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Archives

Vol 6 Year 2023
Vol 5 Year 2022
Vol 4 Year 2021
Vol 3 Year 2020
Vol 2 Year 2019
Vol 1 Year 2018

Search

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VOLUME 06 ISSUE 06 JUNE 2023 []

Vol 6 No 6(2023)

Development of Quizz-Based Creative Thinking Skill Assessment in Thematic Learning of Elementary School Class V Students [https://ijcsrr.org/single-view/?id=10503&pid=10500]

Siti Alfiyah, Sunyono ., Doni Andra
Page No. 3080-3090

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/01-01-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10503&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-01 [https://doi.org/10.47191/ijcsrr/V6-i6-01]

Design and Implementation of a Multi-Channel Temperature Measurement System Based on LabVIEW [https://ijcsrr.org/single-view/?id=10508&pid=10500]

Muhammad Faisal Buland Iqbal, Reshail Abbasi, Faiz Sultan, Shehar Bano
Page No. 3091-3109

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/02-01-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10508&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-02 [https://doi.org/10.47191/ijcsrr/V6-i6-02]

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A Case Report of Limb Dystonia Management in a Nigerian Hospital: Physiotherapy Perspective [https://ijcsrr.org/single-view/?id=10521&pid=10500]

Oti IK, Nwaedozie OC, Ayerite AB, Anyama EU
Page No. 3110-3115

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/03-03-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10521&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-03 [https://doi.org/10.47191/ijcsrr/V6-i6-03]

Physiotherapy Management of Vocal Cord Paralysis in a Nigerian Hospital: A Case Report [https://ijcsrr.org/single-view/?id=10526&pid=10500]

Osuchukwu OIM, Oti IK, Nwaedozie OC
Page No. 3116-3120

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/04-03-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10526&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-04 [https://doi.org/10.47191/ijcsrr/V6-i6-04]

Proposed Design of Performance Management Framework for 3PL (Third-Party Logistics) Aggregator [https://ijcsrr.org/single-view/?id=10531&pid=10500]

Alvin Trianto Atmojo, Dermawan Wibisono
Page No. 3121-3133

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/05-03-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10531&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-05 [https://doi.org/10.47191/ijcsrr/V6-i6-05]

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Archives

Vol 6 Year 2023
Vol 5 Year 2022
Vol 4 Year 2021
Vol 3 Year 2020
Vol 2 Year 2019
Vol 1 Year 2018

Search

Author Name, D(
Select Month
Select Year
Search

Internship Program in Virtual and Modular Instruction as Perceived by Students [https://ijcsrr.org/single-view/?id=10535&pid=10500]

Ronnel V. Mancera
Page No. 3134-3139

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/06-03-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10535&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-06
[https://doi.org/10.47191/ijcsrr/V6-i6-06]

Prevalence Study of Biofilm Marker ica D Gene among Coagulase Positive Staphylococcus of Mastitic Origin [https://ijcsrr.org/single-view/?id=10554&pid=10500]

Shubhangi Warke, Prashant Sonkusale, Sumedha S. Boabde
Page No. 3140-3147

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/07-05-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10554&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-07
[https://doi.org/10.47191/ijcsrr/V6-i6-07]

Implementation of Pancasila Ideology in Indonesian Educational Leadership: A Literature Review [https://ijcsrr.org/single-view/?id=10558&pid=10500]

Margi Jayanti, Sowiyah ., Riswanti Rini, Handoko ., Alifa Soraya Nuryadika
Page No. 3148-3153

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/08-05-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10558&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-08
[https://doi.org/10.47191/ijcsrr/V6-i6-08]

Super Glue Production by Dermatophytes: Review [https://ijcsrr.org/single-view/?id=10563&pid=10500]

Shaimaa Nabhan Yassein
Page No. 3154-3159

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/09-06-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10563&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-09
[https://doi.org/10.47191/ijcsrr/V6-i6-09]

Talent Readiness of PT KAI to Face the Era of Change [https://ijcsrr.org/single-view/?id=10567&pid=10500]

David Gamaliel, Yuni Ros Bangun
Page No. 3160-3178

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/10-06-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10567&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-10
[https://doi.org/10.47191/ijcsrr/V6-i6-10]

The Differences in Toxicity of Pletekan Leaf Extracts (Ruellia tuberosa L.) and Lime (Citrus auraiflia) on Mortality of Culex sp. Mosquito Larvae [https://ijcsrr.org/single-view/?id=10571&pid=10500]

Dwi Wahyuni, Adelia Septi Rihada, Retno Maulidina
Page No. 3179-3187

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/11-06-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10571&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-11
[https://doi.org/10.47191/ijcsrr/V6-i6-11]

Evaluating Encryption Algorithm Method Based on Software Encryption Tools for Information Security [https://ijcsrr.org/single-view/?id=10577&pid=10500]

Dr. Khaled Musa
Page No. 3188-3194

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/12-07-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10577&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-12
[https://doi.org/10.47191/ijcsrr/V6-i6-12]

Implementation of Principal Transformational Leadership and Independent Learning Policy in Improving Education Quality: A Literature Review [https://ijcsrr.org/single-view/?id=10583&pid=10500]

Margi Jayanti, Hasan Hariri, Riswanti Rini, Handoko ., Alifa Soraya Nuryadika
Page No. 3195-3205

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/13-07-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10583&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-13
[https://doi.org/10.47191/ijcsrr/V6-i6-13]

Job Insecurity and Psychological Wellbeing: Is it Necessary to Foster Employee Performance [https://ijcsrr.org/single-view/?id=10587&pid=10500]

Praptini Yulianti, Noorlaily Fitdiarini
Page No. 3206-3211

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/14-07-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10587&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-14
[https://doi.org/10.47191/ijcsrr/V6-i6-14]

Strategic Planning for Drone Company in Indonesia (Case: PT Terra Drone Indonesia) [https://ijcsrr.org/single-view/?id=10591&pid=10500]

Gilang Wirata Pratama Hadi, Donald Crestofel Lantu
Page No. 3212-3222

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/15-08-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10591&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-15
[https://doi.org/10.47191/ijcsrr/V6-i6-15]

Development and Characterization of Solid Dispersion of Rasagilline Mesylate for Improvement of Dissolution Rate Using Hydrophilic Carriers [https://ijcsrr.org/single-view/?id=10607&pid=10500]

Ravi V. Patel, Gazala Y. Ansari, Jitendra O. Bhangale
Page No. 3223-3239

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/16-09-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10607&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-16
[https://doi.org/10.47191/ijcