors of species richness and abundances of some families of large moths and butterflies. However, due to expansion of natural grassland, study showed the decline of moth species (Kamikura and Sakata, 2019).

CONCLUSION AND RECOMMENDATION

Therefore, Mt. Musuan is a home of 29 species of moth belong to twenty-four (24) genera and eleven (11) families. These include family Aganinae, Erebidae, Geometridae, Lassiocampidae, Notodontidae, Sesiidae, Saturniidae, Sphingidae, Uraniidae, Xyloryctidae and Noctuidae. Sixteen (16) species are randomly distributed. The study recommends increasing the number of sampling, and more sampling techniques must be done to increase the number of species present in the study site.

REFERENCES

BADON, J. A., L. LAHOM-CRISTOBAL and A. A. TALAVERA.2019. Philippine Lepidoptera butterflies and moths, Inc. A new online resource for southeast Asian Lepidoptera. News of The Lepidopterists' Society Volume 61, Number 4.

ELLIS, S. 2003. Habitat quality and management for the northern brown argus butterfly Ariciaartaxerxes (Lepidoptera: Lycaenidae) in North East England. Biological Conservation, 113, 285-294.

HORVÁTH, B. V. TÓTH and F. LAKATOS. 2016. Relation between canopy-layer traits and moth communities in sessile oak-hornbeam forests. North-western Journal of Zoology 12 (2): 213-219

KAMIKURA, M. and Y. SAKATA. 2019. Fauna of nocturnal moth species collected in a semi-natural grassland at Kanpu-zan in northern Japan. Biodiversity Data Journal.

KAWAHARAA,A., D. PLOTKINA, M. ESPELANDA, K. MEUSEMANND, E. F. A. TOUSSAINTA, A. DONATHE , F. GIMNICHE , P. B. FRANDSENH, A. ZWICKF , M. DOS REISJ , J. R. BARBERK , R. S. PETERSC , S. LIUL, X. ZHOUM, C. MAYERE , L. PODSIADLOWSKIE , C. STORERA , J. E. YACKN , B. MISOFE and J. W. BREINHOLT. 2019. Phylogenomics reveals the evolutionary timing and pattern of butterflies and moths. PNAS. vol. 116. no. 45. 22657–22663

MCALEECE, N. 1993. Biodiversity Professional Version 2 Devised by PJD Lambhead, GLJ Paterson and DJ Gage. Program Software. The Natural History Museum and the Scottish Assoc. for Marine Science.

LARA-PÉREZ, L., J. CAMPOS-DOMÍNGUEZ, F. DÍAZ-FLEISCHER, J. ADAME-GARCÍA and A. ANDRADE-TORRES. 2017. Species richness and abundance of Saturniidae (Lepidoptera) in a tropical semi-deciduous forest of Veracruz, Mexico and the influence of climatic variables.

SUTRISNO, H. 2008. Moth Diversity at Gunung Halimun-Salak National Park, West Java. Laboratory of Entomology, Zoology Division, Research Center for Biology, Jalan Raya Bogor Km. 46, Cibinong 16911, Indonesia. HAYATI Journal of Biosciences, p 111-117.

TOWNSEND,M. AND P. WARING. 2007. Concise Guide to the moths of Great Britain and Ireland. British Wildlife Publishing.

WALLISDEVRIES, M.F. and VAN SWAAY, C.A.M. 2006. Global warming and excess nitrogen may induce butterfly decline by microclimatic cooling. Glob. Change Biol. 12: 1620-1626.

ZILLIE A. and HOGENES, W. 2002. An annotated list of the fruit-piercing moth genus *Eudocima and billberg*, 1820 (sensu POOLE) with descriptions of four new species (Lepidoptera: *Noctuidae, Catocalinae*). Naturhistorisches Museum Wien, Tokyo, Japan. Pg 30-40.

Table 1

Species composition of macromoth in Mt. Musuan, Maramag, Bukidnon

Mixed Dipterocarp	Grassland	Pine Tree
Aganinae		
Asota	*Asota*	*Asota*
Erebidae		
Amerila	*Amerila*	*Amerila*
Carriola ecnomoda	*Carriola ecnomoda*	*Carriola ecnomoda*
Creatonotos gangis	*Creatonotos gangis*	*Creatonotos gangis*
Nyctemera baulus	*Nyctemera baulus*	*Nyctemera baulus*
Oenistis altica	*Oenistis altica*	*Oenistis altica*
Eudocima sp 1		*Eudocima sp 1*
Oxyodes scrobiculata		*Oxyodes scrobiculata*
Geometridae		
Gastrina cristaria	*Gastrina cristaria*	*Gastrina cristaria*
Pingasa chlora	*Pingasa chlora*	*Pingasa chlora*
Taxeotis sp. 1	*Taxeotis sp. 1*	*Taxeotis sp. 1*
Hypomecis	*Hypomecis*	*Hypomecis*
Pingasa ruginaria	*Pingasa ruginaria*	*Pingasa ruginaria*
Lassiocampidae		
Pinara sp. 1	*Pinara sp. 1*	*Pinara sp. 1*
Noctuidae		
Apsarasa radians	*Persectania dyscrita*	*Apsarasa radians*
Persectania dyscrita	Noctuidae sp. 1	*Persectania dyscrita*
Noctuidae sp. 1		Noctuidae sp. 1
Noctuidae sp. 2	Noctuidae sp. 3	Noctuidae sp. 2
Noctuidae sp. 3		Noctuidae sp. 3
Noctuidae sp. 4	Noctuidae sp. 5	Noctuidae sp. 4
Noctuidae sp. 5		Noctuidae sp. 5
Notodontidae		
Dudusa	*Dudusa*	*Dudusa*
Sesiidae		
Sesiidae sp. 1	*Sesiidae sp. 1*	
Spingidae		
Ambulyx sp. 1		*Ambulyx sp. 1*
Ascomeryx sp.1	*Ascomeryx sp.1*	
Saturniidae		
Cricula sp. 1		*Cricula sp. 1*
Uraniidae		
Ourapteryx	*Ourapteryx*	*Ourapteryx*
Urapteroides sp. 1		*Urapteroides sp. 1*
Xyloryctidae		
Crypthophasa russata		Crypthophasa russata
Total Number of Species 29	20	28
Total number of individuals 314	81	145

Plate 1. Family Geometridae: *Gastrina cristaria* (a), *Pingasa chlora* (b), *Taxeotis* sp.1 (c), *Hypomecis* (d) and *Pingasa ruginaria* (e).

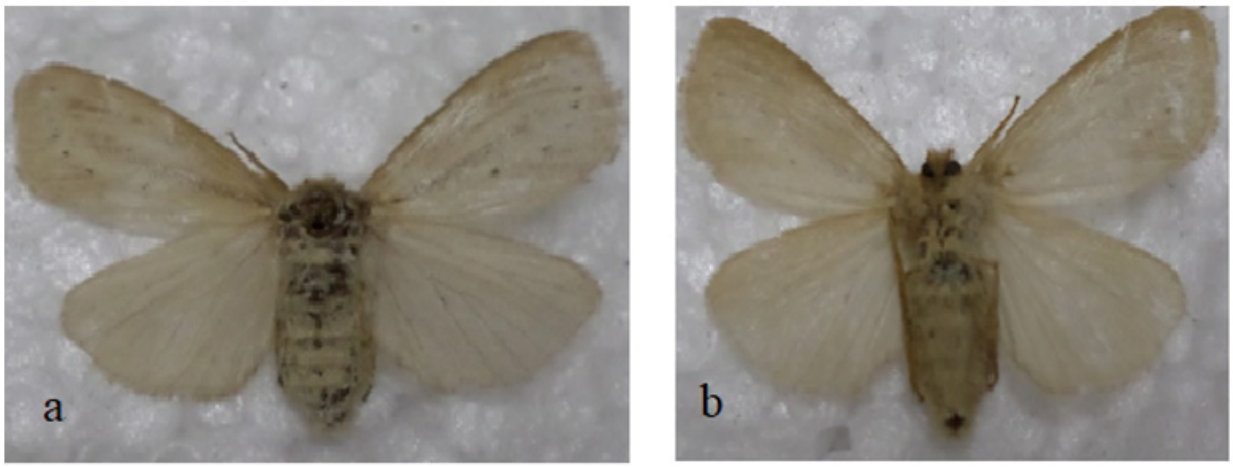


Plate 2. Family Lassiocampidae: *Pinara* sp. 1, dorsal (a) and ventral (b) view.

Plate 3. Family Erebidae: *Carriola ecnomoda* (a) , *Amerila* (b), *Nyctemera baulus* (c) *Creatonotos gangis* (d) *Oenistis altica* (e), *Eudocima sp 1*(f), *Oxyodes scrobiculata* (g)

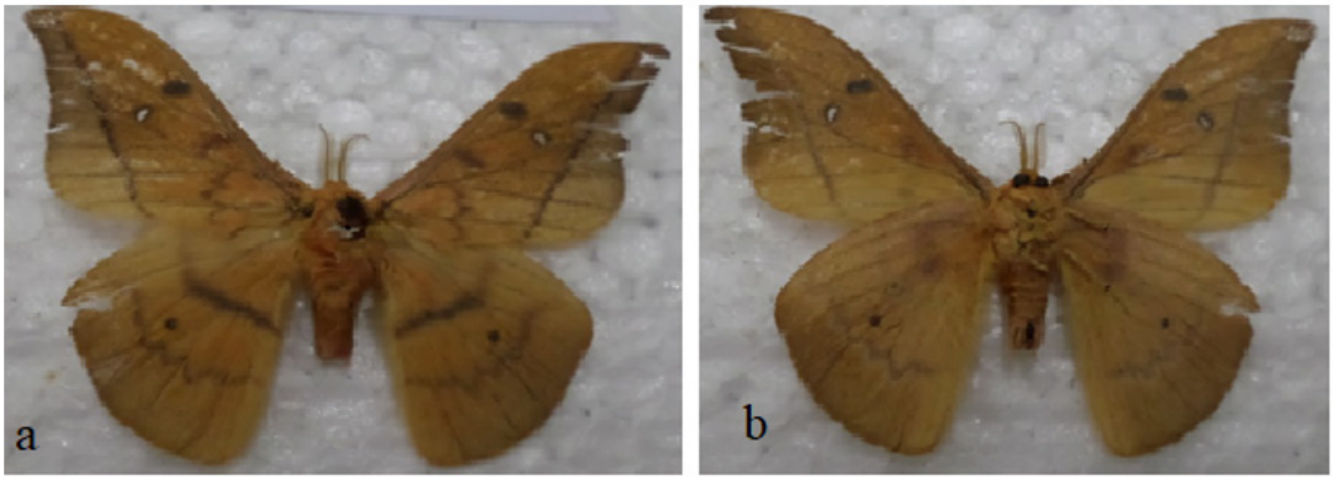


Plate 4. Family Saturnidae: *Cricula* sp. 1, dorsal (a), ventral (b) view.

Plate 5. Family Sesiidae: *Sesiidae* sp. 1

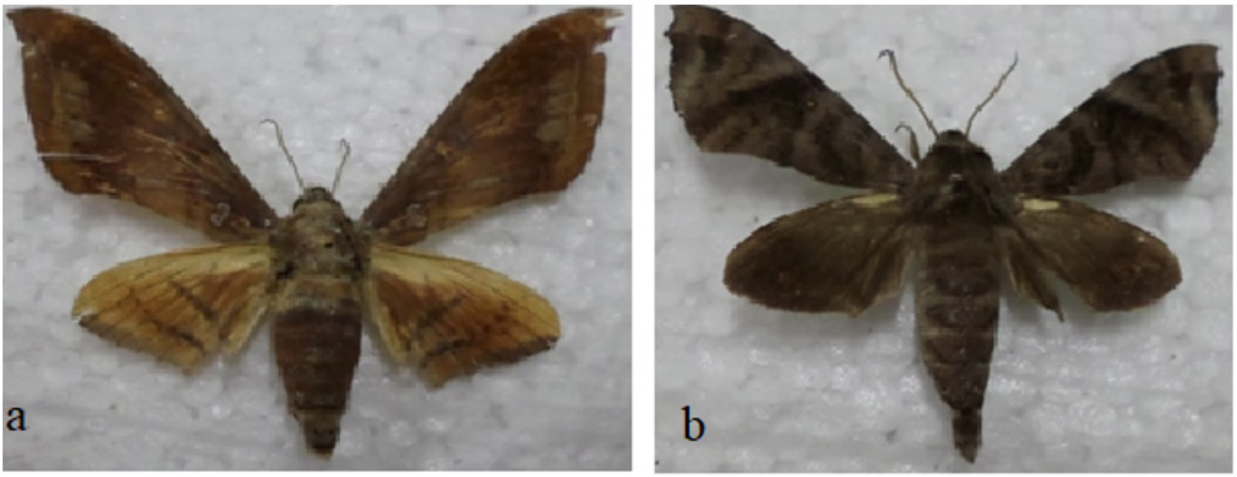


Plate 6. Family Spingidae: *Ambulyx* sp. 1 (a) and *Ascomeryx* sp. 2 (b).

Plate 7. Family Uraniidae: *Ourapteryx* (a), *Lyssa* sp. 1 (b) and *Urapteroides* sp. 1 (c).

Plate 8. Family Xyloryctidae: *Crypthopasha russata*, dorsal (a) and ventral (b) view.

Plate 9. Family Noctuidae: *Apsarasa radians* (a), *Persectania dyscrita* (b), *Noctuidae* sp. 1(c), *Noctuidae* sp. 2 (d) *Noctuidae* sp. 3 (e), *Noctuidae* sp. 4 (f), *Noctuidae* sp. 5 (g)

Plate 10. Subfamily Aganinae: *Asota* (a)

Plate 11. Family Notodontidae: *Dudusa* (a)

Central Mindanao University
Journal of Science
ISSN Print: 0116-7847 ISSN Online: 2704-3703

Phytochemical and Oral Toxicity Studies of *Chromolaena odorata* L. (King and Robinson)Leaf Extract

Anecito G. Juan, Alan P. Dargantes, Kristian Ed M. Serafica,
Kurt Melon C. Calonia, and Rowland M. Awiten

Department of Anatomy, Physiology, and Pharmacology, College of Veterinary Medicine,
Department of Medicine, Surgery and Zoothecnics, College of Veterinary Medicine,
College of Veterinary Medicine, Central Mindanao University, Musuan, Bukidnon, Philippines, 8714

ABSTRACT

In this study, the ethanolic leaf extract of *Chromolaena odorata* was analyzed for qualitative and quantitative composition and evaluated for oral toxicity in Swiss Webster albino mice. The mice were grouped into two and tested for acute (fixed single dose of 2000 and 5000 mg/kg) and sub-acute (daily dose of 250 and 500mg/kg extract for 28 days) toxicity. Animal behavior, body weight, morbidity, and mortality were monitored for 14 days (acute) and 28 days (sub-acute), respectively. Hematologic and blood chemistry parameters (alanine transaminase (ALT), blood urea nitrogen (BUN), creatinine) were measured and analyzed. The mice were sacrificed and necropsied at the end of the study, and organ weights were analyzed. Based on the phytochemical analysis, *C. odorata* contained phenols, flavonoids, tannins, saponins, and anthraquinones. Total phenolic and flavonoids were 146.69±10.25 mg gallic acid equivalent (GAE/L) and 25.75±2.64 mg quercetin equivalent (QE/g). A single dose of the extract (2000-5000mg/kg) is non-lethal but causes temporary toxicity signs. Repeated doses (250-500 mg/kg) caused low ALT, mortality (500 mg/kg), tachypnea, dry hair coat, and alopecia. Both acute and sub-acute toxicity of the leaf extract hampers growth.

Keywords: Chromolaena odorata, ethanolic, phytochemicals, oral toxicity

INTRODUCTION

In rural areas where commercially prepared drugs appear to be scarce and costly, people often resort to herbal medicines to treat illnesses. One plant that is traditionally used to treat ailments is C. odorata. The plant is herbaceous to woody perennial with a bushy habit (Crutwell, 1989; Gautier, 1992). It is popularly known as "hagonoy" in the Philippines. The plant medicinal properties include relief of fever, lethal on mites and ticks, heart tonic, antimicrobial, expel worms, wound healing, relieve muscle spasms, astringent, anti-inflammatory, diuretic, antihypertensive and potential treatment for diabetes mellitus (Iwu et al., 1993; Bunyapraphatsara et al., 2000; Vital & Rivera, 2004; Panda et al., 2010; Kikiowo et al., 2020). Medicinal and toxicological actions are presumed to be produced by the phytochemical components of plants, which, depends upon the variety, growing conditions, cooking, and processing (Saxena et al., 2013). Putri et al. (2019) revealed several antioxidants contained in leaves including new flavonone odoratenin. C. odorata leaf can serve as the potential sources of fatty acids (Alara et al. (2019). Several authors cited plants component high on phenolic and flavonoid content is responsible for its medicinal value (Atmani, 2009; Khodammi, 2013; Tapas, 2008).

Several studies of C. odorata extract reported to decreased ALT, AST, and alkaline phosphatase levels in albino rats (Anyanmu et al., (2017) while Asomugha et al. (2013) and Yakubu et al. (2012) reported elevated liver enzymes. The presence of protease inhibitors and saponins (Asomugha et al., (2015) and pyrrolizidine alkaloids (Fu et al., 2002) makes the extract potentially toxic. Pyrrolizidine alkaloids are carcinogenic and hepatotoxic chemicals. Some authors noted poisonous effects of the plant, such as changes in physical behavior and deaths in rats and destruction of cells in brine shrimps (Ogbonia et al., 2010; Asomugha et al., 2015). Other researchers reported elevated BUN and creatinine and abnormal intestinal histology of albino rats (Anyanwu et al., 2017), and it induces cancer and destructive to liver cells (Fu et al., 2002). Asomugha et al. (2015) reported that C. odorata ethanolic extract is relatively non-toxic with an LD50 of >5000 mg/kg.

Existing studies on C. odorata suggest possible medical use, yet toxic effects are not well documented especially using the standard OECD guidelines. Hence, the present study determined C. odorata ethanolic leaf extract bioactive components and evaluated for acute and sub-acute oral toxicity in Swiss Webster albino mice.

ARTICLE INFORMATION
Anecito G. Juan
Email Address: dokj1910@gmail.com
Received: Dec 1, 2020; Accepted: July 26, 2021
DOI: https://doi.org/10.52751/deju9088

METHODOLOGY

Plant Collection and Preparation

Five (5) kilograms of *C. odorata* mature healthy leaves (dark-green coloration, fully expanded) were collected, clean with flowing water, and rinsed with distilled water. The leaves were air-dried for five days and were subjected to size reduction using a mechanical grinder until the coarse powder was obtained. Powdered leaves were soaked in 95% ethanol for three days. Extracts were then filtered, and filtrates were concentrated in a vacuum using a rotary evaporator. The leaves were selected and collected from Musuan, Maramag, Bukidnon (7.8706°N, 125.0691°E).

Animals

Swiss Webster albino mice (12 females and 15 males; 6-8 weeks;15-30g) were obtained from the Philippine Institute of Tropical Medicine (PITAHC). The animals were kept in plastic and wire cages with wood scrape as beddings, acclimatized for one week, and maintained at room temperature (22±2°C) and relative humidity (45-65%) under 12h night and 12h light cycle. All animals were regularly cleaned, fed with a pelleted diet, and provided with water ad libitum. The research complied with the requirements of the Institutional Animal Care and Use Committee of the College of Veterinary Medicine, Central Mindanao University, Philippines.

Phytochemical analysis

Test for Alkaloids

Three ml of the ethanolic extract was added in a test tube and mixed with 1 ml sulfuric acid, and allowed to stand. The mixture was added with 2-3 drops of Dragendorff's reagent. A yellow-orange precipitate indicates the presence of alkaloids.

Test for saponins

In a test tube, 1 ml of the extract was gradually added with six concentrated sulfuric acid drops. A yellow to a red color reaction within 30 minutes, followed by violet or blue-green, indicates saponins' presence.
Test for steroids and terpenoids

A mixture of 1 ml of the extract and 1 ml of acetic anhydride was made and put in an ice bath in a test tube. Then 1 ml of the concentrated sulfuric acid was gradually added. A red color reaction indicates the presence of terpenoids and bluish green for steroids.

Test for anthraquinones

A mixture of 1 ml of the extract was made in a test tube with 0.5 ml of 5% KOH in methanol. The orange color reaction indicates the presence of anthraquinones.
A mixture of 0.5 ml of the extract was made with 1 ml water in a test tube and added with 1-2 drops of 10% aqueous ferric chloride. The blue color indicates gallic tannins, and the green-black color indicates cathecholic tannins.

Determination of total flavonoid content

The aluminum chloride method using quercetin as standard was used for the determination of the total flavonoid content. A mixture of 4 ml distilled water, and 1 ml of the extract was initially mixed in a volumetric flask, and 0.5 ml of 5% $NaNO_2$ was added. After 5 minutes, the mixture was added with 0.3 ml of 10% $AlCl_3$. After 6 minutes, 2 ml of 1M NaOH was added, and distilled water was added to make 10 ml volume. Absorbance was measured against blank at 430 nm, and total flavonoid content is expressed as quercetin equivalent in mg/g extract (mg QE/g).

Determination of total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu assay (Singleton and Rossi, 1965). The extracts were dissolved in a DMSO (0.1%): methanol: water (15:5:2) solution at 2 mg/ml. A volume of 20 ml of the mixture was then added with Folin-Ciocalteu reagent (1:10) and was shaken. A 5% sodium carbonate solution was added after 30 minutes. The microplate was incubated at room temperature for 2 hours, then the absorbance at 750 nm using spectra Max250 was measured. Gallic acid was expressed as mg gallic acid equivalent (GAE) per liter.

All measurements were triplicated.

Acute oral toxicity test

The extract was dissolved with Dimethyl Sulfide (DMSO, 0.1%). The acute oral toxicity was conducted as per OECD 423 guidelines. Twelve (12) female Swiss Webster albino mice rats were randomly allocated into four (4) groups of three (3) mice each. Each mouse per treatment group was singly gavage with distilled water, DMSO (0.1%), 2000 mg/kg extract, and 5000 mg/kg extract, respectively. The mice were monitored daily for signs of toxicity and mortality for 14 days. Immediate observations for physical behavior changes and mortality were done, commencing at 30 minutes after dosing, every hour for the first day, and daily for two weeks. Animals were weighed twice, initially at the start and finally at the end of the study. At the study's termination, all surviving animals were sacrificed by cervical dislocation, necropsied, and gross examination of organs was performed.

Sub-acute oral toxicity test

The extract was dissolved with 10% Tween-20. Fifteen (15) male Swiss Webster albino mice were utilized for the 28-day oral toxicity test. The mice were randomly distributed into three treatment groups of 5 mice each. The treatment group was gavage daily with 10% tween-20 solution, 250 and 500 mg/kg extract, respectively, for 28 days.

The animals were weighed weekly and observed daily for toxicity signs, e.g., mortality, behavioral, and physical changes. On the 28th day of treatment, animals were given an anesthetic (Tiletamine+Zolazepam), and blood samples were collected via intracardiac puncture. Plasma and serum samples of each mouse were subjected

Table 1

Chromolaena odorata ethanolic leaf extracts bioactive components.

Components	Result
Alkaloids	-
Tannins	+
Saponins	+
Terpenoids and Steroids	-
Anthraquinones	+

Legend: (+) present; (-) absent

Table 2

Chromolaena odorata ethanolic leaf extracts phenolic and flavonoid content.

Components	Result
Total phenolic content	146.69+10.2 mg GAE/L
Total flavonoid content	25.75+2.64 mg QE/g

Legend: gallic acid equivalent (GAE/L); quercetin equivalent (QE/g)

to hematological and biochemical analysis, respectively. Animals were then subjected to euthanasia by cervical dislocation. Gross examination and weighing of the organs (liver, kidney, spleen, heart, and intestine) were done (OECD, 2008). The relative organ weight (ROW) of each animal was calculated as follows:

$$ROW = \frac{absolute\ organ\ w}{t./\ b.\ wt.\ of\ the\ animal\ on\ sacrifice\ day}\ x\ 10$$

Statistical analysis

Measurable data are expressed as mean±SD. In the acute toxicity test, initial and final body weight data were analyzed using a t-test. One way analysis of variance was used to test the difference between treatment groups in acute and sub-acute toxicity tests. Where significant differences were observed, the Tukey's Post Hoc test was used to identify and compare the differences between treatment means. Measured values were considered significant if $p<0.05$.

RESULTS AND DISCUSSION

Phytochemical Analysis

Analysis of the plant ethanolic leaf extract bioactive components revealed the presence of phenols, flavonoids, tannins, and saponins, while alkaloids, terpenoids, and steroids were absent (Table 1). The study demonstrated high levels of total flavonoids and total phenolics (Table 2). The finding on the absence of alkaloids contradicts Fu et al.'s (2002) report that the plant contains toxic pyrrolizidine alkaloid.

Demonstration of phytochemicals in the extract with high phenolic and flavonoid content confirms the plant's medicinal value (Atmani et al., 2009; Khodammi et al., 2013; Tapas et al., 2008). However, saponins' presence makes the extract potentially toxic, as reported by Asomugha et al. (2015).

Acute oral toxicity

Within 30 minutes after a single administration of the extract (2000 mg/kg and 5000 mg/kg), the mice exhibited temporary erection of hairs, tachypnea, lacrimation, excessive saliva, retching, unusual sound, lethargy, and lack of touch response, which disappeared within a day (Table 3). This finding agrees with the study of Ogbonia et al. (2010) and Asomugha et al. (2015) that a high dose of *C. odorata* extracts causes signs of toxicity. However, Ogbonia et al. (2010) toxicity observations were limited to 72 hrs. post treatment, which varied with the 14 days observation time recommended by the OECD.

At two weeks of observation, the mice showed no persistent signs of abnormality in behavior and physical attributes. Morbidity nor mortality was not observed; thus, the LD50 was estimated to be >5000 mg/kg, similar to the findings of Asomugha et al. (2015); study of Ogbonia et al. (2010) revealed LD50 of 16.50 g/kg body weight. Though the mice's body weight initially increased in all groups, it is less pronounced with the extract-treated groups versus the control (Table 4). However, the weight difference among treatment groups is not statistically significant ($p>0.05$). This finding indicates that the extract exhibited a negative effect on growth. Although gross organ lesions were not observed at necropsy and in the absence of histopathologic examination, it is premature to dismiss toxicity suspicion.

These findings indicate the relative safety of a single oral dose of the extract. Based on the rating of toxic chemicals, a single dose of *C. odorata* ethanolic leaf extract is slightly toxic (LD50, >5000 mg/kg).

Table 3

Behavioral and physical observations in mice given with a single oral dose of Chromolaena odorata ethanolic leaf extract.

Observations	DH^2O	DMSO (0.1%)	*C. odorata* ethanolic leaf extract	
			2000 mg/kg	5000 mg/kg
Skin color	n	n	n	n
Piloerection	-	-	+	+
Tachypnea	-	-	+	+
Stool	n	n	n	n
Lacrimation	-	-	+	+
Salivation	-	-	+	+
Retching	-	-	+	+
Vocalization	-	-	+	+
M. membrane	n	n	n	n
Palpebral opening	n	n	n	n
Tremor	-	-	-	-
Staggering gait	-	-	-	-
Grip	n	n	n	n
Opisthotonos	-	-	-	-
Lethargy	-	-	-	+
Touch response	n	n	++	++
Morbidity	-	-	-	-
Mortality	-	-	-	-

Legend: (n) normal; (++) abnormal (-) absent; + (present)

Table 4

Body weight of mice given a single dose of *C. odorata* ethanolic leaf extract.

Weight	DH^2O	DMSO (0.1%)	*C. odorata* ethanolic leaf extract	
			2000 mg/kg	5000 mg/kg
Initial b. wt	26.66 ±1.1	24±2.2	25.33 ±0.5	24.1 ±0.1
Final b. wt. ^{ns} (Pvalue=0.07)	29.67 ±2.1	26±2.3	26.33 ±0.6	24.33±4
T-test on body wt. ^{ns}	0.12	0.28	0.10	0.89
% Difference in body wt.	11.15	8.36	3.93	0.90

Legend: Values are expressed as mean ± SD; ns – not significant

Subacute oral toxicity

Initially, the mice were repeatedly dosed with the extract at 1000-2000 mg/kg; however, several morbidity and mortality were noted after a few days. Based on OECD guidelines, the fixed-dose was then reduced to the next lower level.

Repeated oral administration for 28 days of 250-500 mg/kg *C. odorata* ethanolic leaf extract to the mice showed no marked abnormality on most of the behavior and physical parameters. However, some signs of toxicity were observed (Table 5). After dosing, the mice showed heavy breathing for two days, persistent dry hair coat, and alopecia on the ventral region commencing on day 9 of treatment. One (1) mortality occurred at day 20 to the group that received 500 mg/kg extract, exhibiting liver congestion, and distension of intestines upon necropsy. This finding indicates that repeated administration of the extract caused significant signs of toxicity. It agrees with Asomugha et al. (2015) that *C. odorata* extract causes physical abnormality and mortality. However, no gross lesions were observed in all mice that survived the study's entire duration at necropsy.

The bodyweight of mice's dosed with the extract progressively improved, the increase is much lesser and declined at day 28 compared to the control group (Table 6). However, there was no statistical difference in body weight ($p>0.05$) among treatment groups. The finding indicates that repeated administration of the extract at 250-500mg/

Table 5

Behavioral and physical observations in mice given daily oral doses (28 days) of *C. odorata* ethanolic leaf extract.

Observations	Control	*C. odorata* ethanolic leaf extract	
		250 mg/kg	500 mg/kg
Skin color	n	n	n
Piloerection	-	-	-
Alopecia	-	-	+
Tachypnea	-	+	+
Stool	n	n	n
Lacrimation	-	-	-
Retching	-	-	-
Vocalization	-	-	-
M. membrane	n	n	n
Palpebral opening	n	n	n
Tremor	-	-	-
Staggering gait	-	-	-
Grip	n	n	n
Opisthotonus	-	-	-
Alertness	n	n	n
Touch response	n	n	n
Morbidity	-	-	-
Mortality	-	-	1/5

Legend: (n) normal; (-) absent; (+) present

Table 6

Mean body weights of mice given with repeated oral dose (28 days) of C. odorata ethanolic leaf extract.

Treatment	Mean Body Weight (grams, days)					% Difference in wt.
	0	7	14	21	28	
Control	26.20 ±1.69	28.32 ± 1.66	29.50 ± 1.03	30.15 ± 1.12	30.77 ± 1.18	15.3
250 mg/kg	25.56 ± 0.82	26.43 ± 1.64	27.66 ± 2.33	29.20 ± 2.70	28.43 ± 3.66	10.9
500 mg/kg	25.57 ± 0.89	27.28 ± 1.23	27.56 ± 2.09	27.83 ± 3.77	27.18 ± 3.86	5.89
p-value^{s}	0.642	0.185	0.233	0.448	0.242	

Legend: Values are expressed as mean ± SD; ns – not significant, p>0.05

Table 7

Relative organ weights of mice given with repeated oral dose C. odorata ethanolic leaf extract for 28 days.

Organ	Control	C. odorata ethanolic leaf extract		*p*-value^{ns}
		250 mg/kg	500 mg/kg	
Liver	4.72 ± 0.24	5.05 ± 1.05	4.90 ± 1.55	0.877
Kidney	1.57 ± 0.27	1.71 ± 0.28	1.55 ± 0.22	0.568
Spleen	0.54 ± 0.16	0.57 ± 0.16	0.53 ± 0.42	0.966
Heart	0.60 ± 0.12	0.54 ± 0.13	0.52 ± 0.16	0.621
Intestines	9.71 ± 1.05	10.85 ± 1.17	11.38 ± 0.84	0.087

Note. Values are expressed as mean (grams) ± SD; ns – not significant, p>0.05

Table 8

Serum ALT, BUN, and creatinine values of mice were given with oral doses of *C. odorata* ethanolic leaf extract for 28 days.

Parameter	Control	*C. odorata* ethanolic leaf extract		*p-value*
		250 mg/kg	500 mg/kg	
Creatinine (mg/dL)	0.42 ±0.08	0.43 ± 0.10	0.43 ± 0.06	0.983^{ns}
Urea/BUN (mg/dL)	27.14 ± 6.06	23.32 ± 3.72	28.38± 4.04	0.283 ^{ns}
ALT (IU/L)	70.9 ±13.4a	54.9 ±13.4 ^{ab}	42.78 ±5.4 ^{b}	0.009 **

Note: Values are expressed as mean ± SD; ns – not significant; ** highly significant, p<0.01

Table 9

Blood values of mice given with an oral dose of *C. odorata* ethanolic leaf extract for 28 days.

Parameter	Control	*C. odorata* ethanolic leaf extract		*p*-value^{ns}
		250 mg/kg	500 mg/kg	
RBC (10^6/ uL)	8.60±0.9	8.04±1	7.9±1.2	0.572
Hemoglobin (g/L)	13.8±1.6	14.50±3.7	13.8±2.3	0.891
Hematocrit(%)	46.4±4.4	41.75±7.6	41.8±6.6	0.533
WBC (10^9/L)	7.5±1.8	6.25 ±1.8	4.50±1.1	0.054
Neutrophil (%)	27.4±12	54.40±7.8	31.75±8.5	0.623
Lymphocyte (%)	63.6±9.7	72.60±5.8	63.75±8.9	0.196
Monocyte (%)	1.2±0.5	1.00±0.4	2.00±0.9	0.067
Eosinophils (%)	1.2±0.6	1.50±1.1	0.88±0.5	0.546
Basophils (%)	0.14±0.1	0.22±0.1	0.45±0.4	0.144
Platelet (10^3/uL)	1045.6± 147.87	933.4± 102.86	928.5± 158.40	0.358

Legend: Values are expressed as mean ± SD; ns – not significant, p>0.05

kg for four weeks harmed animals' growth, similar to acute toxicity.

Relative organ weight of the liver, kidney, spleen, heart, and intestines in mice given multiple doses of the extract vary with the control group (Table 7). An increase in the liver and intestines' weights in mice given with the extract was noted and may indicate inflammation. However, the organ weight difference among treatment groups was not significant (p>0.05). Though there is no statistical difference, this finding indicates that the extract induces damage to the liver and intestines. This finding agrees with Anyanwu et al. (2017) study that the extract causes damage to rat intestine histology.

Serum chemistry analysis showed no significant difference in creatinine and BUN values; however, a highly significant decrease in ALT values compared to the control group was observed (Table 8). This finding was corroborated by the study of Anyanmu et al. (2017) that the extract decreased ALT, AST, and alkaline phosphatase levels in albino rats. However, this contradicts the findings of Fu et al. (2002) and Asomugha et al. (2013), who reported elevated liver enzymes in their studies. Ramaty et al. (2014) explained that low ALT values might serve as an independent predictive marker for long-term mortality.

Hematological values (red blood cells (RBC), hemoglobin, hematocrit, white blood cells (WBC), and platelets) of mice given with the extract showed no significant differences than the control group (Table 9). This observation is similar to the oral administration of Carica papaya and Euphorbia hirta extracts in mice, as reported by Ping et al. (2013). However, slight variations in some parameters were observed: RBC, hematocrit, WBC, and platelets slightly decline. This result may indicate minimal toxicity of the extract to the blood cells.

CONCLUSION

Chromolaena ethanolic leaf extract contains bioactive components with potential toxic properties. The absence of morbidity, mortality, and persistent alteration on physical parameters and behavior makes the single oral dose (5000mg/kg) relatively safe. However, caution should be taken on repeated use of the extract, even at a lower dose (250-500 mg/kg), since it can cause significant toxic effects, e.g., alopecia, dry hair, tachypnea, low ALT, and mortality. These findings revealed that multiple oral doses of the extract for four weeks caused significant signs of toxicity. Caution should be taken if used repeatedly in ethno-medicine. Both acute and sub-acute toxicity of the

leaf extract hampers growth.

REFERENCES

Alara, O. R., and Abdurahman, N. H. (2019). GC–MS and FTIR analyses of oils from *Hibiscus sabdariffa, Stigma maydis and Chromolaena odorata* leaf obtained from Malaysia: Potential sources of fatty acids. *Chemical Data Collections* 20.

Asomugha, R. N., Ezejiofor, A. N., Okafor, P. N. & Ijeh, I. I. (2015). Acute and cytotoxicity studies of aqueous and ethanolic leaf extracts of *Chromolaena odorata. Pakistan Journal of Biological Sciences 18*: 46-49.

Anyanwu, S., Iinyang, I. J., Asemota, E. A., Obioma, O. O., Okpokam, D. C. & Aguc, V. O. (2017). Effect of ethanolic extract of C*hromolaena odorata* on the kidneys and intestines of healthy albino rats. *Integrative Medicine Research 6*: 292-299.

Atmani, D., Chaher, N., Atmani, D., Berboucha, M., Debbache, N., & Boudaoud, H. (2009). Flavonoids in human health: From structure to biological activity. *Current Nutrition & Food Science, 5(*4), 225-237.

Bunyapraphatsara, N. & Chokechaijaroenporn, O. (2000). Thai medicinal plants. Faculty of Pharmacy, Mahidol University and National Center for Genetic Engineering and Biotechnology 4: 622-626.

Cruttwell, M. (1989). Siam weed: a new threat to Australia's north. *Plant Protection Quarterly, 4*(1), 3-7.

Fu, P. P., Yang, Y. C., Xia, Q., Chou, M. C., Cui, Y. Y. & Lin, G. (2002). Pyrrolizidine alkaloids-tumorigenic components in Chinese herbal medicines and dietary supplements. *Journal of Food and Drug Analysis 10*(4): 198-211.

Gautier, L. (1992). Taxonomy and tropical distribution of a tropical weed: *Chromolaena odorata* (L.) R. King & H. Robinson. *Candollea*. 645-662.

Iwu, M. M. (1994). African medicinal plants in the search for new drugs based on ethnobotanical leads. In *Ciba Foundation Symposium 185-Ethnobotany and the Search for New Drugs* (pp. 116-129). John Wiley & Sons, Ltd.

Khoddami. A., Wilkes, M. A., & Roberts, T. H. (2013). Techniques for analysis of plant phenolic compounds. *Molecules, 18*(2), 2328-2375.

Kikiowo, B., Ogunleye, J. A., Iwaloye, O. & Ijatuyi, T. T. (2020). Therapeutic potential of *Chromolaena odorata* phyto-constituents against human pancreatic α-amylase, *Journal of Biomolecular Structure and Dynamics* 1-12.

OECD 423, (2001). Guidance document on acute oral toxicity. Environmental health safety monograph series on testing and assessment: 6-24.

Ogbonia, S. O., Mbaka, G. O., Anyika, E. N., Osegbo, O.M., & Igbokwe, N. H. (2010). Evaluation of acute toxicity in mice and subchronic toxicity of hydroethanolic extract of *Chromolaena odorata* (L.) King and Robinson (Fam. Asteraceae) in rats. *Agriculture and Biology Journal of North America, 1*(5), 859-865.

Panda, D., & Dash, G. K. (2010). Qualitative phytochemical analysis & investigation of anthelmintic and wound healing potentials of various extracts of *Chromolaena odorata* Linn. collected from the locality of Mohuda Village, Berhampur (South Orissa) *1*(2): 122-126.

Ping, K. Y., Darah, I., Chen, Y., Sreeramanan, S. & Sasidharan, S. (2013). Acute and Subchronic Toxicity Study of Euphorbia hirta L. Methanol Extracts in Rats. H*indawi Journal*. 1-14.

Putri, D. A. & Fatmawati, S. (2019). A New Flavanone as a Potent Antioxidant Isolated from *Chromolaena odorata* L. Leaves, *Evidence-Based Complementary and Alternative Medicine*, vol. 2019, Article ID 1453612, 12 pages.

Ramaty, E., Maor, E., Peltz-Sinvani, N., Brom, A., Grinfeld, A., Kivity, S., … & Segal (2014). Low ALT blood levels predict the long-term cause of mortality among adults. A historical prospective cohort study. *European Journal of Internal Medicine 25*: 919-921.

Saxena, M., Saxena, J., Nema, R., Singh, D., & Gupta, A. (2013). Phytochemistry of medicinal plants. *Journal of pharmacognosy and phytochemistry, 1*(6).

Tapas, A. R., Sakarkar, D. M., & Kakde, R. B. (2008). Flavonoids as nutraceuticals: a review. *Tropical Journal of Pharmaceutical Research, 7*(3), 1089-1099.

Vital, P. G. & Rivera, W. L. (2009). Antimicrobial activity and cytotoxicity of *Chromolaena odorata* (L.f) King and Robinson and *Uncaria* Perrotti Merr. Extracts. *Journal of Medicinal Plants Research 3*(7): 511-518.

Yakubu, M.T. (2012), Effect of a 60-Day Oral Gavage of a Crude Alkaloid Extract From *Chromolaena odorata* Leaves on Hormonal and Spermatogenic Indices of Male Rats. *Journal of Andrology, 33*: 1199-1207.

Central Mindanao University
Journal of Science
ISSN Print: 0116-7847 ISSN Online: 2704-3703

Species Richness and Status of Pteridophytes in North-Eastern Part of Mount Timolan Protected Landscape, Zamboanga del Sur, Philippines

Liberty Grace L. Calimbo[1], Maricris G. Cudal[2*], Jasper B. Locson[1], Rogelio M. Hayag, Jr.[3], and Jehuel Nathan R. Daculio[1]

[1]Zamboanga del Sur Provincial Government College- Pagadian Campus, Zamboanga del Sur, Philippines, 7016
[2]Pagadian City International College, Zamboanga del Sur, 7016
[3]Ditoray National High School, Tukuran, Zamboanga del Sur, 7019

ABSTRACT

This study was conducted to assess the species composition and conservation status of pteridophytes in the north-eastern portion of Mount Timolan Protected Landscape. The studyrevealed a total of 98 species comprising 52 genera from 21 families. Tectariaceae, Polypodiaceae and Dryopteridaceae was the most represent family with Tectariaceae accounts the highest in terms of genera (9), followed by Polypodiaceae (8) and Dryopteridaceae (6). Whereas, Polypodiaceae has the highest in terms of species record (16 spp.), tailed by Tectariaceae (14 spp.) and Dryopteridaceae (10 spp.). Out of 98 recorded species, 95 were only identified up to species level. High number of accounted family of terrestrial ferns and lycophytes are known as good ecological indicator taxa for habitat destruction and level of human disturbances. In terms of status, eleven (11 spp.) are endemic to Philippines, eighteen (18 spp.) are economically important species, nine (9 spp.) vulnerable, five (5 spp.) classified as other threatened species and two (2 spp.) are non-native species of ferns and lycophytes was recorded. Generally, the high species richness of pteridophytes found in the north-eastern portion of Mount Timolan Protected Landscape are influenced by elevation gradient and susceptibility to human disturbances.

Keywords: endangered, ferns, lycophytes, endemic, assessment

INTRODUCTION

Pteridophytes also known as ferns and lycophytes are group of plants that reproduce through spores and consider as most economically important yet poorly documented non-flowering vascular plants (Amoroso et al., 2016). These group of plants are widely distributed in tropics and temperate regions with higher elevation (Oloyede and Odu, 2011). In Philippines are approximately 1,100 species of ferns and lycophytes belonging to 34 families from 154 genera (Smith et al., 2006). Roughly, this number continues to increase because of the extensive pteridological explorations done by scientists, researchers and other experts which leads to discoveries of new species of ferns and lycophytes (Amoroso et al;. 2020a; Amoroso et al., 2020b; Coritico, 2020). Pteridophytes are relatively information-rich species because the number of scientific studies in their systematics and evolution is relatively large across regions (Ebihara and Nitta, 2019). Some species have known aesthetic and economic importance such as ornamentals (Bharrati et.al. 2013), traditionally used to cure illnesses (Amoroso et al., 2013 and Yong, 2010) and as a source of food and dietary fibers (Amoroso et al., 2014). However, due to the alarming rate of deforestation in the country, many flora and fauna species are undeniably under threat. Among this are the human disturbances, which greatly affects the biodiversity of many vascular plants (Abotsi et al., 2020).

Mount Timolan Protected Landscape (MTPL) is strategically located in the four border towns of Tigbao, Guipos, San Miguel and Lapuyan, Zamboanga del Sur. It is one of the two identified protected area in the province of Zamboanga de Sur covering 2,244.54 hectarehighest elevation of 1,152 meter above sea level. It is known as ecotourism spot in the province and promote as a local tourist destination in the presence of a unique heart-shaped lake- the Lake Maragang. The Mount Timolan Protected Landscape formerly known as Zamboanga del Sur Provincial Park was proclaimed as Protected Area in 2000 by the virtue of Presidential Proclamation No. 354 in pursuance to the provisions of Republic Act 7586 otherwise known as National Integrated Protected Area System (NIPAS) Act of 1992. Moreover, the Mount Timolan Protected Landscape are among the protected areas in the country legislated in the Republic Act 11038 or the Expanded National Integrated Protected Area System (ENIPAS) Act of 2018(PAMB-MTPL, 2020). Despite of the importance of Mount Timolan Protected Landscape as watershed as it primarily forms the headwaters of its neighbouring municipalities and serves as the main source of river systems in lowland municipalities, very limited studies were done in the area.

ARTICLE INFORMATION
Maricris G. Cudal
Email Address: mcrisGcudal@gmail.com
Received: Sept 12, 2020; Accepted: June 29, 2021
DOI: https://doi.org/10.52751/svgb4056

This include among others, Philippine native earthworm fauna (Aspe and James, 2016), slender toad (Sanguila et al. 2011), avifauna (Paguntalan et al. 2011), freshwater fish (Cudal et al. 2019)and angiosperms (Ducot et.al. 2020). Eventually these efforts led to the discovery of two new species namely Polypheretima zamboangensisAspe and James, 2011 and Plagiostachys lourdesiae(Ducot et al., 2020). Surprisingly, the flora and other fauna of Mount Timolan Protected Landscape is poorly identified and remains undocumented, hence this study. This is the time that the species composition, richness and status of pteridophytes and its allies in the north-eastern portion of Mount Timolan Protected Landscape will be reported. The Lake Maragang mentioned in the above text is popularly known as ecotourism site located in the north-eastern part of MTPL. This research was purposely conducted in the north-eastern part of MTPL because it is more susceptible to human disturbance. Thus, this study aims to provide preliminary checklist of pteridophytes primarily ferns as well as lycophytes to determinetheir conservation status which could possibly serve as benchmark data for policy formulation by the Protected Area Management Board (PAMB) in the protection and conservation of Mount Timolan Protected Landscape and its environs.

MATERIALS AND METHODS

Study site

This study was done in an established human trail in the north-eastern part of Mount Timolan Protected Landscape last December 2018(Figure 1). A series of transect walks was conducted from the Lake Maragang to the baseline going to Mount Timolan Protected Landscape peak.

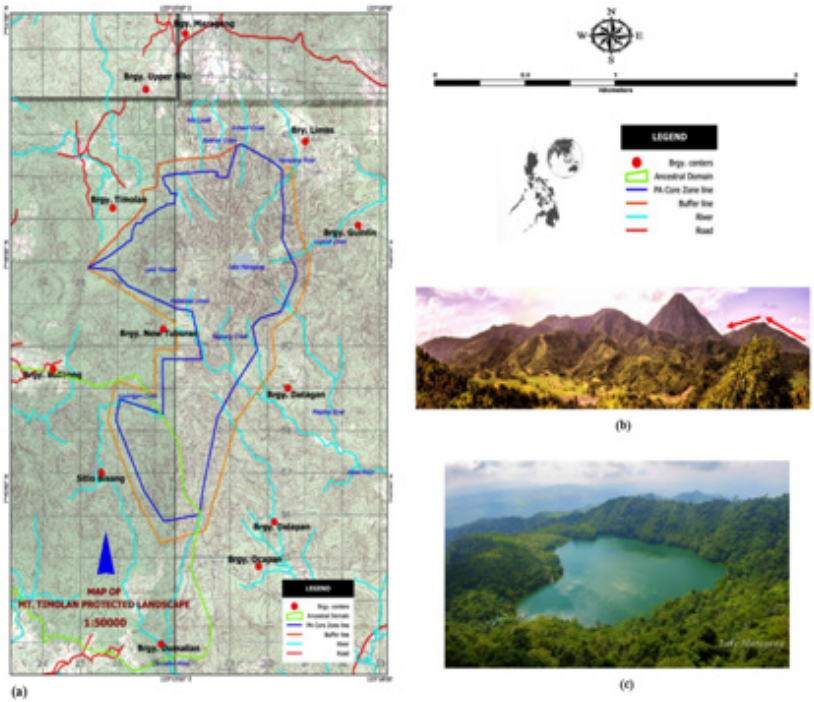
Species collection

In consonance with RA 9147 of 2001 or the Wildlife Resources Conservation and Protection Act, voucher specimens for each species were collected under the Wildlife Gratuitous Permit No. IX-02-2018.Collection of pteridophytes species was done in several transects lines measuring 2 km long and10 m wide. Species found within the transect were initially recorded, photo-documented along with their altitude using Global Positioning System (GPS) device. Voucher specimens for each species were collected for further identification and reference. Initial identification of collected species was done using pictorial handbook (Tandang et al., 2014), available published reports (Amoroso, 2013; Amoroso, 2014; Aya-ay, 2016), expert consultation and online database available in JSTOR's Global Plants website. Voucher specimens were also forwarded to Central Mindanao University for further verification of species identity. For classification of species, Pteridophyte Phylogeny Group I (PPG I 2016) served as the main reference. Conservation status of the recorded species where likewise identified following the criteria set by Department of Environment and Natural Resources – Administrative Order (DENR-DAO Series of 2017-11) and Union for the Conservation of Nature (IUCN 2016). Species richness on the other hand, was based on the total number of species accounted in the survey conducted.

RESULTS AND DISCUSSION

Species Richness

Thefirst pteridological survey in the northeastern portion of Mount Timolan Protected Landscape accounted a total of 98 species comprising 52 genera from 21 families based from the Pteridophyte Phylogeny Group I classification of ferns and lycophytes (PPG I 2016),(Table 1). The most dominant families include Tectariaceae, Polypodiaceae and Dryopteridaceae (Figure 2). Tectariaceae accounts the highest represented genera (9), followed by Polypodiaceae (8) and Dryopteridaceae (6). In terms of species abundance, Polypodiaceae has the highest (16), tailed by Tectariaceae (14) and Dryopteridaceae (10).



Out of the 98 recorded ferns and lycophytes, only three were identified up to genus level (Tectaria sp.Selaginella sp.and Nephrolepis sp.). Most of the recorded pteridophytes are terrestrial (71%), epiphytic (13%), were some are epiphytic/terrestrial (6.3%), mesophytic/terrestrial (3.2%), epiphytic/xerophyte (3.2%), mesophytic (3.2%) and epiphytic/lithophytic (1.1%). The herbaceous family of Selaginella, Lindsaeaseae and Hymenophylaceaeare mostly terrestrial in nature. The high number of accounted terrestrial fernfrom Family Aspleniaceae, Blechnaceae, Cyatheaceae, Dennstaedtiaceae, Dryopteridaceae, Gleicheniaceae, Hymenophyllaceae, Polypodiaceae and Pteridaceae are known as ecological indicator taxa for habitat disturbance due to species variation with regards to habitat adaptation and other climatic factor (Della and Falkenberg, 2019).

Table 1.

List of pteridophytes with conservation status in North-eastern part of Mount Timolan Protected Landscape, Zamboanga del Sur, Philippines.

Family	Species	Status	Habitat
ATHYRIACEAE	D. esculentum (Retz.) Holtt.	EIS	Terrestrial
	D. petiolare C.Presl.		Terrestrial
	Diplazium polypodioides Blume.		Terrestrial
	D. tenuifolium (Copel.)		Terrestrial
ASPLENIACEAE	A. anisodontum C.Presl		Epiphytic/Terrestrial
	A. callipteris Fée, Mém. Foug.	ECS	Epiphytic/Terrestrial
	A. cuneatum Lam., Encycl.		Epiphytic/Terrestrial
	A. nidus (L.)	EIS	Terrestrial
	Asplenium persicifolium J.Sm. ex Mett.,		Epiphytic/Terrestrial
	A. vittaeforme (Cav.)		Epiphytic/Terrestrial
	Blechnopsis finlaysoniana (Wall. ex Hook. & Grev.) C.Presl,	VU	
BLECHNACEAE	C.Presl,		Terrestrial
	Blechnopsis orientalis (L.) C.Presl	EIS	Terrestrial
	Oceaniopteris egregia (Copel.) Gasper & Salino	VU	Terrestrial
	Stenochlaena palustris (Burm.f.) Bedd.	EIS	Terrestrial
CYATHEACEAE	Sphaenopteris glauca (Blume.) R.M. Tryon	EN	Terrestrial
	S. polypoda R.M.Tryon		Terrestrial
DAVALLIACEAE	Davallia solida (G. Forst.) Sw.	OTS	Epiphytic
DENNSTAEDIACEAE	Pteridium aquilinum (L.) Khun	EIS	Terrestrial
DRYOPTERIDACEAE	Bolbitis heteroclita (C. Presl.) Ching		Terrestrial
	Dicranopteris curranii Copel.		Terrestrial
	D. flexuosa		Terrestrial
	D. linearis (Burm. F.) Underw.	EIS	Terrestrial
	E. bellarmanianum		Epiphytic
	E. blumeanum (Fée) J.Sm.		Epiphytic
	Elaphoglossum callifolium (Blume) T.Moore		Epiphytic
	Lomagramma merrillii (Holttum.)		Terrestrial/Epiphytic
	Pleocnemia macrodonta (Fée) Holttum		
	Polystichum acutidens		Terrestrial
GLEICHENIACEAE	Gleichenia truncata (Willd.)		Terrestrial
HYMENOPHYLLACEAE	Cephalomanes atrovirens (C. Presl.)		Terrestrial
	Hymenophyllum angulosum (Christ.)		Terrestrial
HUPERZIACEAE	Huperzia bayanica		Epiphytic
LINDSAEACEAE	L. doryphora K.U.Kramer.		Terrestrial
	Lindsaea fissa (Copel.)		Terrestrial
	L. tenuifolia Blume		Terrestrial
	Sphenomeris - chinensis (L.) Maxon	EIS	Terrestrial
	S. retusa (Cav.) Maxon		Terrestrial
	Tapeinidium pinnatum (Cav.) C. Chr.		Terrestrial
LOMARIOPSIDACEAE	N. biserrata (Sw.) Schott var. furcans	EIS	Terrestrial
	N. brownii (Desv.) Hovenk. & Miyam.		Terrestrial
	N. cordifolia (L.) Presl.	EIS	Terrestrial
	N. falcata (Cav.) C. Chr.	EIS	Terrestrial
	Nephrolepis flexuosa		Terrestrial
	Nephrolepis sp.		Terrestrial

LYCOPODIACEAE	Lycopodiella cernua (L.) Pic. Serm.		Terrestrial
LYGODIACEAE	L. auriculatum (Willd.)		Terrestrial
	L. circinnatum (Burm. F.) Sw.	EIS	Terrestrial
	L. japonicum (Thumb.) Sw.	EIS	Terrestrial
	Lygodium versteegii Christ.		Terrestrial
MARATTIACEAE	A. evecta (G. Forst.) Hoffm.	OTS	Mesophytic/Terrestrial
	Angiopteris palmiformis (Cav.) C. Chr.	OTS	Mesophytic/Terrestrial
OSMUNDACEAE	Osmunda banksiifolia (Presl.) Kuhn	OTS	Terrestrial
	O. bromelifolia (Presl.) Copel.		Terrestrial
POLYPODIACEAE	Aglaomorpha descensa (Copel.)	ECS	Epiphytic/Mesophytic
	A. heraclea (Kunze) Copel.	VU	Epiphytic/Xerophyte
	A. pilosa (J. Sm.) Copel.	VU	Epiphytic/Xerophyte
	A. quercifolia (L.)	EIS	Epiphytic/Mesophytic
	A. rigidula (Sw.)	EIS	Epiphytic/Mesophytic
	A. splendens (Hook. & Bauer) Copel.	VU	Epiphytic/Xerophyte
	Goniophlebium persicifolium (Desv.) Bedd.		Epiphytic
	Leptochilus macrophyllus (Blume)		Terrestrial
	Loxogramme avenia (Blume) C. Presl.		Terrestrial
	Microsorum punctatum (L.) Copel	VU	Epiphytic/lithophytic
	Phymatosorus adnascens (Sw.) Ching	EIS	Epiphytic
	P. commutatum Blume.		Epiphytic
	P. scolopendria (Burm.f.) Pic. Serm.	VU/ECS	Epiphytic
	Platycerium grande (J. Sm. Ex. Fee) Presl.	CR	Mesophytic
	Pyrrosia splendens (Presl.) Ching	VU	Epiphytic
	P. lanceolata (L.) Farw.		Epiphytic
PSILOTACEAE	Psilotum nudum (L.) P. Beauv.		Epiphytic
PTERIDACEAE	Adiantum mindanaoense Copel.		Terrestrial
	A. philippense (Linn.)		Terrestrial
	Antrophyum callifolium Blume, Enum. Pl. Javae		Terrestrial
	A. reticulatum (Forst.) Kaulf.		Terrestrial
	Coniogramme macrophylla (Blume.) Hieron		Terrestrial
	Haplopteris elongata (Sw.)		Terrestrial
	Pityrogramma calomelanos (L.) Link	NNS	Terrestrial
	Pteris ensiformis Burm.f.	EIS	Mesophytic
	P. pacifica Hieron.		Mesophytic
SELAGINELLACEAE	Selaginella cupressina (Willd.) Spring		Terrestrial
	S. delicatula (Desv.)		Terrestrial
	S. jagori (Warb.)	ECS	Terrestrial
	S. plana (Desv. ex Poir.) Hieron.	NNS	Terrestrial
	Selaginella sp.		Terrestrial
TECTARIACEAE	Christella dentata (Forssk.) Brownsey & Jermy		Terrestrial
	C. parasitica (L.) H. Lev.		Terrestrial
	Coryphopteris pubirachis var. philippinensis Holttum	ECS	Terrestrial
	Nannothelypteris inaequilobata Holttum	ECS	Terrestrial
	Parathelypteris beddomei var. eugracilis (Copel.) Holttum		Terrestrial
	Pneumatopteris laevis (Mett.) Holttum	ECS	Terrestrial
	Pronephrium nitidum (Holttum.) Holttum		Terrestrial
	P. xiphioides (Christ.) Holttum	ECS	Terrestrial
	Pseudophegopteris aurita (Hook.) Ching		Terrestrial
	Sphaerostephanos unitus (L.) Holttum		Terrestrial
	Tectaria. athyriosora M.G.Price	ECS	Terrestrial
	T. decurrens (Presl.) Copel.		Terrestrial/Mesophytic
	T. devexa (Kunze ex Mett.) Copel.	OTS	Terrestrial
	Tectaria sp.		Terrestrial

Note: VU (vulnerable); EN (endangered); CR (critically endangered); EIS (economically important species); ECS (endemic species); OTS (other threatened species); NNS (non-native species).Source: DENR-DAO Series of 2017-11 and IUCN, 2016.

The species richness recorded in this study, resembles that of Mt. Pinamantawan, Bukidnon with 121 spp. (Sumagaysay, 2012) but quite higher than Mt. Iraya, Batanes (121 *spp*; Barcelona, 2003), Mt. Pangasugan, Leyte (94 *spp*.; Belonias and Banoc, 1994), Pasonanca Natural Park, Zamboanga del Sur (72 *spp*.; Andas, 2015), Mt. Malukot, Batangas (40 *spp*.; Catapang et al., 2012).

Different pteridophyte taxadiversity also vary in topography with different range of elevations (Abotsi et al., 2020 and Nervo et al., 2019). In this study, some species of Aglaomorpha (A. heraclea, A. splendens and A. pilosa) which are epiphytic xerophyte often found in area with lower elevation (670 masl). The accessibility of the area is also a contributing factor that may influence species richness (Coritico et al., 2017). The filmy species of Hymenophyllaceae and other epiphytic species are frequently encountered in the mid-elevation forest (830 masl).Since, the mid-elevation forest of the area sampled are characterized by steep slopes and more ravine, not much of climbers are using the area as trail during their climb going to peak, thus, making the area less susceptible to disturbances.

Conservation status

Conservation status was based on the national

Figure 2. Some representative species from dominant families of Tectariaceae, Polypodiaceae and Dryopteridaceae: (A) D. linearis; (B) G. persicifolium; (C) L. avenia; (D) L. macrophyllus; (E) M. punctatum; (F) P. xiphioides; (G) P. nitidum; (H) C. parasitica; (I) L. merilli; (J) C. pubirachis var. philippinensis

list of threatened Philippine plant species (DENR-DAO 2017). It was carried out to identify beleaguered ferns and lycophytes species for protection, conservation and monitoring studies (Amoroso et. al., 2011). Markedly, the critically endangered P. grandeand endangered species of S. glauca were found respectively around the Lake Maragang and in theestablished open human trail of Mount Timolan Protected Landscape. Since the area is popularly known as tourist attraction, the identified two threatened species are susceptible to human disturbances. Whereas, a total of 11 spp. Philippine endemic, 18 spp. of economically important species, 9spp. vulnerable, 5spp. classified as other threatened species and 2 spp. non-native species of ferns and lycophytes was recorded (Table 1). Another notable species found along the open human trail is the vulnerable species of whisk fern, the P. nudum attached on the trunks of the Sphaeropteris polypoda.

Pteridophytes classification are poorly known especially to the locals and only little attention are given to this group. Based on the interview with some villagers, they only know some of Diplazium species, locally known as "pako" and Lygodium or "nito", both with respective value as food and for handicraft use. Nevertheless, some notable species listed as threatened species have unknown value and were simply considered as weeds.

CONCLUSIONS

Generally, the species richness of pteridophytes found in the north-eastern portion of Mount Timolan Protected Landscape is high. Mostly, all pteridophytes family were listed as potential ecological indicator for habitat quality degradation and forest destruction. However, fern species diversity to habitat disturbance relationship and other microenvironment factors requires further studies. Likewise, additional pteridological exploration in other part of the landscape is needed inestablishing a real profile on the ferns and lycophytes species found in Mount Timolan Protected Landscape.

In interim, effective forest conservation should be prioritised especially to the identified highly threatened species found in the side of established open human trails. Similarly, proper information dissemination through trainings about basic biodiversity research to the locals and other stakeholders of the Mount Timolan Protected should be conducted to conserved the area and mitigate measures in the survival of beleaguered ferns and lycophytes species as well as the other flora and fauna of Mount Timolan Protected Landscape.

REFERENCES

Abotsi, K.E., Bose, R., Adjossou, K., Deblauwe, V., Rouhan, G., Segla, K.N., Atsri, K.H., Kokou, K. (2020). Ecological drivers of pteridophyte diversity and distribution in Togo (West Africa). Ecol. Indic. (108):1 [CrossRef].

Adeleye, M., Akinsoji, A., and Adeonipekun, P. A. (2017). Survey of Vascular Epiphytes of Oil Palms (Elaeisguineensis JACQ.) in Lekki Conservation Centre, Lagos, Nigeria. FUW Trends in Science and Technology Journal (74):1.

Amoroso, V.B., Cariño, Y.L.L., Nobleza, J.C., and Coritico, F.P., (2020a). Ophioderma subsessile (Ophioglossaceae), a New Snake Tongue Fern Species from Mindanao, Philippines. Philippinr Journal of Science, 150(1): 215-221. Special Issue on Bioddiversity.

Amoroso, V.B., Coritico, F.P., and Fritsch, P.W. (2020b). Actinostachys minuta, a new species of grass fern from Mindanao, Philippines. Phytokeys, 151:59-66.

Amoroso, V.B. (2013).Philippine Medicinal Ferns and Lycopods. Central Mindanao University, Musuan Bukidnon.

Amoroso, V.B., Lagumbay, A. J. and Villalobos, A. P. (2014). Edible Ferns and Fern Recipe Book. Central Mindanao University, Musuan Bukidnon., Philippine Journal of Science, 142(2):127-137

Amoroso, V.B., Lagumbay, A. J., Mendez, R., Dela Cruz, R., & Villalobos, A. P. (2014). Bioactives in Three Edible Fern. Central Mindanao University, Musuan Bukidnon. Asia Life Sciences, 23(2):445.

Amoroso, V.B., Acma, F.M., Dela Cruz, R.Y., Coritico, F.P., Nietes, A.D., Hamo, G.B., Lumista, H.P. (2015). Diversity of herbaceous pteridophytes in four Mindanao long term ecological research (LTER) sites, Philippines. Asia Life Sci 24: 69–85.

Amoroso, V.B., Laraga, S.H., Calzada, B.V. (2011). Diversity and assessments of plants in Mt. Kitanglad Range Natural Park, Bukidnon, Southern Philippines. Garden's Bulletin Singapore 63(1&2): 219–236.

Andas, S.T. (2015). Diversity of Trees and Pteridophytes in Pasonanca Natural Park, Zamboanga City. [Dissertation]. Musuan, Bukidnon, Philippines: Central Mindanao University 326 p.

Aya-ay, M. (2016). Ethnobotany of Ferns and Fern Allies in Mount Macabol, Marilog District, Davao City, Philippines. University of Immaculate Conception, Father Selga, Davao City. IJaber, Vol. 14, No 2: 1127-1137

Barcelona, J.F. (2003). The taxonomy and ecology of the pteridophytes of Mt. Iraya & vicinity, Batan Island, Batanes Province, Northern Philippines.

Belonias, B.S., Banic, L.M. (1994). Species diversity and distribution of pteridophytes in Mt. Pangasugan. Ann Trop Res 16: 30–38.

Bharati, S. K., Manabendra, D.C. and Behari, M.P. (2013). In Vitro propagation in Pteridophytes: A Review. International J. Res. Ayurveda Pharm. 4(2):297-303.

Catapang, MV.L, Reyes, PJ.D., Medecilo, M.P. (2012). Factors influencing species diversity of ferns in Mt. Makulot, Cuenca, southern Luzon, Philippines. In: 2nd International Conference on Environment and Industrial Innovation; 2-3 June 2012; Hongkong, China:

IACSIT. Press, Singapore. IPCBEE 35: 98–102.

Coritico, F.P., Amoroso, V.B., Acma, F.M., Cariño, Y.L.L and Fritsch, P.W. (2020). Philippine Journal of Science. 149(3-a):733-790.

Della, A.P. and Falkenberg, D.B. (2019). Pteridophytes as ecological indicators: an overview. Hoehnea 46: e522018. http://dx.doi.org/10.1590/2236-8906-52/2018.

Ebihara, A. and Nitta, J.H. (2019). An update and reassessment of fern and lycophyte diversity data in the Japanese Archipelago. Journal of Plant Research132:723–738. https://doi.org/10.1007/s10265-019-01137-3.

International Union for the Conservation of Nature [IUCN] (2016). The IUCN Red List of Threatened Species, Version 2016–2.

Kessler, M., Lehnert, M. (2009).Do ridge habitats contribute to pteridophyte diversity in tropical montane forests? A case study from southeastern Ecuador. J. Plant Res. 122,:421–428. [CrossRef].

Nervo, M.H., Andrade, B.O., Tornquist, C.G., Mazurana, M., Windisch, P.G., Overbeck, G.E. (2019). Distinct responses of terrestrial and epiphytic ferns and lycophytes along an elevational gradient in Southern Brazil. J. Veg. Sci. 30:55–64. [CrossRef].

Oloyede, F.A., and Odu, E.A. (2011). Taxonomic Evaluationof Homosporous Leptosporangiate Ferns In Southwestern Nigeria. Journal of Current Research 2(1): 009-017.

PPG I. (2016). A community-derived classification for extant lycophytes and ferns. Journal of Systematics and Evolution 54: 563–603. doi:10.1111/jse.12229.

Sumagaysay, CJ.L. (2012). Pteridophyte diversity of Mount Pinamantawan, Tangkulang Range, Quezon, Bukidnon. [MS thesis]. Musuan, Bukidnon, Philippines: Central Mindanao University. 198 p.

Yong, J., Tan, P.Y., Hassan, N.H., Tan, S.N. (2010). A Selection of Plants for Greening of Waterways and Waterbodies in the Tropics. Singapore: Chung Printing. 480 pp.

Central Mindanao University
Journal of Science
ISSN Print: 0116-7847 ISSN Online: 2704-3703

EFFECT OF BOILING TIME AND STORAGE CONDITION (FROZEN AND UNFROZEN) ON THE PHYSICO-CHEMICAL PROPERTIES OF FLACOURTIA JANGOMAS (LOUR) RAUESCH FRUIT

Manuel Anthony C. Contridas[2], Noah Patrick K. Madrazo[2], Marvin A. Mangogtong[2], Denheart O. Andrade[2], Zairev Oliveros[2] , Lynette C. Cimafranca[1*] and Julious B. Cerna[1]

[1]Department of Food Science and Technology, Visayas State University, Visca, Baybay City, Leyte
[2]VSU Integrated High School, Visca, Baybay City, Leyte

ABSTRACT

Flacourtia jangomas, locally known as *Seriales* is an indigenous fruit tree in the Philippines. It potentially contains important nutrients and bioactive compounds, but it is a less known commodity, with no economic value. Moreover, the fruit is highly prone to enzymatic browning reaction, resulting to undesirable discoloration once exposed to air. Hence, this study was conducted to determine the changes in the physico-chemical properties of ripe *Seriales* fruit as affected by boiling time and storage conditions. Ripe *Seriales* fruits of known weight were exposed to upto 10 minutes of boiling water. Representative samples from each treatment were analysed of its color, total soluble solids, pH, and % titratable acidity. The same procedures were done to samples that had been stored for 19 days in the freezer. The results of the experiment revealed that storage conditions (frozen and not frozen) only affected the b* of the skin of the ripe Seriales fruits, while boiling time affected all other parameters, excepts pH, %TTA, and b* values of the fruit flesh.

Keywords: Flacourtia jangomas, physico-chemical, frozen, unfrozen, boiling

INTRODUCTION

Seriales is the local name for *Flacourtia jangomas* in the Visayas and Mindanao, and is one among other less-known native fruit commodities in the Philippines. It is a plant that belongs to the family of Flacourtiaceae grown in various parts of the world. It is commonly known as Indian coffee plum (Hossain and Sisodia, 2011; Kumar et al., 2018, Sasi et al., 2018), Indian sour cherry (Kumat et al., 2018), Indian cherry (Hossain et al., 2011), Paniala (Hossain and Sisodia, 2011; Sasi et al., 2018), Chinese plum (Hossain and Sisodia, 2011), among others.

The plant is considered underutilized, with little use such as food for birds and by some people leaving in the rural areas where it is mostly found growing. It can also be utilized as lumber, and is considered important in India due to its medicinal properties. The ripe fruits have high fibre content together with good protein content, low fat and higher amount of monosaturated fatty acids as compared to polysaturated fatty acids. It contains significant amount of β-carotene followed by lutein and zeaxanthene, retinol and phylloquinone (vitamin K) which are important in the regulation of haemoglobin and fibrinogen in human body (Srivastava et al., 2009).

Specifically, fruits are used for treatment of biliousness, fever and digestive disorder (Kitikar and Basu, 1993 as cited by Kumar et al., 2018). The medicinal capability of the commodity is attributed to the chemical components and its bio-functional properties. Parvin et al. (2011) reported that extracts from plant and plant parts are effective against both gram-positive and gram-negative bacteria, which is comparable with the standard antibiotic called Amoxicillin. The fruit itself has a diverse array of compound classes including terpenoids, alkaloids, flavonoids and tannins, lignans and flavanolignans, glucosides, coumarins and isocoumarins; as well as xanthones, quinones, limonoids and phenazines (Parvin et al., 2011). Specifically, the ripe fruits contain alkaloids, flavonoid, phenolic compounds and tannins which is proven to have high antioxidant potential (Neeharika and Pandey, 2013; Sinha et al., 2018). However, some of these components and other unmentioned components may have been degraded during its processing. For example, Cimafranca (2017) reported that different pre-treatments on *Seriales* fruit were noted to have significant variation in terms of bioactive compound content as well as on physico-chemical attributes. In terms of boiling and time of exposure, many studies had proven significant effect on many quality parameters of the commodity. For instance, boiling the Bactrijasipaes for 30 minutes did not affect total carotenoid content of the commodity (Jatunov et al., 2010), but in eggplant, it causes dry matter loss (Scalzo et al., 2016). PassoTsamo et al. (2015) reported that total phenolics decreased after boiling of whole banana fruit. These patterns serve to illustrate that

ARTICLE INFORMATION
Visayas State University, Visca, Baybay City, Leyte
Email Address: lynette.cimafranca@vsu.edu.ph
Received: Jan 7, 2020; Accepted: June 29, 2021
DOI: https://doi.org/10.52751/vacr1044

there is no applicable trend when it comes to the effect of heat treatment. Meanwhile, storage duration may also cause significant effect to crops. In apples for example, an increase in the total soluble solid, total sugar, pH and TSS/ Acid ratio was evident according to Jan and Rab (2012). It shall be noted that known quality parameters sometimes are dependent on specific commodities. Hence, this study aimed to determine the effect of boiling time on the physico-chemical properties of Seriales fruit, as well as the difference due to variation on storage conditions.

METHODOLOGY

Experimental Material

Seriales fruits were obtained from Brgy. Igang, Baybay City, Leyte. The whole batch of fruits were sorted, and only the good quality ripe fruits were used. These purplish-red skin colored fruits were washed thoroughly and then drained.

Heat Treatment

The boiling method employed in the experiment was adopted from the report of Ahmed and Ali (2013), with modifications. Distilled water (3000 ml) was poured into a stainless steel vessel with cover, and was heated to boiling. Seriales fruits (whole) weighing 650 g were submerged into the boiling water. The fruits were boiled at varying time of exposure (2, 4, 6, 8, and 10 minutes) (Table 1). After desired exposure time, samples were taken out quickly from the heat, drained in a stainless sieve, and air cooled for 30 minutes. Treatment samples labelled T1 to T6 were subjected to analyses, while the rest (T7to T12) were packed and sealed in polyethylene bags, stored in the freezer (Biobase BDF-40H200) for 19 days, and were analysed thereafter.

Experimental Design

The study was a six (6) treatment experiment arranged in a Completely Randomized Design (CRD) with varying time of exposure to boiling temperature (Table 1). Separate studies were conducted for freshly heat-treated Seriales samples, and the frozen heat-treated fruits.

Physico-chemical Analyses

Color Determination

Color of samples (skin and flesh) was measured using Lovibond LC 100 Spectrocolorimeter, and measurements were recorded as L, a and b values. Color values were gathered and computed from means of three (3) measurements of the skin color and the color of flesh exposed after the fruit was mashed.

Total soluble solids (TSS)

Representative fruits were mashed to fine pieces, placed it in a filter paper, massaged gently by hand until the juice was extracted. A drop of extracted juice from the fruit (25°C) was placed onto the prism of calibrated digital refractometer (ATAGO ATC-IE model, Japan), and TSS reading was taken.

pH

Determination of pH was carried out using a digital pH meter (pHeps) that was calibrated with pH 4 and 7 buffer solutions. The sample for pH determination was prepared from a 2:1 ratio of sample and distilled water that was blended with the aid of an Osterizer blender. The sample was then placed in a clean and dry plastic cup container, mixed with a plastic spoon, followed by submersion of the pH meter electrode onto the prepared sample. The stable pH reading from the display of the meter was taken and recorded. Three replications were made per treatment sample.

Total titratable acidity (TTA, expressed as % citric acid)

Total titratable acidity was determined by employing standard titration method using 0.1 N NaOH

Table 1

Different time exposure of Seriales fruit to boiling temperature

TREATMENTS	BOILING TIME (mins)	STORAGE CONDITION
T_1	0	No freezing
T_2	2	No freezing
T_3	4	No freezing
T_4	6	No freezing
T_5	8	No freezing
T_6	10	No freezing
T_7	0	Frozen
T_8	2	Frozen
T_9	4	Frozen
T_{10}	6	Frozen
T_{11}	8	Frozen
T_{12}	10	

solution following AOAC (2010). Percent citric acid was computed using the formula:

$$\% \text{ citric acid} = \frac{\text{Volume of titrant used (mL) x Normality (N) of titrant x mEq.Wt. of acid (0.064 for citric acid)}}{\dfrac{\text{Fresh weight of sample (g)}}{\text{Volume of water added + Fresh weight of sample}} \text{ x Volume of aliquot}} \text{ x } 100$$

Statistical analysis

Data were statistically analysed using Microsoft Excel for analysis of variance (ANOVA) and determination of the difference between treatments.

RESULTS AND DISCUSSION

Color of the Flesh of Seriales Fruit

Food acceptance by the consumers is initially affected by color. It is also an indicator to some important beneficial component such as anthocyanin. The colors red, purple, and blue in fruits are relatively associated to high anthocyanin content. F. jangomas (Seriales) is one example of these fruits containing anthocyanin due to its characteristic purplish red coloration (Cimafranca, 2017) or dull brownish red or purple to blackish skin color, with greenish-yellow pulp (Sasi et al 2018).These anthocyanins among other important antioxidants are needed in the body in the prevention and/or treatment of diseases.

As indicated in the results presented in Table 2a, the color analysis revealed that the luminance (L*) ranges from 36.15 (T_1) to 53.98 (T_{12}) regardless whether the fruit is frozen or not. These values are generally higher compared to the values reported by Cimafranca (2017) on ripe seriales fruit. Samples that did not undergo freezing has lowest L value (36.15 for T_1), and the highest (53.10) was observed at 6 minutes exposure to boiling water. However, the latter result was statistically the same with T_3, T_5 and T_6. L* values higher than 50, co-notes that the samples are of lighter hue as compared to values within 0-50 such as T1, T_2 and T_9. ANOVA on the other hand, revealed statistical difference among these data sets for luminance (Table 2a).

Other important parameters are a* and b*, which projects the red versus green color, as well as the yellow to blue hue of the sample tested, respectively. The mean values for a* ranges from 21.28 (T_5) to 29.30 (T_9), while mean b* values ranges from 17.52 (T_9) to 26.30 (T_1). The ANOVA revealed similar results to luminance, that freezing and non-freezing conditions are not affecting the a* value of the samples, but are significantly affected by boiling time (Table 2a). The results of the multiple comparison difference test revealed that T_2, T_3, T_4, T_9 and T_{11} are not significantly different from each other. Other groups that are statistically the same are T_4, T_7, T_{11} and T_{12}, and the lowest a* values are grouped namely T1, T5, T6 and T7 (Table 2a). On the other hand, statistical analysis on the b* discloses that both the storage condition and the time of boiling do not affect the b* values of the treatment samples (Table 2a). Moreover, a significant increase in the luminance value is generally observed in unfrozen boiled Seriales fruit. Table 2a also presents that there are significant differences between treatment values of the samples analysed.

Table 2a

Color characteristics of the flesh of ripe frozen and unfrozen Seriales (F. jangomas) fruit as affected by varying time of exposure to boiling

TREATMENT	SERIALES CONDITION	BOILING TIME (MINUTES)	COLOR VALUES		
			*L**	*a**	*b**
T_1	No freezing	0	36.15 ± 3.25^{d}	16.25 ± 2.21^{d}	26.30 ± 3.93^{a}
T_2	No freezing	2	45.17 ± 1.02^{bc}	27.58 ± 0.55^{ab}	21.22 ± 0.75^{b}
T_3	No freezing	4	50.40 ± 1.04^{ab}	26.28 ± 0.91^{abc}	20.63 ± 1.49^{b}
T_4	No freezing	6	53.10 ± 1.53^{a}	26.12 ± 2.78^{abc}	20.88 ± 0.79^{b}
T_5	No freezing	8	53.05 ± 0.72^{a}	21.28 ± 2.18^{cd}	20.90 ± 0.37^{b}
T_6	No freezing	10	52.70 ± 0.34^{a}	21.43 ± 1.28^{cd}	18.40 ± 0.47^{b}
T_7	Frozen	0	45.72 ± 3.12^{bc}	22.77 ± 1.90^{bc}	20.12 ± 0.46^{b}
T_8	Frozen	2	51.73 ± 2.29^{a}	21.28 ± 1.40^{cd}	21.82 ± 1.21^{b}
T_9	Frozen	4	42.15 ± 0.40^{c}	29.30 ± 1.18^{a}	17.52 ± 0.60^{b}
T_{10}	Frozen	6	53.70 ± 2.08^{a}	21.57 ± 1.71^{c}	20.12 ±1.97^{b}
T_{11}	Frozen	8	52.88 ±1.21^{a}	26.08 ± 1.56^{abc}	19.05 ± 1.19^{b}
T_{12}	Frozen	10	53.98 ±2.49^{a}	23.43 ± 2.65^{bc}	21.28 ± 1.75^{b}

Values are means of triplicate determination ± standard error (SE)
Means of 6 fruit per treatment using a Lovibond LC 100 Spectrocolorimeter. L* = lightness, a* = bluish/green/red-purple hue component, b* = yellow/blue hue component,
Mean values followed by different letter superscripts in the same column are significantly ($p<0.05$) different.

Color of the Skin of Seriales Fruit

The seriales fruit had different colors of its peel (skin) and pulp (flesh). Specifically, the fruit skin was darker red than the pulp (low L* and b* values; and positive a* values). Moreover, means of a* and b* values reflected in Table 2b were all positive, indicating that the color plays around red (positive a*) and yellow (positive b*).
The higher value denotes lighter hue than the other. Generally speaking, all values indicate darker hue since L* ranges within 0-50 (Table 2b). The lowest L value is that of T_9 (24.10), while T_{10} got the highest luminance of 36.82. The ANOVA revealed that the treatment samples are significantly affected by boiling time and not on the storage condition of the fruit. Further, the test of difference between treatments show that T_2 is significantly different from the rest of the treatment samples. Specifically, T_1, T_3 and T_9 possesses the lowest L values, while T_2 had the highest.

Similar with the results of the ANOVA for luminance, the ANOVA for the a* values revealed that storage condition did not affect the forenamed parameter, but boiling time was. The test to determine the difference between treatments as displayed in Table 2b denotes that the highest a* values are observed on treatment samples T_4, T^{10}, T_{11}, and T_{12}. On the contrary, low a* values are denoted in T_5 and T_7, but these two treatment samples are statistically of no significant difference with T_1 and T_8.

Meanwhile, the b* as another important parameter to color characteristics were noted to range from 11.18 (T_1) to 24.65 (T_6). The former is not statistically different from T_9 and T_{11}, while T_6 was statistically the same with T_4, T_5, T_8 and T_{12}. Storage condition and boiling time were found to cause significant effect on the aforesaid parameter as shown in Table 2b.

Total Soluble Solids (TSS)

Total soluble solids is the measure of sugar content of the fruit, which includes the carbohydrates, organic acids, proteins, fats and minerals. The Seriales fruits in Leyte had higher TSS values as compared to other countries (Cimafranca, 2017). The forenamed author reported a mean TSS value of 14.93^0B, which is within the range of TSS values noted in this experiment (11.67 to 18.00^0B). However, processing sometimes reduces the TSS content due to dilution effect, and often results to nutrient degradation (Hwang et al. 2012). That is why a decreasing trend in the TSS values was reflected in Table 3. Similar result was reported on vegetables namely broccoli, cabbage, cauliflower, spinach and watercress, where significant reduction was evident due to leaching of soluble sugars and organic acids into the water (Vinha et al., 2015). The statistical results in this experiment indicates that the total soluble solids of the seriales were affected by boiling at different time variation, similar with the results on boiled Khilek young flowers (Teangpook et al., 2012). PassoTsamo et al. (2015) on contrary, suggested that boiling may not affect the soluble sugar contents of the pulp and the peel in bananas.

pH

Table 3 presents the pH values of the different treatment samples. The range of values was 3.12 (T10) to 3.32 (T4). These values are in agreement to the pH values

Table 2b

Color characteristics of the skin of ripe frozen and unfrozen seriales (F. jangomas) fruit as affected by varying time of exposure to boiling

TREATMENT	SERIALES CONDITION	BOILING TIME (MINUTES)	COLOR VALUES		
			*L**	*a**	*b**
T_1	No freezing	0	25.68 ± 0.74^{ef}	19.25 ± 3.38^{def}	11.18 ± 1.38^{fg}
T_2	No freezing	2	43.02 ± 4.72^{a}	21.83 ± 0.81^{cde}	21.05 ± 0.89^{abcde}
T_3	No freezing	4	28.13 ± 1.34^{def}	24.22 ± 2.38^{bcd}	17.42 ± 2.67^{de}
T_4	No freezing	6	32.82 ± 1.06^{bcd}	28.18 ± 0.87^{ab}	24.12 ± 4.84^{ab}
T_5	No freezing	8	32.47 ± 1.04^{bcd}	16.17 ± 0.40^{f}	23.27 ± 1.75^{abcd}
T_6	No freezing	10	35.32 ± 1.30^{bc}	23.28 ± 2.25^{bcd}	24.65 ± 0.86^{a}
T_7	Frozen	0	30.07 ± 1.21^{cde}	15.80 ± 0.63^{f}	18.05 ± 0.56^{cde}
T_8	Frozen	2	33.70 ± 2.40^{bc}	17.72 ± 0.44^{ef}	23.52 ± 2.90^{abc}
T_9	Frozen	4	24.10 ± 1.08^{f}	15.72 ± 1.55^{f}	08.25 ± 1.91^{g}
T_{10}	Frozen	6	36.82 ± 0.93^{b}	28.03 ± 0.46^{ab}	18.55 ± 0.60^{bcde}
T_{11}	Frozen	8	32.03 ± 1.90^{bcd}	29.48 ± 2.80^{a}	16.57 ± 0.34^{ef}
T_{12}	Frozen	10	34.23 ± 1.10^{bc}	25.13 ± 1.99^{abc}	20.97 ± 1.44^{abcde}

Values are means of triplicate determination ± standard error (SE)
Means of 6 fruits per treatment using a Lovibond LC 100 Spectrocolorimeter. L* = lightness, a* = bluish/green/red-purple hue component, b* = yellow/blue hue component,
Mean values followed by different letter superscripts in the same column are significantly ($p<0.05$) different.

gathered by Cimafranca (2017) on the same commodity. In comparison with F. jangomas from other countries, the pH of the seriales fruit in Leyte are comparably lower than 3.64±0.01 pH obtained on similar fruit from Bangladesh (Ara et al. 2014). With respect to boiling time, Table 3 revealed that an increase in pH was noted up to 6 minutes of exposure to boiling water, then dropped down in the subsequent heating period. Significant decrease of the pulp pH of bananas was observed after boiling. PassoTsamo et al. (2015) cited Vaclavik and Christian (2014), stating that the release of organic acids from the cell walls to the cytoplasm caused pH to lower upon cooking. Relative to storage condition, a generally downward trend was observed. However, it should be noted that statistically, all these values do not differ from each other, a result dissimilar to the trend exhibited on boiled Khilek.

Total titratable acidity (TTA)

Titratable acidity of fruit juices is an important parameter in determining fruit maturity and sour taste in citrus fruits. It is a measure of the amount of acid present in a solution, which is expressed as grams/liter (g/L). The total titratable acidity of F. jangomas deliberates on the fruit's total acidity but not the measurement of the strength of acids.

In the experiment, the % TTA ranges from 0.01 to 0.02% (Table 3), which is in disagreement to the %TTA values gathered by Cimafranca (2017) and Ara et al. (2014). This could probably be due to variability of the fruit. According to Robertson et al. (1990), titratable acidity decreased with increased maturity, and this could be one reason of the variability between the results of the previous studies from various authors.

Results of statistical analysis on the other hand revealed that both storage condition and boiling time did not affect the % TTA of the treatment sample.

CONCLUSION

It was concluded that boiling time significantly affected the TSS values of the fruits, the L* and a* values of the flesh, and all color parameters of the skin. TSS was significantly affected by boiling time, while pH and % TTA were unaffected by both storage condition and boiling time.

REFERENCES

Ahmed, F.A., & Ali, R.F.M. (2013). Bioactive compounds and antioxidant activity of fresh and processed white cauliflower. BioMed Research International 2013.

AOAC. (2000). Official Method of Analysis. Vol. I and II. Association of Official Analytical Chemist. Washington, D.C.

Ara, R., Jahan, S., Abdullah, A.T.M., Fakhruddin, A.N.M., Saha, B.K. (2014). Physico-chemical properties and mineral content of selected tropical fruits in Bangladesh. Bangladesh Journal of Scientific and Industrial Research 49(3):131-136.

Cimafranca, L.C. (2017). Physico-chemical properties, phytochemical components, antioxidant and antimicrobial activities of fresh And processed seriales

Table 3

TSS, pH and % TTA of the ripe frozen and unfrozen seriales (F. jangomas) fruit as affected by varying time of exposure to boiling

TREATMENT	SERIALES CONDITION	BOILING TIME (MINUTES)	PHYSICO-CHEMICAL PROPERTIES		
			TSS*	pHns	%TTA
T_1	No freezing	0	18.00 ± 0.73^{a}	3.20 ± 0.16	0.02 ± 0.003^{ab}
T_2	No freezing	2	17.33 ± 0.49^{ab}	3.23 ± 0.04	0.01 ± 0.001^{c}
T_3	No freezing	4	15.00 ± 0.52^{cd}	3.27 ± 0.06	0.01 ± 0.001^{c}
T_4	No freezing	6	14.00 ± 0.89^{cde}	3.32 ± 0.03	0.01 ± 0.001^{bc}
T_5	No freezing	8	14.67 ± 0.42^{cde}	3.27 ± 0.05	0.01 ± 0.001^{bc}
T_6	No freezing	10	13.17 ± 0.60^{def}	3.18 ±0.06	0.02 ± 0.012^{a}
T_7	Frozen	0	17.50 ± 0.67^{ab}	3.30 ± 0.13	0.02 ± 0.001^{abc}
T_8	Frozen	2	15.67 ± 1.38^{bc}	3.27 ± 0.04	0.01 ± 0.001^{abc}
T_9	Frozen	4	17.83 ± 0.48^{a}	3.17 ± 0.03	0.01 ± 0.001^{bc}
T_{10}	Frozen	6	12.67 ± 0.80^{ef}	3.12 ± 0.12	0.01 ± 0.001^{c}
T_{11}	Frozen	8	12.67 ± 0.76^{ef}	3.13 ± 0.02	0.01 ± 0.001^{bc}
T_{12}	Frozen	10	11.67 ± 0.67^{f}	3.13 ± 0.03	0.01 ± 0.001^{c}

Values are means of triplicate determination ± standard error (SE)
Means of 6 fruits per treatment using a Lovibond LC 100 Spectrocolorimeter. L* = lightness, a* = bluish/green/red-purple hue component, b* = yellow/blue hue component,
Mean values followed by different letter superscripts in the same column are significantly (p<0.05) different.

[Flacourtiajangomas(Lour) Raeusch] fruit. PhD Dissertation. University of the Philippines – Los Baños, College, Laguna.

Hossain, K.S. &Sisodia. P. (2011). Paniala (Flacourtiajangomas) plant extract as eco- friendly inhibitor on the corrosion of mild steel in acidic media. Rasayan J. Chem. 4(3):548-553.

Hwang, I.G., Shin, Y.J., Lee, S., Lee, J.,Yoo, S.M. (2012). Effects of different cooking methods on the antioxidant properties of red pepper (Capsicum annuum L.). Preventive Nutrition and Food Science, 17(4): 286–292.

Jan I., &Rab, A. (2012). Influence of storage duration on physic-chemical changes in fruit apple cultivars. The Journal of Animal and Plant Science 22(3):708-714.

Jatunov, S., Quesada, S., Diaz, C., & Murillo, E. (2010). Carotenoid composition and antioxidant activity of the raw and boiled fruit mesocarp of six varieties of Bactrisgasipaes. ArchovosLatinoamericanos de nutrition 60(1):99-104

Kumar, R.S., Kumar, V.S.., Lathigg, A.M.K.M., Krithika, U.S., &Sudhakar, P. (2018). Antioxidant and anti-inflammatory activities of leaf extracts of Flacourtiajangomas (Lour) Raeusch: An in vitro study. Advance Pharmaceutical Journal 3(6):169-176.

Neeharika, D., & Pandey, V.N. (2013). Ethnobiological importance of Flacourtia jangomas (Lour.) Raeusch. Trends in Biosciences, 6(5):532-534.

Parvin, S., Kader, A., Sarkar, G.C., Bin Hosain, S. (2011). In-vitro study of antibacterial and cytotoxic properties of Flacourtiajangomas.International Journal of Pharmaceutical Sciences and Research, 2(11): 2786-2790.

Robertson, J.A., Meredith, F.I., Horvat, R.J., &Senter, S.D. (1990). Effect of cold storage and maturity on the physical and chemical characteristics and volatile constituents of peaches (cv. Cresthaven). Journal of Agricultural and Food Chemistry, 38(3):620-624.

Sasi, S., Anjum, N., &Tripathi, Y.C. (2018). Ethnomedicinal, phytochemical and pharmacological aspects of FlacourtiaJangomas: A review. International Journal of Pharmacy and Pharmaceutical sciences, 10(3):9-15.

Scalzo, R. L., Fibiani, M., Francese, G., D'Alessandro, A., Rotino, G. L., Conte, P., &Mennella, G. (2016). Cooking influence on physico-chemical fruit characteristics of eggplant (Solanum melongena L.). Food chemistry, 194, 835-842.

Senthil Kumar, R., Vinoth Kumar, S., Abdul Lathiff, M.K.M., Uma Krithika, S., & Sudhakar, P. (2018). Antioxidant and anti-inflammatory activities of leaf extracts of Flacourtia jangomas (Lour.) Raeusch: An study in vitro. Advance Pharmaceutical Journal, 3(6):169-176.

Sinha, D., Srivastava, A.K., Prabhuji, S.K. and Srivastava, G.K. (2018). Antioxidant potential of the ripe fruits of Flacourtia jungomas (Lour.) Raeus., Medicinal Plants, 10(1): 84-86.

Srivastava, D, Prabhuji, SK and Rao, GP (2009). Taxonomic and ethnobiological status of Flacourtia jungomas (Lour.) Raeus.: an endemic nutraceutical plant of eastern U.P. Medicinal Plants, 1(1): 49–53.

Teangpook, C., Puminat, W., Titatarn, Y., Onhem, S. &Paosangtong, U. (2012). Effect of boiling on anhydrobarakol content of Khilek (Siamese cassia) young flowers. Pakistan Journal of Nutrition 11(12):1125-1130.

Thompson, A.K. 2014. Fruit and Vegetables: Harvesting, Handling and Storage. John Wiley and Sons-Vol 2.

PassoTsamo, C., Herent M.F., Tomekpte, K., HappiEmaga, T. Quertin-Leclercg, J., Rogez, H., Larondelle, Y., & Andre, C. (2015). Effect of boiling on phenolic profiles determined using HPLC/ESI-LTQ-Orbitrap-MS, physico-chemical parameters of six plantain banana cultivars (Musa sp). Journal of Food Composition and Analysis, 44:158-169.

Vinha, A. F., Alves, R. C., Barreira, S. V., Costa, A. S., & Oliveira, M. B. P. (2015). Impact of boiling on phytochemicals and antioxidant activity of green vegetables consumed in the Mediterranean diet. Food & function, 6(4), 1157-1163.

Central Mindanao University
Journal of Science
ISSN Print: 0116-7847 ISSN Online: 2704-3703

Factors affecting the nodulation of *Pongamia pinnata* (L.) Pierre inoculated with *Rhizobium mesoamericanum* (Lopez-Lopez et al., 2012)

Phoebe S. Nemenzo[1]*, Marianita B. Santos[1], Melanie M. Garcia[2], and Jhonnel P. Villegas[3]

[1]Biology Program, Arts and Sciences Department, San Pedro College, Davao City
[2]Ph.D in Environmental Science-Graduate Student, School of Environmental Science and Management (SESAM) and Professional School for Agriculture and the Environment (PSAE) University of the Philippines-Los Baños, 4031, Philippines
[3]Institute of Education and Teacher Training, Davao Oriental State University, Mati City, 8200, Philippines

ABSTRACT

Pongamia pinnata or "bani" in the Philippines is a non-food crop that can grow on marginal land where food crops do not grow. Its seeds are used for biofuel production such as biodiesel and aviation fuel. It is resilient against abiotic stresses such as drought, salinity and acidity. It can also grow in nitrogen-limited soils. It is capable of nitrogen-fixation activity through its root nodules developed by symbiosis with rhizobia. Rhizobium mesoamericanum (Lopez-Lopez et al., 2012) promotes the nodulation of Pongamia as confirmed by strain symbiotic effectiveness testing where shoot length, number of nodes, number of leaflet ts, nodule number, and plant dry weights were analysed. Variability test was carried out to determine any variability of nodulation in the seeds coming from one mother tree. A time course of nodulation revealed that there was an increasing pattern of nodule number, leghemoglobin concentration, area of zone of infection and dry weights. Nodulation increased when nitrate concentration was increased from 2 to 5 mM, but was inhibited in 10 mM nitrate. Nodulation decreased as salinity increased from 1% to 3%. R. mesomaericanum improved the growth and nodulation in Pongamia, whether as a group or as individual inoculants, provided that a sufficient concentration of the inoculant was achieved.

Keywords: Nodulation, symbiotic nitrogen fixation, Pongamia pinnata, Rhizobium mesoamericanum, legume tree

INTRODUCTION

Pongamia pinnata (L.) belongs to family Leguminosae, subfamily Papilionoidae and Millettieae tribe. It is a medium-sized arboreal legume tree indigenous to the Indian subcontinent, Northern Australia and Southeast Asia including the Philippines. Pongamia is considered a tropical plant and can be found in coastal areas or close to marin environments use as inlets, river mouths and sea fronts, along limestone and rock coral outcrops, edges of mangrove forests and tidal streams and rivers (Calica, 2017; Gresshoff et al., 2017). In the Philippines, Pongamia is commonly called "bani" especially in Pangasinan, Zambales, Pampanga, Bataan and Cotabato. It is locally known as "balikbalik" in Tagalog, "balobalo" in Zambales and Basilan; "balukbaluk", "balutbalut" or "magit" in Cotabato, "baobao" in Agusan, "kudet" in Tayabas, "m arokbarok" in Camarines, and, "salingkugi" in Zamboanga (NFTP-EP, 2021). It can grow up to 15 meters and a diameter of about 0.5 meter. It has alternate and compound leaves with three to seven leaflets, which are smooth, pointed at the apex, rounded at the base, and seven to 10 cm in length. The flowers are pink to purplish, 1.5 cm in length, and borne in raceme. The somewhat flattened pods have oval outline or shape with a single seed. Pongamia is distributed from northern Luzon to southern Mindanao. It is used in the Philippines in crafts and furniture making. The bark and bast are used for making strings and ropes (NFTP-EP, 2021).

Pongamia form association with bacteria present in soil which are generally called rhizobia resulting in root nodulation and symbiotic nitrogen fixation. To optimize the nitrogen fixation activity in Pongamia, the best or superior rhizobia must first be isolated for Pongamia symbiosis. Baiting technique is an indirect technique to isolate rhizobia from soil by using a host plant as bait and then later on isolate from the surface-sterilised nodule using one-nodule-one-drop technique (Nemenzo-Calica et al., 2016). This uses the nodulation process as effective trap for selective enrichment and allows the isolation of superior strain of rhizobia. Once the best rhizobia is isolated, it can be inoculated to Pongamia for efficient nodulation and nitrogen fixation. Nemenzo-Calica et al (2016) previously established that Rhizobium mesoamericanum previously isolated from Queensland, Australia, is a superior rhizobial strain for Pongamia.

Pongamia nodulation can be affected by several factors which can further influence the efficiency of biological nitrogen fixation. The symbiosis between Pongamia and R. mesoamericanum is maximized when niche requirements are met for both host and rhizobia species. Factors that limit plant health and photosynthetic

ARTICLE INFORMATION
Phoebe S. Nemenzo
Email Address: f.phoebe.nemenzo@cmu.edu.ph
Received: May 6, 2021; Accepted: June 24, 2021
DOI: https://doi.org/10.52751/eqse9859

capacity will likewise limit nitrogen fixation potential. However, even when optimal conditions are met for Pongamia growth and development, the establishment of R. mesoamericanum can be independently inhibited by factors including nutrient excess or deficiency, moisture, acidity, element toxicity, native microbial competitors and problems with inoculation (Kasper et al., 2019). Pongamia nodulation studies were previously done using different rhizobia strains including Bradyrhizobium species (Samuel et al., 2013). Biswas et al (2013) reported that nitrate inhibited the nodulation and autoregulation of nodulation in Pongamia when inoculated with Bradyrhizobium japonicum CB1809 and nodulation was decreased with increasing salinity. Gresshoff et al (2017) also reported that low NaCl concentration stimulates growth of Pongamia seedlings compared with no NaCl. However, there has been no published study on the factors affecting the nodulation of Pongamia when inoculated with R. mesoamericanum. Hence, this study was conducted to determine the factors affecting the nodulation of Pongamia inoculated with R. *mesoamericanum*.

METHODOLOGY

Isolation, identification and strain symbiotic effectiveness of R. mesoamericanum

Soil samples were collected from different locations across Queensland, Australia. The samples were characterized for their physico-chemical properties such as physical appearance, pH, EC, and total nutrient levels. LECO TruSpec analyzer was used to analyze the total Carbon and Nitrogen level. Total nutrient analysis was done using a Varian Vista Pro ICPOES instrument. Baiting technique and one drop-one nodule technique were done to isolate rhizobia from randomly selected nodules following the protocol of Nemenzo-Calica et al (2016). The isolates were morphologically characterized based on their colony size in diameter, color, shape, margin, elevation and texture. Growth curves in liquid culture of all isolates were also determined. The antibiotic resistance of each isolate was also determined.

Strain symbiotic effectiveness tests were carried out to confirm if the isolates were indeed capable of nodulating Pongamia using uniform growth medium (sterile vermiculite) under uniform growth conditions in the glasshouse. The pure cultures of rhizobia isolated from different soil samples and from Pongamia nodules were inoculated into yeast extract mannitol broth for 48 hours with shaking. Bradyrhizobium strains: USDA110 (U.S. Department of Agriculture, U.S.A.), CB1809 and CB564 (Commonwealth Scientific and Industrial Research Organisation or CSIRO Division of Tropical Agronomy, Brisbane, Australia)were included in this test to compare with the isolates. Meanwhile, several Pongamia seeds were sown into sterile vermiculite in 15cm pots which were thinned out later into one plant per pot. Three to five replicate plants were chosen for each treatment. Plants were maintained for 12 weeks. The data gathered include: Shoot length, Number of nodes, Number of leaflets, Number of nodules, Root, Shoot, Nodule and Total Plant dry weights, Nodule fresh weight (preserved by refrigeration), and Nodule morphology.

The selected superior isolates previously identified in the strain symbiotic effectiveness test were further characterized and grown in various growth conditions to determine their tolerance in different temperature (22°C, 37°C and 45°C), pH (4.0, 7.0 and 9.0), and salinity (0.1%, 0.5% and 1% (w/v) NaCl).

The selected superior isolated were genetically identified by using nif genes and nod C. The isolates were grown in yeast extract mannitol broth (YMB) in flasks and then transferred into 1.5 ml centrifuge tubes. Tubes were centrifuged at 16,000 g for 10 min. The resulting supernatant was decanted. The rhizobia were resuspended in 467 µl of 10 mmol/L Tris-HCL, pH 8 and 1 mmol/L EDTA. 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added into the tubes. Tubes were incubated for 1 hour at 37°C. 500 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added. Tubes were centrifuged at 16,000 g for 2 min. 0.1 ml of 3 M Sodium Acetate, pH 5.2 and 0.6 ml of isopropanol was added to precipitate the DNA. Tubes were incubated at least 30 minutes on ice. The tubes were centrifuged for 15 minutes at 16,000 g. The DNA pellet were washed in 500 µl of 70% ethanol. The tubes were centrifuged for 15 minutes. The DNA were dried and resuspended in 100 µl of sterile MilliQ water. Success of DNA extraction was confirmed by loading 5 uL of each rhizobium with 2uL of loading dye into the wells of agarose gel mixed with ethidium bromide. Molecular ladder used was 1kB (brand) and run for 60 minutes at 100 volts. One kb molecular ladder was loaded on the first well. The gel electrophoresis was done using 100 volts in 60 minutes. Identification of the rhizobia up to the species level was done by amplifying the nif genes and nod C (Samuel et al., 2013) using a thermocyler machine (S1000 Bio-Rad Thermal Cycler). The PCR condition was based on (Nemenzo et al., 2016). All amplicons were confirmed using gel electrophoresis and excised using QIAquick Gel Extraction Kit, Qiagen). The amplicons were sent to Australian Genome Research Facility (AGRF) for sequencing. Genetic sequences were run in BLAST for identification.

Variability test

Fifty pots were sterilized and were filled with sterile vermiculite. Fifty sterile Pongamia seedlings were planted to pots and inoculated with R. mesoamericanum. Plants were maintained in the glasshouse and were watered once with 150-ml nitrogen-free nutrient solution, Broughton & Dilworth (B&D solution) and twice weekly with sterile distilled water. After 12 weeks, plants were uprooted and observed. Statistical analysis such as ANOVA and Tukey's Honestly Significant Difference was done to analyse data including leaflets arrangement, number of nodes, number of leaflets, nodule number, nodule morphology, and plant dry weights using R software. An assumed value of variance for each of the parameters: plant structure, nodulation and nitrogen fixation was determined. Hypothetically, if the variance for each parameter will be below 0.5, then it can be concluded that there is no significant variation among the 50 seedlings in terms of the parameters tested. Fifty seedlings were used as sampling size to obtain around 90-95% statistical power to provide reliable inference in supporting the hypothesis.

Time course of nodulation

Time course of nodulation was done with R. mesoamericanum which were incubated with shaking for 48 hours until turbid and inoculated to sterile Pongamia seedlings at 40 ml per plant (108 cells/ml). Plants were watered with B&D solution and tap water for 12 weeks. Plants were randomly harvested per week from week 4 to week 8 with four replicates per harvest. Date gathered include nodule number and fresh weight, leghemoglobin concentration, area of zones of infection, plant dry weights, and N level content in roots and shoots.

Leghemoglobin concentration was quantified following the method by Keilin and Wang (1945). Nodules from individual plants were weighed (fresh weight) and homogenised in 5 ml of 0.1 N KOH using a mortar and pestle. Maximum of 1g of homogenized plant nodule was used as sample. The suspension was centrifuged for 10 min at 10000 g. A 1.5 ml aliquot of the supernatant was mixed with 1 ml distilled water and 0.5 ml 5 N KOH. After reduction with 0.1 g sodium sulfate, the optical density or absorbance was determined using spectrophotometer at 600 nm. The leghemoglobin concentration of the seedlings was derived from the standard curve using prepared leghemoglobin of various concentrations as standards (0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, and 1.5 mg/ml).

The area of the zones of infection were determined by cutting the largest nodule per week of harvest into half and observed using a compound microscope with attached camera. NIS-element software (4.20.00 64- bit program) was used to automatically highlight and measure the area of infection.

Effect of nitrate and salinity in nodulation of Pongamia

Four replicate Pongamia seedlings previously sterilised as seeds were inoculated with R. mesoamericanum. Treatments include 2 mM KNO_3, 5 mM KNO_3, 10 mM KNO_3, 1% NaCl, 2% NaCl, 3% NaCl, positive control (inoculated) and negative control (uninoculated and not treated). The seedlings were watered with the treatments only once at 250 ml per plant. Plants were maintained under uniform glasshouse conditions and watered with sterile distilled water for 12 weeks and data gathered included shoot length, number of nodes, number of leaflets and plant dry weights.

Synergistic effect of rhizobia in nodulation

Pongamia seedlings were inoculated with either R. mesoamericanum strain PR-UQ-03, PR-UQ-05 only or combination of PR-UQ-03 and PR-UQ-05 strains to demonstrate any synergistic effect of strains to nodulation of Pongamia. There were five replicates per treatment. Plants were maintained for eight weeks under glasshouse conditions and nodule number, shoot length, number of nodes and leaflets and plant dry weights were gathered and analysed statistically using ANOVA and Tukey's HSD test.

RESULTS AND DISCUSSIONS

Strain symbiotic effectiveness and identification of R. mesoamericanum

There were 21 different locations that were sampled. A total of 42 samples were characterized (2 replicates per sample). The sampling was done in December to February where the weather is sunny and dry. In the baiting experiment using saline soils, it was observed that there were different zones of nodulation in the baited seedlings using the 5 different soil samples from Darbalara. The soil samples used corresponded to the gradient in the field (S2 least saline, S5, S8, S11 and S13 as highly saline). S2 soil sample contained rhizobia that nodulated the Pongamia seedlings at the early stage as evident in the nodules located close to the base of the roots, whereas, the S5, S8, S11 and S13 nodules tend to form farther from the base of the roots.

In several studies, strain symbiotic effectiveness testing is also referred to as the authentication test for rhizobia. The uninoculated control plants did not produce nodules, which confirmed that there was no contamination in the glasshouse set-up. The highest shoot length was observed in plants inoculated with isolate PR-UQ-03, with a mean value of 42 cm. The highest number of nodules was observed in plants inoculated with PR-UQ-05 with a mean value of 143 per plant and with a range of 70-240 per plant. All nodules were lateral with some distinct clustered nodules found in PR-UQ-05 inoculated plants. The shape of the nodules was globular to coralloid, having a range in size of 1-5 mm, and light to dark brown in color. Using Tukey's HSD test, the treatments had significant effects (at 0.01 and 0.05 levels) on biomass (shoot dry weight), number of nodules and other tested parameters.

Based from the results of the strain symbiotic effectiveness test, two superior rhizobia isolates were identified: PR-UQ-03 and PR-UQ-05. A single molecular band was produced from all the PCR products. Restriction enzyme digestion showed that the pattern of band fragments differed among the selected two superior rhizobia isolates, which means that they belong to different strains. BLAST results (e values=0) revealed that PR-UQ-03 and PR-UQ-05 are R. mesoamericanum (at 99% certainty). When, PR-UQ-05 was aligned with PR-UQ-03, the BLAST alignment revealed that these two isolates were highly similar (>99%), although the restriction nuclease digestion pattern, antibiotic profile and symbiotic tests results differ between them. Hence, they could be the same species but different strains.

Variability test

The nodulation studies are dependent on the differences in growth patterns among the different treated plants, aside from the nodule number and morphology. The leaflet arrangement, number of nodes, number of leaflets, shoot length, nodule number and plant dry weights of all 50 sterilised seeds from a single mother tree were not significantly different. Figure 1-3 show the results of the variability test for the 50 Pongamia from a single mother tree. There was no variation in the data for all parameters tested among all the 50 seedlings. All of the 50 Pongamia seedlings had a pinnately trifoliate leaflets arrangement,

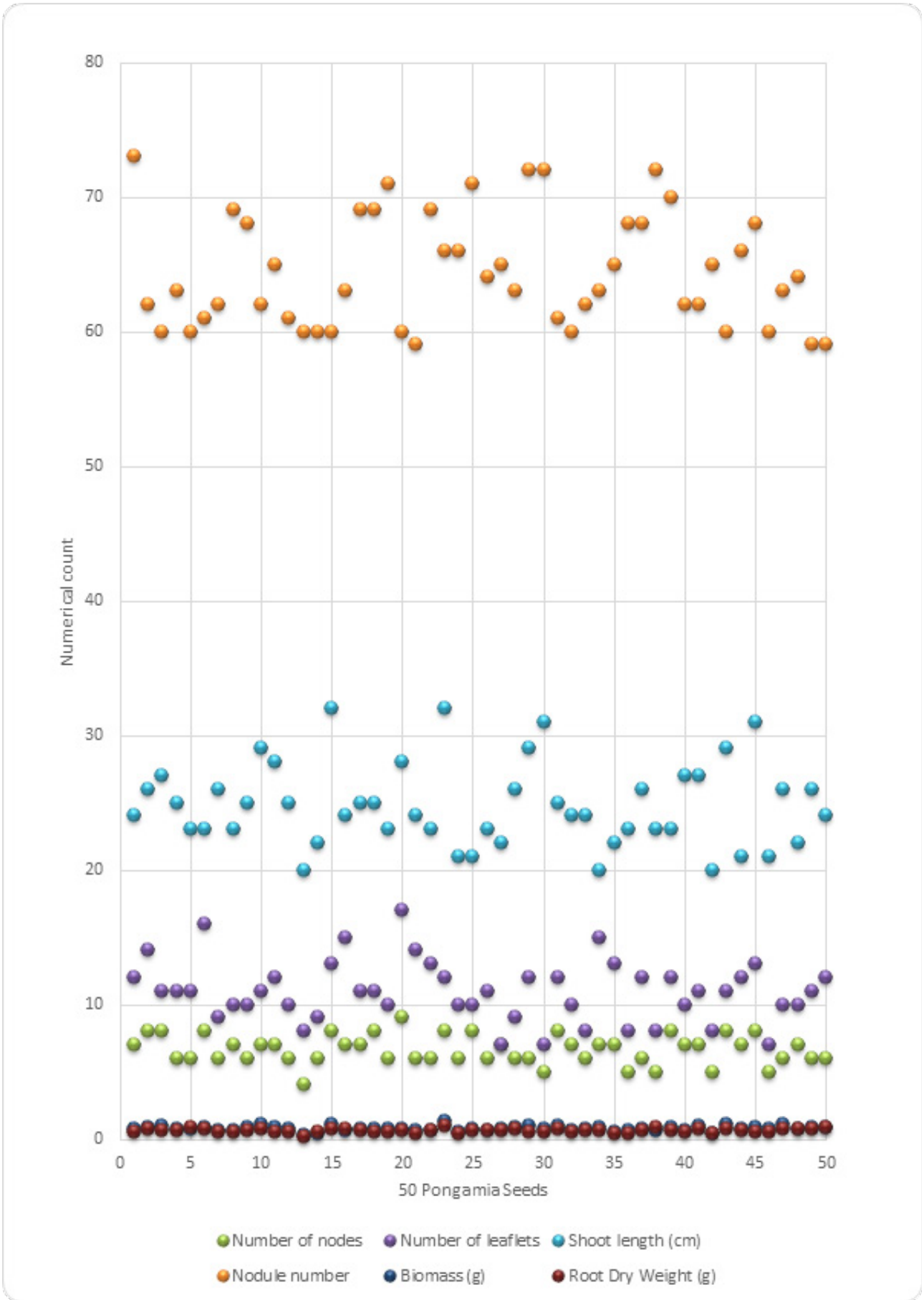


Figure 1. Scatter plot for the 50 *Pongamia* seedlings across all parameters.

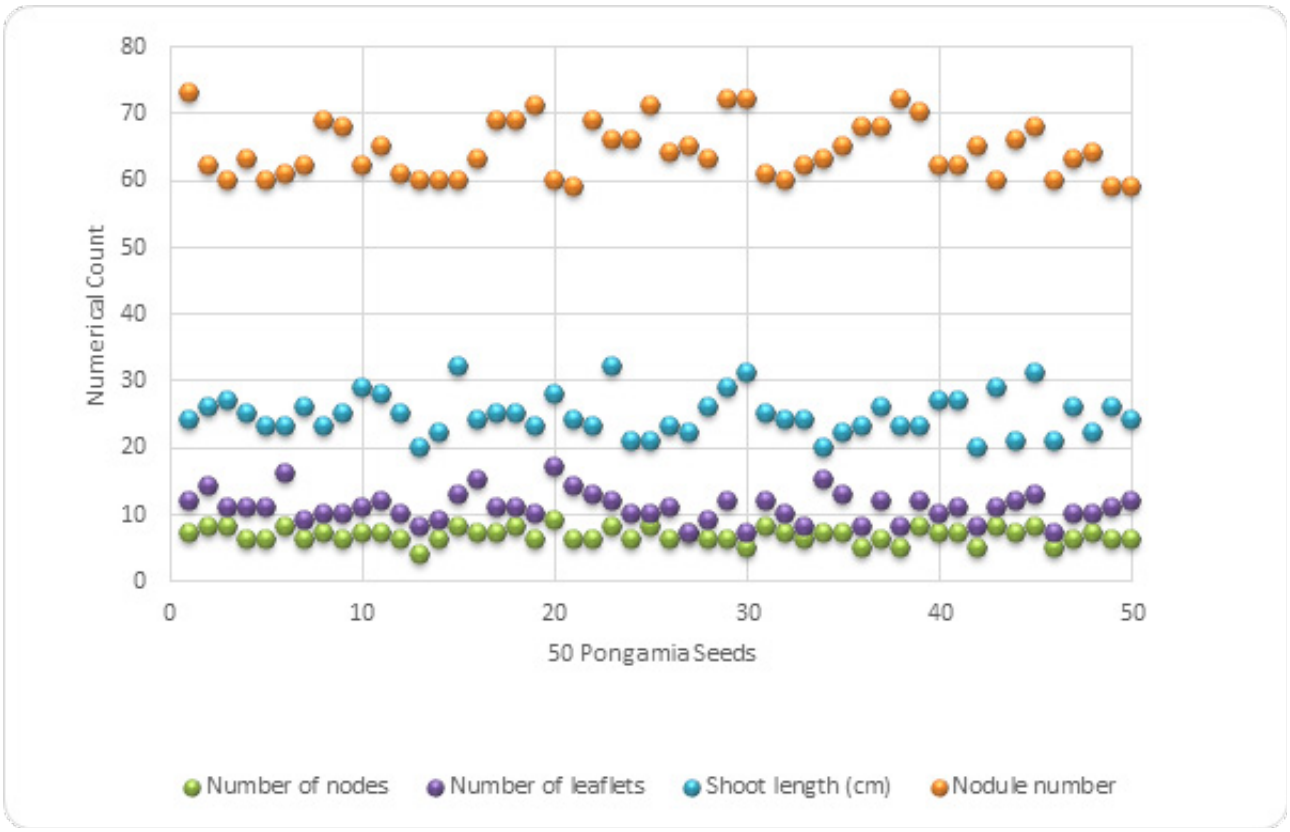


Figure 2. Scatter plot for number of nodes, number of leaflets, shoot length (cm) and nodule number.

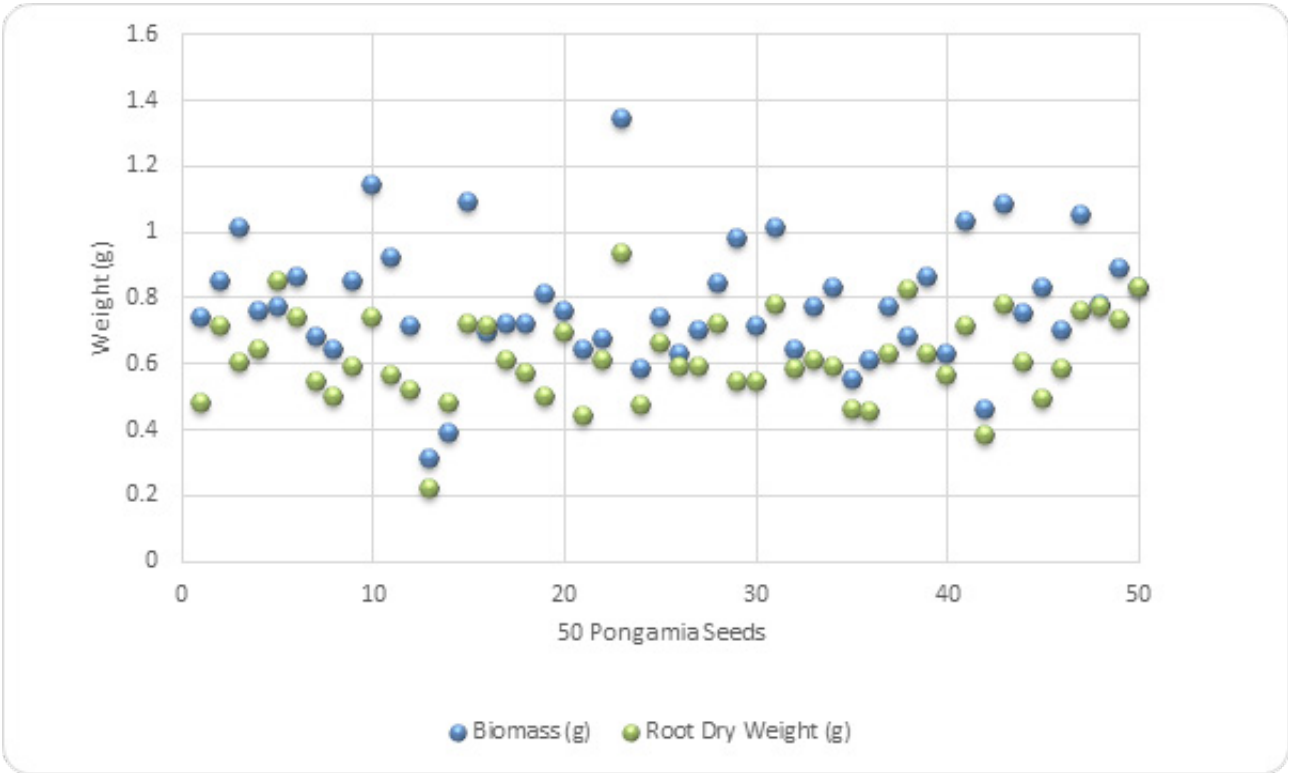


Figure 3. Scatter plot for biomass (g) and root dry weight (g).

spherical to coralloid and brown nodules.

Time course of nodulation

For the time course of nodulation using R. mesoamericanum strains, all uninoculated control plants had no nodules from weeks 4 to 8. Total plant dry weight increased from 1.23 g in week 4 up to 2.11 g in week 8. The total N level in shoots and roots increased from 5.12% Wt in week 4 to 6.22% Wt in week 7 but decreased to 5.89% Wt in week 8. Strain 1 (PR-UQ-03) plants nodulated in week 4 with 38 nodules per plant and continued to produce nodules until week 8 with 80 nodules per plant. The total plant dry weight increased from week 4 at 1.859 g to week 6 at 2.406 g but there were almost no changes in weeks 7 and 8. Leghemoglobin concentration at week 4 was 0.132 mg/ml and increased to 0.640 mg/ml in week 8. The area of the zone of infection of the biggest active nodule was 1.58 mm2 in week 4 and this continued to increase up to 3.89 mm2 at week 8. This is measured as the cross-section of the nodule. The total N level in shoots and roots was 6.56% Wt in week 4, with no significant change until week 8. The Strain 2 (PR-UQ-05) plants produced 56 nodules per plant in week 4, which continued to increase until week 8 with 78 nodules per plant. The total plant dry weight also doubled from 1.139 g in week 4 to 2.283 g in week 8. Leghemoglobin concentration increased from 0.235 mg/ml in week 4 to 0.663 mg/ml in week 8. The area of the zone of infection of the biggest nodule started at 1.86 mm2 in week 4 and increased to 3.08 mm2 in week 8. The total N level in shoots and roots was 4.30% Wt in week 4, increasing to 5.12% Wt in week 8.

The inoculation of PR-UQ-05 showed a significant increase in nodule number, total plant dry weight, leghemoglobin concentration, area of zone of infection, and total N level in shoots and roots, from week 4 to week 8, while inoculation with PR-UQ-03 increased nodule number, leghemoglobin concentration and area of zone of infection from week to week 8, but there was no significant increase in total plant dry weight and total N level in shoots and roots between weeks 4 and 8.

Effects of nitrate and salinity on Pongamia nodulation

R. mesoamericanum was inoculated to Pongamia seedlings which were then subjected to different nitrate concentrations (2 mM KNO3, 5 mM KNO3 and 10 mM KNO3) and NaCl concentrations (1% NaCl, 2% NaCl, and 3% NaCl). Negative control (uninoculated) and positive control (inoculated but without added nitrate and NaCl) were included. The results showed that treatment plants inoculated with R. mesoamericanum but without nitrate and NaCl added had 65+1.58 nodules and a biomass of 0.85+0.09 g. Plants grown with 2 mM KNO3 has higher number of nodules (72+-1.29) which further increased to 83+-1.44 for plants with 5mM KNO3. However, nodule count decreased significantly (33+-1.03) at 10mM KNO3. Nodules significantly decreased at 10 mM KNO3 with a mean value of 33+1.03. The addition of 1% NaCl had no significant effect relative to the positive control but decreased significantly when increased to 2% (NaCl) (from 63+0.71 nodules to 27+3.5). Addition of 3% NaCl significantly inhibited nodulation with only 1 replicate having three nodule while the rest has none. Biomass had no significant difference among all the treatments. The uninoculated plants (negative control) had no nodules and had significantly the lowest shoot and biomass measurements among all the treatments.

Synergistic effect of rhizobia on nodulation

Table 2 shows that there was no significant difference in all the parameters tested except for the nodule number and nodule dry weight in which the combined strains had significantly lower mean values at 0.1 level than either R. mesoamericanum strains PR-UQ-03 or PR-UQ-05. There was no synergistic effect of combined strains to Pongamia nodulation.

RESULTS AND DISCUSSIONS

Rhizobia nodulating Pongamia were found to

Table 1

Nodulation results of Pongamia seedlings inoculated with R. mesoamericanum subjected to different nitrate and NaCl concentrations.

Treatment	Shoot Length	Number of Nodules	Number of Nodes	Number of Leaflets	Biomass (g)	Root Dry Weight (g)
Positive Control (inoculated)	26.50^{a}	65.00^{bc}	6.75a	12.00a	0.85a	0.64a
Negative Control (uninoculated)	23.25^{a}	0.00^{e}	5.75a	8.00a	0.54a	0.45a
2 mM KNO3	26.25^{a}	72.00^{b}	6.00a	8.75a	0.85a	0.66a
695 mM KNO3	27.75^{a}	82.75^{a}	7.00a	12.25a	0.94a	0.62a
10 mM KNO3	27.00a	32.50^{d}	7.25a	10.50a	0.83a	0.50a
1% NaCl	25.25^{a}	63.00^{c}	7.25a	10.25a	0.80a	0.63a
2% NaCl	25.75^{a}	26.75^{d}	6.75a	10.00a	0.74a	0.52a
3% NaCl	24.75^{a}	0.75^{e}	7.00a	8.75a	0.87a	0.53a
Treatment Mean	25.81^{ns}	42.84**	6.72^{ns}	10.06^{ns}	0.80^{ns}	0.57^{ns}

Means having the same letter superscripts are not significantly different at .05 level using Tukey's HSD test
*** Mean is highly significant at .01 level*
ns = not significant at .05 level

Table 2.

Nodulation results of single inoculant R. mesoamericanum strains PR-UQ-03 and PR-UQ-05 versus multiple/ combined inoculants to determine synergistic effect of rhizobia.

Treatments	Shoot Length	Number of Nodes	Number Leaflets	Nodule Number	Biomass (g)	Root Dry Weight (g)	Nodule Dry Weight (g)	Total Dry Weight (g)
Combined Strains	29.00[a]	7[a]	16[a]	51[b]	1.41[a]	0.70[a]	0.04[b]	2.15[a]
PR-UQ-03	42.20[a]	6a	13[a]	84a[b]	2.02[a]	0.94[a]	0.16[a]	3.13[a]
PR-UQ-05	35.60[a]	5a	11[a]	143[a]	1.33a	0.76[a]	0.15[a]	2.24[a]
Treatment Mean	36.07[ns]	6ns	13[ns]	96*	1.60[ns]	0.81[ns]	0.12*	2.53[ns]

Means having the same letter superscripts are not significantly different at .05 level using Tukey's HSD test
** Mean is significant at .01 level*
ns = not significant at .05 level

be present in any soil at different sites in Queensland. These unique bacteria established symbiotic nitrogen fixation with Pongamia to different degrees, from very efficient nodules formed in the baiting techniques to less efficient ones. The superior rhizobia were identified as R. mesoamericanum. The establishment of effective Pongamia-rhizobia symbiosis is crucial in the optimization of nitrogen fixation in Pongamia, which greatly depends on the rhizobia's adaptability and ability to form efficient nodulation, leading to an efficient nitrogen-fixation process. This study revealed that there were 95 potential rhizobia in the soil that nodulated Pongamia. This helps prove that Pongamia is a promiscuous legume, based on the wide range of rhizobia that formed nodules during the baiting experiments. The presence of rhizobia nodulating Pongamia is shown in this study to be widely distributed geographically. Across Queensland, rhizobia were found to be present even in those areas where Pongamia was not previously grown. The geographical distribution of rhizobia nodulating Pongamia shown in this study provides hope for people or institutions who may want to grow Pongamia in their agricultural or marginal lands as source of biofuel. Moreover, it was established in the study that these rhizobia are able to grow in soils which are low in nutrients, acidic to basic pH values, and even in high saline environments.

However, it was shown that highly saline soil samples inhibited nodule formation of Pongamia, as in the case of the Darbalara soil samples. Darbalara is a farm in the University of Queensland Gatton Campus where the soils are saline. Irrigation-induced and dryland salinity have become major issues for the Lockyer Valley where Darbalara is located. This alluvial plain is associated with excessive clearing of the uplands and an expansion of irrigation after World War II. Dryland salinity expands during and after wet years when rising water tables get close to the surface. By contrast irrigation salinity is exacerbated during drought years, as water levels, depressed by increased irrigation, encourage the migration of saline waters from the overlying sandstone beds into the alluvium (Queensland Water Resources Commission, 1982). Additionally farmers may draw on deeper aquifers, which are closer to the sandstone and invariably higher in salinity. Salinity has been shown to interfere with nodule initiation in chickpea, cowpea and mung bean, and to also cause a reduction in number, weight as well as nitrogen fixing efficiency of nodules (Balasubramanian and Sinha, 1976). Salinity causes a significant decrease in leghemoglobin content up to 125 decasiemens, the effect decreasing with aging of the nodules probably, because of irreversible oxidation of leghaemoglobin. Tu (1981) observed that inhibition of colonization of the root by the rhizobium strain was the main reason for poor nodulation under NaCl stress. Presence of high salt concentration causes root hairs to shrink. Martin and Ruiz-Torres (1992) found that nitrate accumulation was significantly higher in plants using C3 photosynthesis (barley and wheat) than C4 (maize and sorghum).

Salinity showed a significant inhibitory effect on the nodulation of chickpea even at 4.0 dS m-1. NaCl stress decreases shoot and root dry weight, total number of nodules per plant, nodule weight and average nodule weight (Elsheikh and Wood, 1990; Mudgal, 2004). Although, nodules were observed in inoculated plants grown at 6 dS m-1, nitrogen fixation was completely inhibited. These findings indicate that symbiosis is more NaCl sensitive than both the rhizobium and the host plant.

Another important finding in the baiting experiment using saline soils was the observation of the different zones of nodulation in the baited seedlings using the 5 different soil samples from Darbalara. The soil samples used corresponded to the gradient in the field (S2 least saline, S5, S8, S11 and S13 as highly saline). S2 soil sample contained rhizobia that nodulated the Pongamia seedlings at the early stage as evident in the nodules located close to the base of the roots, whereas, the S5, S8, S11 and S13 nodules tend to form farther from the base of the roots. Early nodulation means that the rhizobia infect the roots immediately after inoculation while delayed nodulation means that rhizobia infect the roots few weeks after inoculation. In early nodulation, the nodules are formed near the base of the roots while in delayed nodulation, the nodules formed towards the lateral roots far from the base of the roots or crown.

Rhizobia were shown to be so physiologically versatile that they were isolated even in those soil types in which other bacteria cannot survive, such as in the case of Meandu Mine soil samples. This finding implies that the

rhizobia can be applied in infertile soils, including areas undergoing rehabilitation in mining sites or unproductive areas, to convert them into productive agricultural land.

The nodule morphology of Pongamia was described as being laterally located in the roots, with shapes ranging from spherical, cylindrical or coralloid, their color being dark brown, reddish/pinkish or brown, and a size ranging from 1 mm to 10 mm in diameter. The nodule anatomy of Pongamia in this study was similar to that described by Samuel et al. (2013). Pongamia nodule exhibited several lobed infection zones surrounded by a thicker layer of cells called the inner nodule cortex. The lobed infection zones were filled by non-infected interstitial cells which were located towards the center of the cortex. Large areas of the cortex consisted of parenchyma cells surrounded by a sclerenchyma layer which separated the outer cortex from the rest of the nodule. Within the cortex were several tannin cells. The vascular traces were dichotomous and branched several times to encapsulate the nodule and were located close to the infected zone within the cortex. This close proximity has been reported previously and is most likely present to increase symbiotic efficiency (Walsh, et al., 1992; Guinel, 2009).

R. mesoamericanum, a fast-grower displayed highest nodule number, shoot length and biomass. The variability test shows that there was no significant variation in the nodulation and growth parameters of all the 50 seedlings which came from one source or mother tree. Thus, the results in all the experiments were sufficient to make the conclusion that a particular treatment is most effective when based on different parameters when using seeds coming from same mother tree. There was no genetic effect or variability noted in the seeds from the same source. Therefore, nodulation can be improved when seeds from best performing tree are used for germination. Nodule number of the 50 seedlings was shown to have no variation.

The time course of nodulation was relevant in filling in the gaps of early Pongamia nodulation. Growth pouches enabled the observation of root development and nodule formation at its earliest stage until 6 weeks. Radicles formed at 5 days, while nodules started to become visible at 2-3 weeks after inoculation. Unlike in soybeans where nodules can be counted after 2 weeks, nodulation in Pongamia tends to be delayed or slower than that of soybeans. New nodules continued to be formed even at 8 weeks after inoculation, while old nodules became coralloid. Pot studies were found to be effective for the study of the time course of nodulation for later stages of growth, since growth pouches cannot support the root mass of Pongamia at 6-8 weeks. Also, the roots tend to go outside the pouches, thereby damaging the setup.

Therefore, growth pouches may be good when observing early root and nodule development in Pongamia but not during its later stages of growth. In addition, nodule number was shown to decrease or be inhibited in the growth pouches setup but not in pots where several nodules were observed. The time course of nodulation in pots showed that there was no significant difference in the data among R. mesoamericanum strains. The size of nodules was less than 1 mm in week 3 and increased up to 5-10 mm in week 8. This is directly proportional to the nodule dry weights.

The leghemoglobin concentration tended to increase from the week that the nodules started to appear but then levelled off in later weeks. This implies that Pongamia nodules started to become less active or less efficient during weeks 3 and 4 as the bacteroids inside the nodules invaded the whole nodule, thereby increasing the leghemoglobin concentration. This finding is consistent with the area of zone of infections computed for the largest nodule found in replicate plants in the week of harvest. Unlike in Bradyrhizobium species cross-sections where it is possible to see many interstitial cells inside the nodules, Pongamia nodules infected with R. mesoamericanum were observed to have been fully occupied by bacteroids (reddish area in cross-sections) and that the area of zone of infections increased over the course of time. The use of a dissecting microscope attached to the computer greatly enhanced the cross-section observations, while with the aid of the software, the area was conveniently obtained based on the highlighted image (the zone of infection inside the nodule can be traced using the mouse cursor and values are generated which can be converted into the actual size of the nodule).

Nitrate and salinity were also found to have effects on nodulation in Pongamia. The addition of nitrate resulted in no significant difference from inoculated plants (without nitrate added), although the mean values of nodule numbers suggested that 2 mM maybe less significant or had no effect on nodulation, as it is close to the mean value of inoculated plants. However, the nodule number at 5 mM nitrate showed an increase, which implies that Pongamia nodulation was not inhibited by the increased nitrate concentration. However, at 10 mM there was a significant decrease in nodule number (to half the values at 2 and 5 mM), which means that the presence of high nitrate concentration of 10 mM can inhibit Pongamia nodulation. In order for Pongamia to have improved nodulation, the nitrate level in the soil or growth medium must be controlled to low amounts such as less than 2 mM – the optimum nitrate level which is best for nodulation. This finding was supported by the study of Eaglesham et al. (1983) which showed that legumes only require low amounts of nitrate for optimum nodulation.

In the case of the salinity test, 1% NaCl did not have significant effect on nodulation but at 2% NaCl nodule number was reduced by 50%, while at 3% NaCl nodulation seemed to be almost completely inhibited. Although these results suggest that Pongamia nodulation is affected by the presence of NaCl, nodule formation, even at 2% NaCl, is proof that rhizobia nodulating Pongamia can still survive at that high saline concentration but not at higher levels (at 3%). Moreover, Pongamia still grew well even at 3% (although with no nodules), which suggests that Pongamia is indeed NaCl tolerant as indicated in the literature.

Nitrate inhibition of nodulation was previously demonstrated by Samuel et al. (2103) in a split-root system but different rhizobia were used (CB1809/USDA110 vs R. mesoamericanum in this study). Also, the results of the former authors showed that at 5 mM nitrate, nodulation

was severely inhibited and at 10 mM nitrate nodules were reduced by 90%. In the current study, 5 mM appears to be a good concentration for Pongamia nodulation, while at 10 mM the nodules were partially inhibited (50%). Therefore, it is apparent that different rhizobia can have different effects on the nodulation of Pongamia at varying nitrate levels.

Lastly, the synergistic effect of combined rhizobia was shown to be not significant or absent in the tested plants. When combined strains was inoculated to baited plants (used to represent synergy), the nodule number and nodule dry weight were significantly lower than that of the single inoculants R. mesoamericanum strains PR-UQ-03 and PR-UQ-05. Therefore, a combination of both fast and slow growing rhizobia has no relevance for Pongamia nodulation. However, the strain symbiotic effectiveness test showed that the fast-grower inoculants to be more effective than slow-growers. The slow-growers replicate at a slower rate, thus they need longer time to reach certain concentration to initiate infection, which is the reason why the fast-growers outcompete them. Also, infected nodules may have lower zones of infection because they tend to invade the nodule at slower rate than the fast-growers. This is the reason why in the strain symbiotic test, plants inoculated with slow-growers tended to have less efficient nodules, while the fast-growers produced better plants more efficiently over the 8-week growth period in the glasshouse.

CONCLUSION AND RECOMMENDATION

Salinity affects nodulation in an inversely proportional relationship, with a decline in nodulation as the NaCl concentration increases. Pongamia is NaCl-tolerant and can still withstand 3% NaCl concentration. R. mesoamericanum, a fast-grower, is considered the superior strain of rhizobia among the 95 isolates from across Queensland. It can survive at 2% NaCl and can still nodulate Pongamia but at a lower rate. Nodules formed within 3-4 weeks after inoculation and continued to increase in size and number over the 8-week growth period, while the area of zone of infection became wider until it fully occupied the whole nodule. Although the leghemoglobin content initially showed an increase over time, it then tended to level off in later weeks. The combination of rhizobia inoculants had no synergistic effect on the nodulation of Pongamia. The results from the study may imply that Pongamia can be grown commercially as biofuel feedstock and its growth can be improved with R. mesoamericanum through the process of biological nitrogen fixation. It is recommended to test legume like Pongamia for NaCl sensitivity on sandy and rich soils and do a soybean comparison. Soil nitrate level must be controlled to a maximum of 5 mM concentration to prevent potential inhibition of nodulation.

REFERENCES

Balasubramanian, V., Sinha, S. K. (1976). Effects of salt stress on growth, nodulation and nitrogen fixation in cowpea and mung beans. Physiol Plant 36:197.

Biswas, B., Kazakoff, S. H., Jiang, Q., Samuel, S., Gresshoff, P. M., & Scott, P. T. (2013). Genetic and genomic analysis of the tree legume Pongamia pinnata as a feedstock for biofuels. *The Plant Genome, 6*(3), plantgenome2013-05.

Calica, P. N. (2017). Nodulation and nitrogen fixation of Pongamia pinnata. *Journal of Tropical Crop Science Vol, 4*(1).

Eaglesham, Hassouna, S., and Seegers, R. (1983). Fertilizer-N effects on N2 fixation by cowpea and soybean. *Agronomy journal 75*, 61.

Elsheikh E. A. E. and Wood, M. (1990). Effect of Salinity on Growth, Nodulation and Nitrogen Yield of Chickpea (Cicer arietinum L.). J. Exp. Bot. (1990) 41 (10): 1263-1269 doi:10.1093/jxb/41.10.1263.

Gresshoff, P. M., Rangan, L., Indrasumunar, A., & Scott, P. T. (2017). *A new bioenergy crop based on oil-rich seeds from the legume tree Pongamia pinnata. Energy Emission Control Technol, 5*, 19-26.

Guinel, C. F. (2009). Getting around the legume nodule: 1. The structure of the peripheral zone in four nodule types. *Botany. 87*, 1117-1138.

Kasper, S., Christoffersen, B., Soti, P., & Racelis, A. (2019). Abiotic and biotic limitations to nodulation by leguminous cover crops in South Texas. *Agriculture, 9*(10), 209.

Keilin, D., & Wang, Y. L. (1945). *Haemoglobin in the root nodules of leguminous plants. Nature, 155*(3930), 227-229.

Martin, B. and Ruiz-Torres, NA. (1992). Effects of water-deficit stress on photosynthesis, its components and component limitations, and on water use efficiency in wheat (Triticum aestivum L.). *Plant Physiology 100*, 733-739.

Mudgal, V., Madaan, N., Mudgal, A., Singh, A., & Kumar, P. (2010). Comparative study of the effects of salinity on plant growth, nodulation, and legheamoglobin content in kabuli and desi cultivars of Cicer arietinum (L.). *Atlas Journal of Biology, 1*(1), 1-4.

Nemenzo-Calica, P., Indrasumunar, A., Scott, P., Dart, P., & Gresshoff, P. M. (2016). Nodulation and symbiotic nitrogen fixation in the biofuel legume tree Pongamia pinnata. *Atlas Journal of Biology*, 274-291.

Samuel, S., Scott, P.T., and Gresshoff, P.M. (2013). Nodulation in the legume biofuel feedstock tree Pongamia pinnata. *Agricultural Research 2*, 207-214.

Tu, J. C. (1981). Effect of salinity on Rhizobium-root-hair interaction, nodulation and growth of soybean. *Canadian Journal of Plant Science, 61*(2), 231-239.

Walsh, K. B., Atkins, R. S., & Low, C. S. (1992). Vascular anatomy of fabaceous nodules of determinate growth. *Plant, Cell and Environment. 15*, 849-854.

Central Mindanao University
Journal of Science
ISSN Print: 0116-7847 ISSN Online: 2704-3703

Anti-microbial Properties of Selected Plant Leaf Extracts Against *Aspergillus niger* (van Tieghem), *Pseudomonas aeruginosa* (Schroeter) and *Staphylococcus aureus* (Rosenbach)

Melanie M. Garcia[1,2]*, Maria Luisa M. Antao[2], Chloe Lynn M. Gotera[2], Paulyn Jane P. Hermoso[2], Rizzarttein V. Samblaceño[2], Russel D. Valencia[2], Mary Grace C. Budias[2], Farrah V. Teodoro[2], Nicole F. Olayvar[2], Jhonnel P. Villegas[3] and Phoebe S. Nemenzo[4]

[1]Ph.D in Environmental Science-Graduate Student, School of Environmental Science and Management (SESAM) and Professional School for Agriculture and the Environment (PSAE) University of the Philippines-Los Baños, 4031, Philippines Davao Doctors College, Davao City, 8000, Philippines
[3]Institute of Education and Teacher Training, Davao Oriental State University
[4] Biology Program, Arts and Sciences Department, San Pedro College, Davao City

ABSTRACT

With growing reports of multidrug-resistant pathogens causing untreatable human infections, the need for new antimicrobial therapies is becoming increasingly important. This study was conducted to investigate the antimicrobial properties of the leaf extracts of *Premna odorata* Blanco, *Petersianthus quadrialatus* Merr., *Shorea astylosa* Foxw., and *Tridax procumbens* Linn. The medicinal importance of these plants remains understudied despite their abundant distribution and endemism in the Philippines. A disk diffusion assay was utilized to test the antimicrobial properties of *T. procumbens* leaf extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. On the one hand, the fungal colony and spore germination assay was used to test *P. odorata, P. quadrialatus*, and *S. astylosa* leaf extracts against Aspergillus niger. Different concentrations of leaf extracts were prepared and compared with positive and negative controls. *T. procumbens* minimally inhibits the growth of *P. aeruginosa* (10 mm) and *S. aureus* (10 mm), while *S. astylosa* leaf extracts revealed the most significant inhibition on colony growth (5.38 mm) and spore germination (15). P. odorata and *P. quadrialatus* showed the least (28.35 mm; 82.17) and moderate (10.97 mm; 49.5) inhibitory potentials, respectively. The discovery of new antimicrobial compounds from these plant extracts is seen as a potential resolve to the pressing problem of antimicrobial resistance.

Keywords: Anti-microbial, Tridax procumbens, Staphylococcus aureus, Pseudomonas aeruginosa, Premna odorata, Petersianthus quadrialatus, Shorea astylosa, Aspergillus niger

INTRODUCTION

Improper use of antibiotics and antifungal drugs remains the foremost factor for antimicrobial resistance (AMR) and multi-drug resistance (MDR), compromising medical efficacy and human health. There is a rapid emergence of AMR and MDR among pathogens, which necessitates discovering novel antimicrobial therapies and alternative clinical approaches (Aslam et al., 2018; Kumar et al., 2020). This global crisis has refocused scientific attention on drug discovery from traditional medicine species (Prakoso et al., 2018).

Selected pathogens of concern, such as the *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Aspergillus niger*, have exhibited drug resistance over the years. *S. aureus*, the leading cause of skin and soft tissue infections, is resistant to methicillin (Stapleton & Taylor, 2002). Another opportunistic pathogen causing healthcare associated infections (HAI), infections, *P. aeruginosa*, is reported to be resistant to antibiotics (Pang et al., 2019; Wu & Li, 2015). Likewise, the black mold disease-causing *A. niger* demonstrated resistance against specific antifungal agents (Van Der Linden et al., 2011; Baker, 2006). The discovery of new antimicrobial compounds from plant extracts is seen as a potential resolve to this pressing problem. Novel antimicrobials such as ceftolozane exhibits specific activity against *Pseudomonas aeruginosa* in patients with hospital-acquired and ventilator-associated pneumonia and ceftazidime with the novel β-lactamase inhibitor avibactam produce an additive effect in patients infected with Gram-negative bacteria (Cheesman et al., 2017).

P. odorata is an endemic and a well-known medicinal plant in the Philippines. Various applications of the plant include its use as analgesic, antipyretic, and anti-inflammatory. *P. quadrialatus* is an endemic Philippine tree of the family Lecythidaceae, locally known as Toog, Magtalisai, and Kapullan. (Dayan, et. al., 2005). Yakal (*Shorea astylosa* Foxw.) is a medium to large tree about 25 to 30 meters tall. It is one of the species of family Dipterocarpaceae, native in the Philippines, commonly

ARTICLE INFORMATION
Melanie M. Garcia
Email Address: mmgarcia14@up.edu.ph
Received: June 24, 2021; Accepted: Sept 10, 2021
DOI: https://doi.org/10.52751/cnfq9322

found in Luzon, particularly Quezon and Camarines; Samar; Negros; and Mindanao, particularly Zamboanga, Agusan, and Davao. *Tridax procumbens* belong to the family-Asteraceae, tribe-Heliantheae, genus-Tridax, is a perennial plant. The extracts of *Tridax procumbens* have been reported to have various pharmacological effects, antimicrobial activity against both gram-positive and gram-negative bacteria, and stimulate wound healing.

Several endemic plants such as *Premna odorata* (Common Name: Alagao), *Petersianthus quadrialatus* (Common Name: Toog), and *Shorea astylosa* (Common Name: Yakal) are widely used as traditional medicine in the Philippines (Fernando et al., 2008). These are traditionally used to treat microbial diseases, although pharmacological evidence is scarce. Their ethnomedical applications as an analgesic, antipyretic, anti-inflammatory, antinociceptive, or anti-viral indicate their homeopathic properties (Dianita & Jantan, 2017; Nunez et al., 2021; Ecosystem Research and Development Bureau (ERDB), 2012; Dayan et al., 2005). Reports indicated that *Tridax procumbens*, an invasive alien plant species (Shabana et al., 2020), have medicinal properties and may be used to treat wounds, viral infections, inflammations, diabetes, and arthritis (Suseela et al., 2002; Kumar et al., Diwan et al., 1982; Diwan et al., 1983; Taddel & Rosas, 2000; Udupa et al., 1991).

This study investigates the antimicrobial potentials of the ethanolic leaf extracts of *P. odorata*, *P. quadralatus*, and *S. astylosa* against *A. niger* and *T. procumbens* against *P. aeruginosa* and *S. aureus*. The results aimed to provide baseline data and preliminary evidence of their pharmacological applications.

METHODOLOGY

Plant samples

P. odorata is an endemic and a well-known medicinal plant in the Philippines. Various applications of the plant include its use as analgesic, antipyretic, and anti-inflammatory. Compounds associated with anti-inflammatory and anti-nociceptive properties were previously isolated from its leaves. Preliminary phytochemical screening of *P. odorata* crude leaf extracts showed the presence of steroids, terpenoids, flavonoids and hydrolysable tannins (Montoya, 2012). It is one of the species of family Lamiaceae, native in the Philippines, Nepal, India to Myanmar, China, Taiwan, Indo-China, Thailand, Japan, Malaysia and Australia. This plant is also referred to several names such as Fragrant Premna, Alagaw, Alagao, and Adiyo (Philippine Medicinal Plants, ND) (Fig. 1).

P. quadrialatus is an endemic Philippine tree of the family Lecythidaceae, locally known as Toog, Magtalisai, and Kapullan. (Dayan, et. al., 2005). It is a deciduous, medium-sized to fairly large tree that grows up to 40 m tall and 100 cm in diameter. It has been found to strong as akle, ipil and molave. Because of its appearance and high quality, Toog is now recognized in the local and world market under the trade name Philippine Rosewood. However, it is considered a vanishing timber (Philippine Flora, 2009). It is

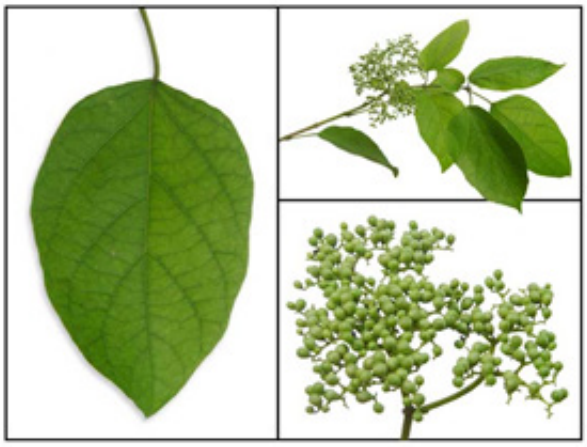


Figure 1. Alagao (*Premna odorata Blanco*) leaves and fruits (Stuart, 2015).

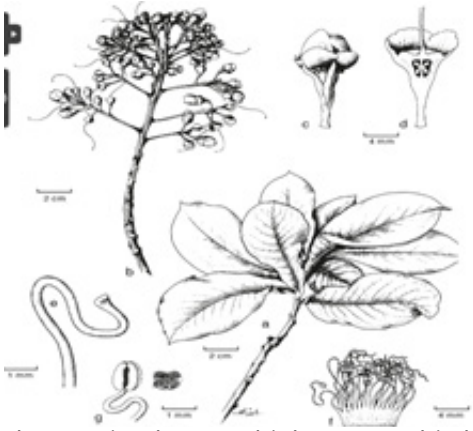


Figure 2. Philippine Rosewood (*Petersianthus quadrialatus*) a. Habit; b. inflorescence; c. flower bud with winged calyx tube; d. longitudinal section of ovary; e. style and stigma; f. stamen; g. anther (Flora Malesiana, n.d.)

Figure 3. Yakal (*Shorea astylosa*) tree and leaves from Tree Facts *Shorea astylosa* Foxw. (Energy Development Corporation, 2012).

Figure 4. *Tridax procumbens*
Source: https://lithophytes.files.wordpress.com/2012/12/dscn4440edited.jpg

fairly common and grows scattered in primary rainforests, near riverbanks or on hillside, in swampy and cool places and grows in an elevation that ranges from sea level up to about 400 m. It thrives in an area where rainfall is evenly distributed throughout the year. It requires well-drained, clayish, sandy and loamy soils (Philippine Flora, 2009). Isolation of stigmasterol, unsaturated triglycerides, β-amyrin fatty acid ester and α-amyrin fatty acid ester from the leaves of *P. quadrialatus*. (Ragasa, et. al., 2014).

Yakal (*Shorea astylosa* Foxw.) is a medium to large tree about 25 to 30 meters tall. It is one of the species of family Dipterocarpaceae, native in the Philippines, commonly found in Luzon, particularly Quezon and Camarines; Samar; Negros; and Mindanao, particularly Zamboanga, Agusan, and Davao. It can also be found in primary forests at low altitudes (Philstar, 2012). This plant is also referred to as Yamban, Gisok, and Dungon-dungon (Energy Development Corporation, 2012).

Tridax procumbens belong to the family-Asteraceae, tribe-Heliantheae, genus-Tridax, is a perennial plant. It is available in all seasons. It has been known by several names like coat buttons in English, ghamra in Hindi, Jayanti veda in Sanskrit, herbecaille in French, vettukaayapoondu in Tamil. It is a weak straggling herb about 12-24cm long with few leaves 68cm long and grows on road sides, hedges and in wastes globally. The leaves of this plant including other aerial parts except flowering tops have been claimed to be useful in the treatment of inflammatory conditions and has a tendency to heal wounds, anti-diabetic activity, anti-arthritic activity, preventing hair loss, diarrhea and serve as insect repellent. (Kumar et. al. 2012) Tridax procumbens Linn (Compositae) is common grass found in tropical southern part of Nigeria, growing primarily during raining season.

The extracts of *Tridax procumbens* have been reported to have various pharmacological effects, antimicrobial activity against both gram-positive and gram-negative bacteria, and stimulate wound healing. Traditionally, the local Yoruba population of Western States of Nigeria uses the leaf of the plant as treatment to reduce blood pressure. (Taddel and Rosas, 2000, Udopa et al;1991, Diwan et al; 1982, and Diwan et al; 1983). *Tridax procumbens* is known for several potential therapeutic activities like antiviral, antibiotic efficacies, wound healing activity, insecticidal and anti-inflammatory activity (Suseela et al 2002).The Ethno pharmacological and traditional use of plants often results in the discovery of new biologically active molecules (Alisi et al., 2008). Plants have a long history of use in the treatment of cancer (Mohammad et al., 2006).

Fresh and healthy leaves of the plant, *P. odorata*, *P. quadrialatus*, and *S. astylosa* and *T. procumbens* were

collected between August and October, 2017 at Cadayona Farm, Tugbok District, Mintal, Davao City. The taxonomy of the studied plant species: P. odorata, P. quadrialatus, S. astylosa, and T. procumbens were certified and verified by a botanist. The researchers also ensured that the plant specimens were healthy and free from any discoloration in any part.

Microbial cultures

Pure cultures of *P. aeruginosa* (ATCC27853) and *S. aureus* (ATCC25923) were obtained from the Department of Science and Technology Regional Office XI (DOST XI) located in Friendship cor. Dumanlas Rds., Bajada, Davao City. Also, A. niger samples were identified by a plant pathologist. Experiments using the plant and microbial cultures were conducted at the Davao Doctors College (DDC), Davao City, Philippines.

Leaf extract preparation

Fresh leaves of *P. odorata*, *P. quadrialatus*, *S. astylosa* and *T. procumbens* were washed with tap water and distilled water separately. After washing, the leaves were air-dried at room temperature for seven days prior to cutting into smaller pieces. A commercial mechanical grinder was used to powderize the leaves. Next, 250 g of powdered leaves of *P. odorata*, *P. quadrialatus*, *S. astylosa* and *T. procumbens* were soaked in 800 ml of 80% ethanol for 48-72 hours. Ethanol was used in this study because this solvent was able to extract hydrophilic bioactive compounds due to its polarity (Do et al., 2014; Ehiowemwenguan et al., 2014). The extracts were filtered using Whatman filter paper (No.1) while hot, concentrated in vacuum under reduced pressure using rotary flask evaporator, and dried in a desiccator. The extracts were then kept in sterile bottles, under refrigerated conditions (2^0 to 4^0 C) until further use for antimicrobial assays. The dry weight of the plant extracts was obtained by the solvent evaporation and used to determine concentration in mg/ml (Bhalodia et al.,2011).

Preparation of Different Concentrations of Plant Extracts

Different concentrations (100%, 75%, 50% and 25%) of *P. odorata, P. quadrialatus, S. astylosa*, 40 ml of the P. odorata, P. quadrialatus, and S. astylosa extracts (40 ml) were prepared in sterilized vials at room temperature (Guevara, 2005). The prepared 40 ml leaf extracts of *P. odorata, P. quadrialatus, and S. astylosa* were diluted by adding 80 ml distilled water (1:1 ratio) to obtain the 50% concentration.

T. procumbens leaf extracts were prepared in different concentrations such as 25 µg/ml, 50 µg/ml, 75µg/ml, and 100 µg/ml with dimethyl sulfoxide (DMSO). One hundred grams (100 g) of leaves were washed, air-dried, and macerated. The leaves were soaked in 100ml ethanol for 24-72 hours (Guevara, 2005). The macerated leaves were separated from the extract mixed with ethanol. Afterwhich, pure extract from the ethanolic extract was derived via rotaevaporation (RE100-Pro Digital Rotary Evaporator, 3500 rpm for 30 minutes). The beaker in rotaevaporation was rotated at a variable speed of 0 – 220 rpm. Finally, the beaker was placed in a water bath without exceeding the boiling point. After the evaporation process, pure *T. procumbens* leaf extract was obtained.

Phytochemical Screening

The extracts were subjected for a phytochemical screening in order to detect bioactive compounds. Air dried and powdered plant materials were brought and screened to a private laboratory institution of the University of the Immaculate Concepcion-Science Resource Center for the presence of saponins, tannins, alkaloids, flavonoids, triterpenoids, steroids, glycosides, anthraquinones, coumarin, saponins, gum, mucilage, carbohydrates, reducing sugars, starch, protein, and amino acids (Kumar et al.,2009; Nariya et al., 2011).

Antimicrobial property

Media preparation

A mixture of 5.7 g nutrient agar and 9.5 g Muller-Hinton Agar were placed on a 500 ml Erlenmeyer flask. It was added with 250 ml distilled water then heated using a hot plate until both agar mixtures were clear. Next, the Erlenmeyer flasks containing the cooked agar were covered with cotton and aluminum foil before its placement in the autoclave for 45 minutes to 1 hour at 121°C. After autoclaving the agar, it was cooled and poured unto the sterile Petri dishes to solidify.

Meanwhile, 39 g of powdered Potato Dextrose Agar (PDA) was suspended in 1 L distilled water. The solution was thoroughly mixed and boiled until it was homogenized. The mixture was sterilized in the autoclave at 121°C for 15 minutes. Then, it was cooled down to room temperature and was poured into each Petri plate (Aryal, 2017).

Agar Disk diffusion method

The agar disk diffusion method was used to determine the antibacterial activities of T. procumbens against Pseudomonas aeruginosa and Staphylococcus aureus. The test was performed by applying a bacterial inoculum of approximately 1-2x108 CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton Agar plate and Nutrient Agar by spreading. Replicates (N=3) and control set-ups with fixed concentrations and paper antibiotic disks, respectively were prepared and incubated at 37°C for 24 hours. For the control set-up, the antibiotic Vancomycin served as the positive control while distilled water served as the negative control. The test disks were soaked in the Petri dishes containing different concentrations of leaf extracts for 15 minutes before being placed on the agar plates. The zones of inhibition around each of the antibiotic disks were measured in millimeters (mm) using a caliper.

Colony diameter method

A 2 ml volume of leaf extracts with 50% concentration was aseptically poured onto Petri plates (per plant sample), followed by the addition of 18 ml of melted Potato Dextrose Agar (PDA) (Raji & Raveendran,

2013). Before the agar medium congealed, the plates were swirled gently to achieve thorough mixing of the contents. The plant extracts were substituted with Fungicide X as positive control and distilled water as the negative control. In a solid medium, the inoculum discs of *A. niger* were inoculated at the center of each Petri dish. The inoculated Petri dishes were stored at room temperature. Then, the diameters of the fungal colonies were measured on the 1st, 3rd, 5th, and 7th day of incubation with the aid of a Vernier caliper (Rao & Srivastava, 1994). For evaluation, the mean values of the three readings and fungi's radial growth per day were divided by the total number of observation days (Brancato et al., 1953).

Spore germination assay

A 0.1 ml of leaf extracts with 50% concentration was placed in the cavity slide with the spores from the prepared subculture. Each slide was stored in a moist Petri dish at room temperature to maintain enough humidity (Das et al., 2009). Six replicates were maintained for each treatment, including the controls. The slides were examined at an interval of three hours for a total six-hour duration. Percent spore germination of each treatment was calculated using this formula (Kiraly et al., 1974):

$$\text{Percent spore germination} = \frac{\text{no. of spores germinated}}{\text{total no. of spores examined}} \times 100$$

The leaf extracts of *P. odorata*, *P. quadrialatus*, and *S. astylosa* were tested against *A. niger*. The fungal growth of A. niger was compared among the five treatments: 50% ethanolic extract of P. odorata, P. quadrialatus, S. astylosa, fungicide X (positive control), and distilled water (negative control). The treatments were replicated six times and incubated for seven days. The fungal colonies' diameter was measured on the 1st, 3rd, 5th, and 7th day of observation.

Data analysis

Descriptive statistics were included in the data analyses. The parameters, treatments, replicates were clearly described. The descriptive statistics included mean values, standard deviation or variance, minimum and maximum values of the range. One-way Analysis of Variance (ANOVA) was used to compare the variance among different treatments observed. It is used when one categorical independent variable has three or more levels and one continuous dependent variable (Boduszek, 2017). The data obtained from the growth of *A. niger* through PDA and spore germination was statistically analyzed using R and R Studio at $p \leq 0.05$ level of significance.

Biological safety

The microorganisms were treated as potential pathogens. All the experiments were done in a biosafety cabinet. Proper handling procedures, storage, and disposal of microorganisms and plant materials were established to prevent contamination and infection. Decontamination and disposal of plant materials were done by use of autoclave (121°C, 15 psi for 15 minutes). All the standard microbiology laboratory safety protocols were followed.

RESULTS AND DISCUSSION

Figure 5 shows the fungal colony growth on PDA with the different treatments.

In Table 1, the Tukey HSD pairwise comparison among the treatments on fungal growth revealed that the S. astylosa leaf extract is not significantly different from the positive control (Fungicide X). On the 7th day, P. odorata (T1) had a mean value of 2.84 cm, P. quadrialatus (T2) had a mean value of 1.1 cm, S. astylosa (T3) had a mean value of 0.54 cm, distilled water (T4) had a mean value of 3.21 cm,

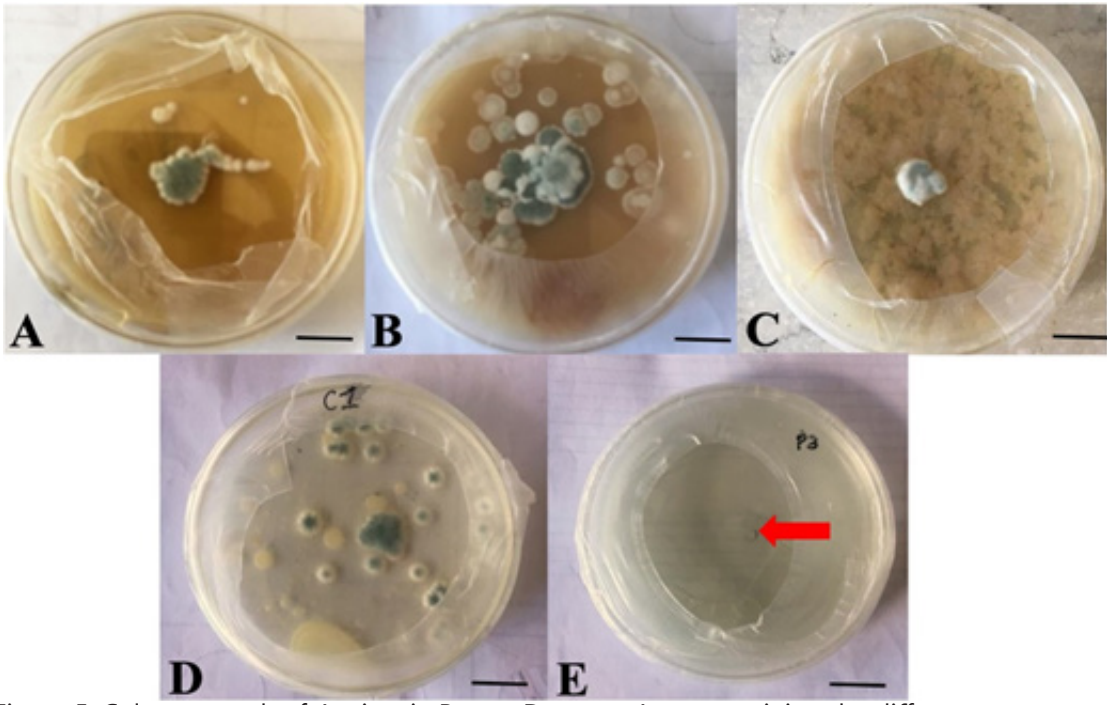


Figure 5. Colony growth of *A. niger* in Potato Dextrose Agar containing the different treatments: A – *P. odorata* leaf extract (50%); B – *P. quadrialatus* leaf extract (50%); C – *S.astylosa* leaf extract (50%); D - Distilled water; and, E - Fungicide X. (Red arrow: inoculum disk). Bar = 10 mm.

Table 1

Antifungal activity P. odorata, P. quadrialatus, and S. astylosa leaf extracts against Aspergillus niger after 7 days.

Treatment	n	Colony Diameter Growth (mm) of A. niger			Spore Germination Count		
		Mean ± SD	Min	Max	Mean ± SD	Min	Max
T^1- P. odorata	6	28.35^{e} ± 5.58	21	36	82.17^{c} ± 11.48	66.00	99.00
T^2 - P. quadrialatus	6	10.97 ± 1.9	7.9	12.9	$49.5^{b,d}$ ± 6.12	42.00	57.00
T^3 - S. astylosa	6	5.38 ± 0.59	4.5	6.1	$15.0^{c,f}$ ± 5.22	7.00	21.00
T^4 - Distilled Water	6	32.13^{b} ± 1.25	30.1	33.9	349.0 ± 48.62	276.00	401.00
T^5 - Fungicide X	6	0.00 ± 0.00	0.00	0.00	0.00^{d} ± 0.00	0.00	0.00
p-value[a]		0.000004*			0.000189*		

a. One-Way ANOVA: Significant *p*-value (*) at 0.05 level
Tukey HSD Post-Hoc Test: Not significantly different to b. T^1, c. T^2, d. T^3, e. T^4, and f. T^5 at 0.05 level

and Fungicide X (T5) had no growth recorded. This data indicates that *S. astylosa* leaf extract effectively inhibited the colony growth of *A. niger* at a 50% concentration.

The total number of spores, number of spores germinated, and percentage of spore germination per treatment were recorded. Results revealed that the lowest spore count and spore germination percentage was observed in S. astylosa leaf extract. The spore germination indicated that the activity of S. astylosa leaf extract and fungicide X were significantly different and significantly lower compared to other treatments, as shown in Table 1. The phytochemical screening showed that S. astylosa contains flavonoids, saponins, and tannins, providing insight into its antimicrobial activity.

Aspergillus niger is a naturally ubiquitous, filamentous fungus of the Phylum Ascomycota, which can cause the black mold disease on certain fruits such as grapes and peanuts, and vegetables such as onions (Baker, 2006). Rotting caused by black mold is the primary cause of rotting in stored yellow onions in Texas, USA, between 1974 and 1976 (Miller and Dillon, 1979). In Japan, onions stored over the summer had losses of 33% (Tanaka and Nonaka, 1981). In the Philippines, onion bulbs exhibit clusters of black fungal spores from along veins and on or between the outer papery scales of the bulbs (Philippine Council for Agriculture, Forestry and Natural Resources Research and Development, 2018). *A. niger* can grow in a wide range of temperatures (6-47°C), making it ubiquitous in warm and humid places (Palacios-Cabrera et al., 2005). Also, Bui-Klimke and Wu (2015) reported that A. niger could produce ochratoxin, a mycotoxin linked with the human diseases Balkan endemic nephropathy (BEN) and chronic interstitial nephropathy (CIN), and other renal diseases.

In this study, three Philippine endemic plants were investigated. *P. odorata* belongs to the Family Lamiaceae, locally known as Alagao (Philippine Medicinal Plants, 2021). Various plant applications include its use as an analgesic, antipyretic, and anti-inflammatory. Compounds associated with anti-inflammatory and antinociceptive properties were previously isolated from its leaves. Initial phytochemical screening of *P. odorata* crude leaf extract showed steroids, terpenoids, flavonoids, and hydrolyzable tannins (Montoya, 2012). In the study of Lirio et al. (2014), P. odorata crude methanolic extract and sub-extracts showed that 1-heneicosyl formate present in the leaf extracts has significant inhibitory activity against *M. tuberculosis* H37RV (MIC= 8 mg/ml). It also has antimicrobial, anti-inflammatory, and chemopreventive activities (Pinzon et al., 2011).

Secondly, *P. quadrialatus* belongs to the Family Lecythidaceae, locally known as "Toog" (Dayan et al., 2005). It is a deciduous tree that grows up to 40 m tall and 100 cm in diameter. Because of its appearance and high lumber quality, it is now recognized in the local and world market under the trade name Philippine Rosewood (Philippine Flora, 2009). However, it is considered vanishing timber, calling for comprehensive conservation actions and sustainable use. The study of Ragasa et al. (2014) revealed that *P. quadrialatus* leaf extracts yield unsaturated triglycerides and a mixture of beta and alpha-amyrin fatty acid ester.

The third endemic tree, *S. astylosa*, is locally called "Yakal," "Yamban," "Gisok," and "Dungon-dungon" (Energy Development Corporation, 2012). It is a medium to large-sized species about 25 to 30 meters tall. It belongs to the family Dipterocarpaceae and is widely distributed in Quezon and Camarines, Samar, Negros, Zamboanga, Agusan, and Davao. Among the endemic plants investigated, *S. astylosa* leaf extract (50%) inhibited the growth and spore germination of A. niger.

In this study, the phytochemical screening (see Supplemetal Data) of the leaf extracts revealed flavonoids, saponins, and tannins. Flavonoids are secondary metabolites in class polyphenol and present in several plants and diets (Wang et al., 2018). Aboody and Mickymaray (2020) reported that flavonoids show various pharmacological functions, including antioxidant, anti-diabetic, anti-obesity, anti-hyperlipidemic, anti-inflammatory, antiosteoporotic effects, antiallergic and antithrombotic, hepatoprotective, neuroprotective, renoprotective, chemopreventive and anticancer, antibacterial, antifungal, and anti-viral

activities. They are recognized as antioxidants and possess free radical quenching properties. Also, they perform as chelators of divalent cations. It has free radical scavenger properties that inhibit lipid peroxidation, capillary permeability, platelet aggregation, and fragility. Flavonoids regulate biological systems by inhibiting many enzymes, such as hydrolase, lipase, α-glucosidase, aldose reductase, cycloxygenase, xanthine oxidase, hyaluronidase, alkaline phosphatase, arylsulphatase, lipoxygenase, Ca+2-ATPase, cAMP phosphodiesterase, and several kinases.

Flavonoids from *Erythrina burtii* showed antifungal activity (Yenesew et al., 2005). Aquilegia vulgaris leaf extracts contain 4-methoxy-5,7-dihydroxyflavone-6-C-glucoside (isocytisoside) and antifungal activity against A. niger (Bylka et al., 2004). Thus, this study's findings provide preliminary evidence of S. astylosa's pharmacological benefits upon discovering flavonoids and antimicrobial activities.

Meanwhile, saponins are secondary metabolites in plant species. They act as inactive precursors and are readily converted into active biological antibiotics against pathogens. They are glycosylated compounds and can be divided into three major groups: triterpenoids, steroid or steroidal glycoalkaloids, and triterpene saponin. These compounds were found in *Capsicum frutescens* and showed antifungal activity against sixteen different fungal strains (Renault et al., 2003). Deterioration of cooked foods caused by yeast and fungi is restrained by plant extracts containing saponins (Tamura et al., 2012).

On the other hand, *T. procumbens* leaf extract at 100% concentration partially inhibited the growth of P. aeruginosa and S. aureus. T. procumbens is a perennial plant that belongs to the family Asteraceae. The leaves of this plant have been reported to have anti-viral, anti-inflammatory, anti-diabetic, and anti-arthritic activities. It has been reported to aid in wound healing, prevent hair loss and serve as an insect repellent. It has antimicrobial activity against gram-positive and gram-negative bacteria and stimulates wound healing (Suseela et al., 2002; Kumar et al., 2012).

Tannin, flavonoids, luteolin, terpenoid, and alkaloids were isolated from *T. procumbens* leaves (Kethamakka and Deogade, 2014; Cushnie and Lamb, 2005; Cowan, 1999). Tannin can inhibit the extracellular microbial enzyme, deprive substrates required for microbial growth, and directly inhibit oxidative phosphorylation (Fenner and Freeman, 2020). Luteolin can inhibit DNA topoisomerase I and II activity, which further inhibits nucleic acid and

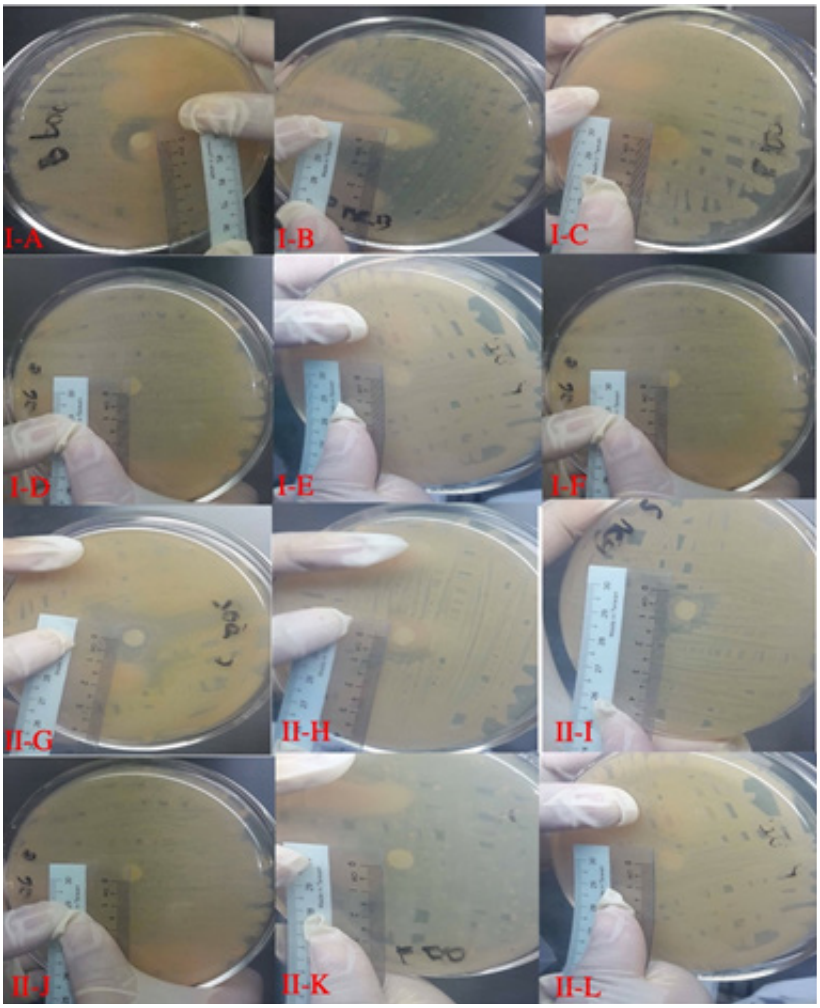


Figure 2. *T. procumbens* tested against I. *P. aeruginosa*: A. Positive Control Vancomycin; B. Negative Control (dH2O); C. 100% *Tridax procumbens* leaf extract; D. 75% *Tridax procumbens* leaf extract; E. 50% *Tridax procumbens* leaf extract; and, F. 25% *Tridax procumbens* leaf extract; and II. *S aureus*: G. Positive Control Vancomycin; H. Negative Control (dH2O); I. 100% *Tridax procumbens* leaf extract; J. 75% *Tridax procumbens* leaf extract; K. 50% *Tridax procumbens* leaf extract; and, L. 25% *Tridax procumbens* leaf extract.

protein synthesis, especially against *S. aureus* (Wang and Xie, 2010). In addition, terpenoid is effective against gram-positive and gram-negative pathogens by interfering in bacterial membrane permeability (Sathya Bama et al., 2012). Flavonoids inhibit cytoplasmic membrane function and energy metabolism (Cushnie and Lamb, 2005). Lastly, the alkaloids can affect the virulence gene regulatory systems such as quorum sensing and virulence factors such as sortases, adhesins, and secretion systems. It also inhibits the transcriptional regulator in bacteria (Cushnie et al., 2014).

On the other hand, *T. procumbens* was tested against P. aeruginosa and S aureus. Table 2 shows the zones of inhibition (ZOI) of the different concentrations of T. procumbens leaf extract. The 100% leaf extract had a partially active result (ZOI=10 mm). Based on the equivalent antibacterial activity level by Guevarra (2005), T. procumbens leaf extract at 100% concentration was partially active in inhibiting the growth of *Pseudomonas aeruginosa* and *S. aureus*.

In this study, *S. aureus* was found to be susceptible to the ethanolic leaf extract of *T. procumbens*. The antibacterial activity of ethanolic extract of *Tridax procumbens* can be attributed to the presence of flavonoids and tannins which are substances known to have several mechanisms of action such as inhibition of DNA gyrase, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, etc. (Cushnie and Lamb, 2005). It is a common opportunistic bacteria in human skin and has been known to cause severe problems due to its methicillin resistance. Its major route of infection includes open wounds, especially excisional wounds that may harbor bacterial species causing delayed wound healing. Also, *S. aureus* is the leading cause of skin and soft tissue infection resulting in significant morbidity and mortality from septic shock, endocarditis, pneumonia, and bacteremia (Prakoso et al., 2018; Stapleton & Taylor, 2002).

Meanwhile, the leaf extracts of *T. procumbens* also demonstrated inhibition activity against *P. aeruginosa*. This data presents clinical importance as the nosocomial pathogen is known to have increasing antibiotic resistance. As it is opportunistic, it exploits immunocompromised hosts to mount an infection. It can cause disease in the urinary tract, respiratory system, skin, soft tissue, bone and joint, gastrointestine, and blood, particularly in patients with severe burns, tuberculosis, cancer, and Acquired Immunodeficiency Syndrome (AIDS) (Pang et al., 2019; Wu and Li, 2015). *P. aeruginosa* has high intrinsic antibiotic resistance, enabling it to survive in various environmental settings, including medical facilities (Balcht and Smith, 2010). Multi-drug resistant strains are emerging, such as carbenicillin-resistant, cephalosporins-resistant, ceftazidime-resistant, and ciprofloxacin-resistant (Public Health Agency of Canada, 2012). With the severity of P. aeruginosa infections and the limited antimicrobial interventions used to treat them, finding alternative prevention and treatment strategies is an urgent priority (Gellatly & Hancock, 2013).

Traditionally, indigenous people in other countries like Nigeria use the leaves of this plant as a treatment to reduce blood pressure (Taddei and Rosas, 2000, Udopa et al.,1991; Diwan et al., 1982, and Diwan et al., 1983). The Philippine Medicinal Plants website (2021) mentioned that Indians utilized it as an anticoagulant, repellent, antidiarrheal, and anti-dysenteric. The leaf extract can be used on fresh wounds to stop bleeding and as a hair tonic. In Ayurveda, it is used for liver disorders, arthritis, and heartburn. Local uses in Guatemala revealed that the leaf extract is used for colds, inflammation, vaginitis, stomach pains, and diarrhea. The whole plant is used for protozoal infections, treatment of chronic ulcers. The whole dried plant is used in Africa for fever, cough, backache, diarrhea, and epilepsy. Dried leaves are used for malaria, gastrointestinal mycosis, and dressing wounds.

CONCLUSION

S. astylosa leaf extract demonstrated the most significant inhibition of fungal growth (5.38 mm) and spore germination (15) of *A niger*. However, *P. odorata* and *P. quadrialatus* showed the least (28.35 mm; 82.17) and moderate (10.97 mm; 49.5) inhibitory potentials, respectively. *T. procumbens* minimally inhibits the growth of *P. aeruginosa* (10 mm) and *S. aureus* (10 mm). In the qualitative phytochemical screening of S. astylosa, results indicated presence of flavonoids, saponins, and tannins. Among these phytochemical components, flavonoids may act as the antifungal component. Meanwhile, 100% ethanolic leaf extract of *T. procumbens* inhibited *P. aeruginosa* and *S. aureus*. There is a significant difference (Significant p-value (*) at 0.05 level) in the mean zone of inhibition (ZOI) of *P. aeruginosa* and *S. aureus* compared with the positive and negative controls. It is recommended that further studies be made on method development such as extraction procedure and processing of samples to potentially increase the antimicrobial activities of these

Table 2

The Zone of Inhibition (ZOI) of T procumbens leaf extract against P. aeruginosa and S. aureus after 24 hours of incubation.

Test Organism	n	Zone of Inhibition (mm) Tridax procumbens leaf Extract				Positive Control (mm)	Negative Control (mm)
		100%	75%	50%	25%		
Pseudomonas aeruginosa	1	10	0	2	4	18	2
Staphylococcus aureus	1	10	0	0	8	20	16

plant samples against more indicator strains.

ACKNOWLEDGMENT

The authors would like to thank the Davao Doctors College for offering its laboratory, equipment, and reagents; the Department of Science and Technology XI for the provision of bacterial cultures; and the University of the Immaculate Conception for the phytochemical screening of S. astylosa leaf extract. Special mention to Dr. Reynaldo G. Abad and Dr. Majella D. Bautista for validating the plant and fungal species used in this study.

REFERENCES

Aboody, M. S. A., & Mickymaray, S. (2020). Antifungal efficacy and mechanisms of flavonoids. *Antibiotics*, 9(2), 45.

Aryal, S. (2015). Potato Dextrose Agar (PDA)—Principle, uses, composition, procedure and colony characteristics. *Retrieved on February, 18*, 2017.

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Nisar, M. A., Alvi, R. F., Aslam, M. A., Qamar, M. U., Salamat, M. K., & Baloch, Z. (2018). Antibiotic resistance: A rundown of a global crisis. *Infection and Drug Resistance, 11*, 1645-1658. https://doi.org/10.2147/idr.s173867

Baker, S. E. (2006). Aspergillus niger genomics: past, present and into the future. *Medical mycology, 44*(Supplement_1), S17-S21.

Bhalodia, N., & Shukla, V. (2011). Antibacterial and antifungal activities from leaf extracts of Cassia fistula l.: An ethnomedicinal plant. Journal of Advanced Pharmaceutical Technology & Research, 2(2), 104. doi:10.4103/2231-4040.82956

Boduszek, D. (2017). Non-parametric test in SPSS (between subjects). University of Huddersfield. Retrieved on (23/02/2017) *from http://webzoom. freewebs. com/danielboduszek/documents/Nonparametric% 20tests% 20between% 20subjects% 20SPSS*.

Brancato, F. P., & Golding, N. S. (1953). The diameter of the mold colony as a reliable measure of growth. *Mycologia, 45*(6), 848-864.

Bui-Klimke, T. R., & Wu, F. (2015). Ochratoxin A and human health risk: A review of the evidence. *Critical reviews in food science and nutrition, 55*(13), 1860-1869.

Bylka, W., Szaufer-Hajdrych, M., Matławska, I., & Goślińska, O. (2004). Antimicrobial activity of isocytisoside and extracts of Aquilegia vulgaris L. *Letters in applied microbiology, 39*(1), 93-97.

Cheesman, M. J., Ilanko, A., Blonk, B., & Cock, I. E. (2017). Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution?. *Pharmacognosy reviews, 11*(22), 57.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical microbiology reviews, 12*(4), 564-582.

Cushnie, T. T., & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International journal of antimicrobial agents, 26*(5), 343-356.

Das, M. P., Dhanabalan, R., Doss, A., & Palaniswamy, M. (2009). Phytochemical screening and antibacterial activity of aqueous and methanolic leaf extracts of two medicinal plants against bovine mastitis bacterial pathogens. *Ethnobotanical leaflets, 2009*(1), 15.

Dayan, M., Reaviles, R.S, And Abarro, V. (2005). Forest tree seeds: a phenological guidebook. Ecosystems Research and Development Bureau-Department of Environment and Natural Resources, College, Laguna (Philippines).

Dianita, R., & Jantan, I. (2017). Ethnomedicinal uses, phytochemistry and pharmacological aspects of the genus Premna: A review. *Pharmaceutical Biology, 55*(1), 1715-1739. https://doi.org/10.1080/13880209.2017.1323225

Diwan Pv, Tilloo Ld And Kulkarni D 1982: Influence of Tridax procumbens on wound healing. Indian J. Med Res. Mar. 75; 450-4.

Diwan Pv, Tilloo, L.D. And Kulkarni D.R. 1983: Steroid depressed wound healing and Tridax procumbens. Indian J. Physiol Pharmacol 27:1, 32-6.

Ecosystems Research And Development Bureau. (2012). Philippine Country Report on Forest Genetic Resources, ERDB, College, Laguna. 162 p. Retrieved at http://erdb.denr.gov.ph/wp-content/uploads/2015/06/country_report.pdf.

Energy Development Corporation. (2012). Yakal (*Shorea astylosa*) tree and leaves from Tree Facts *Shorea astylosa* Foxw. http://binhi.ph/database/future/tree facts/result/Shorea+astylosa# tree-facts-tabs=0.

Fernando, E. S., Co, L. C., Lagunzad, D. A., Gruezo WSm, B. J., Madulid, D. A., & Zamora, P. M. (2008). Threatened plants of the Philippines: a preliminary. *Asia Life Sci, 3*, 1-52.

Gellatly, S. L., & Hancock, R. E. (2013). Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathogens and disease, 67*(3), 159-173.

Guevarra, 2004. *A guidebook to plant screening: phytochemical and biological*. University of Santo Tomas Publishing House.

Kiraly, Z., Klement, S.J., Voros And Solymosy, K. (1974). Methods in plant pathology with special reference to breeding for resistance to breeding for resistance. Elsevier scientific publishing company, New York, 212 PP.

Kumar A, Ilavarasan R, Jayachandran, Decaraman M, Aravindhan P. Phytochemicals Investigation on a

tropical plant in South India. Pak J Nutr 2009;8:83-5.

Kumar, S. B., Arnipalli, S. R., & Ziouzenkova, O. (2020). Antibiotics in food chain: The consequences for antibiotic resistance. *Antibiotics, 9*(10), 688. https://doi.org/10.3390/antibiotics9100688

Kumar, P., & Khanna, P. (1986). Chemical investigation of aerial plant parts and tissue culture of Solanum dulcamara Linn. *Acta Botanica Indica, 14*, 136-8.

Lirio, S. B., Macabeo, A. P. G., Paragas, E. M., Knorn, M., Kohls, P., Franzblau, S. G., ... & Aguinaldo, M. A. M. (2014). Antitubercular constituents from Premna odorata Blanco. *Journal of ethnopharmacology, 154*(2), 471-474.

Miller, M. E., & Dillon JR, R. C. (1979). Survey of bulb diseases on onions in South Texas. *Journal of the Rio Grande Valley Horticultural Society*.

Montoya, I. J. M. (2012). *The Antinociceptive and Anti-inflammatory Activities of the Methanolic Extract of the Leaves of Premna Odorata Blanco (verbenaceae)* (Doctoral dissertation, UST Graduate School).

Nuneza, O., Rodriguez, B., & Nasiad, J. G. (2021). Ethnobotanical survey of medicinal plants used by the Mamanwa Tribe of Surigao Del Norte and Agusan Del Norte, Mindanao, Philippines. *Biodiversitas Journal of Biological Diversity, 22*(6). https://doi.org/10.13057/biodiv/d220634

Palacios-Cabrera, H., Taniwaki, M. H., Hashimoto, J. M., & Menezes, H. C. D. (2005). Growth of Aspergillus ochraceus, A. carbonarius and A. niger on culture media at different water activities and temperatures. *Brazilian Journal of Microbiology, 36*(1), 24-28.

Pang, Z., Raudonis, R., Glick, B. R., Lin, T., & Cheng, Z. (2019). Antibiotic resistance in pseudomonas aeruginosa: Mechanisms and alternative therapeutic strategies. *Biotechnology Advances, 37*(1), 177-192. https://doi.org/10.1016/j.biotechadv.2018.11.013

Patwardhan, B., Vaidya, A. D., & Chorghade, M. (2004). Ayurveda and natural products drug discovery. *Current science*, 789-799.

Philippine Council For Agriculture, Aquatic And Natural Resources Research And Development (Pcarrd). (2021). http://www.pcaarrd.dost.gov.ph/home/portal/.

Philippine Flora. (2012). Philippine Rosewood (Toog Tree) Philippine Medicinal Plants. http://www.stuartxchange.com/Alagaw.html.

Philippine Medicinal Plants. (2021). http://www.stuartxchange.org/CoatButtons.

Pinzon, L. C., Uy, M. M., Sze, K. H., Wang, M., & Chu, I. K. (2011). Isolation and characterization of antimicrobial, anti-inflammatory and chemopreventive flavones from Premna odorata Blanco. Journal of Medicinal Plants Research, 5(13), 2729-2735.

Prakoso, Y. A., Setiyo Rini, C., & Wirjaatmadja, R. (2018). Efficacy of Aloe vera, Ananas comosus, and Sansevieria masoniana Cream on the Skin Wound Infected with MRSA. *Advances in pharmacological sciences, 2018*.

Ragasa, C. Y., Torres, O. B., Tongco, J. V. V., Razal, R. A., & Shen, C. C. (2014). Chemical constituents of Petersianthus quadrialatus (Merr.). *Research Journal of Pharmaceutical, Biological and Chemical Sciences, 5*(4), 181.

Raji, R., & Raveendran, K. (2013). Antifungal activity of selected plant extracts against phytopathogenic fungi Aspergillus niger. *Asian Journal of Plant Science and Research, 3*(1), 13-15.

Renault, S., De Lucca, A. J., Boue, S., Bland, J. M., Vigo, C. B., & Selitrennikoff, C. P. (2003). CAY-I, a novel antifungal compound from cayenne pepper. *Medical mycology, 41*(1), 75-82.

Sathya Bama, S., Jayasurya Kingsley, S., Sankaranarayanan, S., & Bama, P. (2012). Antibacterial activity of different phytochemical extracts from the leaves of T. procumbens Linn.: identification and mode of action of the terpenoid compound as antibacterial. *Int J Pharm Pharm Sci, 4*(1), 557-564.

Sharma, C., Rokana, N., Chandra, M., Singh, B. P., Gulhane, R. D., Gill, J. P. S., ... & Panwar, H. (2018). Antimicrobial resistance: its surveillance, impact, and alternative management strategies in dairy animals. *Frontiers in veterinary science, 4*, 237.

Stapleton, P. D., & Taylor, P. W. (2002). Methicillin resistance in staphylococcus aureus: Mechanisms and modulation. *Science Progress, 85*(1), 57-72. https://doi.org/10.3184/003685002783238870

Shabana, H. A., Gairola, S., & Mahmoud, T. (2020). Tridax procumbens L.(Asterales Asteraceae), a new record to the flora of the United Arab Emirates. *Biodiversity Journal, 11*(4), 889-896.

Suseela, L., Saraswathy, A., & Brindha, P. (2002). Pharmacognostic studies on Tridax procumbens L.(Asteraceae).

Taddei, A., & Rosas-Romero, A. J. (2000). Bioactivity studies of extracts from Tridax procumbens. *Phytomedicine, 7*(3), 235-238.

Tamura, Y., Miyakoshi, M., & Yamamoto, M. (2012). Application of saponin-containing plants in foods and cosmetics. *Alternative medicine*, 85-101.

Tanaka, K., & Nonaka, F. (1981). Synergistic action of oxalic acid and pectolytic enzyme on the rot of onion bulb caused by Aspergillus niger. *Japanese Journal of Phytopathology, 47*(2), 166-174.

Udupa, S. L., Udupa, A. L., & Kulkarni, D. R. (1991). Influence

of Tridax procumbens on lysyl oxidase activity and wound healing. *Planta medica, 57*(04), 325-327.

Van Der Linden, J. W., Warris, A., & Verweij, P. E. (2011). Aspergillusspecies intrinsically resistant to antifungal agents. *Medical Mycology, 49*(S1), S82-S89. https://doi.org/10.3109/13693786.2010.499916

Wang, Q., & Xie, M. (2010). Antibacterial activity and mechanism of luteolin on Staphylococcus aureus. *Wei sheng wu xue bao= Acta microbiologica Sinica, 50*(9), 1180-1184.

Wang, T. Y., Li, Q., & Bi, K. S. (2018). Bioactive flavonoids in medicinal plants: Structure, activity and biological fate. *Asian Journal of Pharmaceutical Sciences, 13*(1), 12-23.

Wu, M., & Li, X. (2015). Klebsiella pneumoniae and Pseudomonas aeruginosa. In *Molecular medical microbiology* (pp. 1547-1564). Academic Press.

Yenesew, A., Derese, S., Midiwo, J. O., Bii, C. C., Heydenreich, M., & Peter, M. G. (2005). Antimicrobial flavonoids from the stem bark of Erythrina burttii. *Fitoterapia, 76*(5), 469-472.

Table of CONTENTS

CENTRAL MINDANAO UNIVERSITY
University Town, Musuan, Maramag, Bukidnon, Philippines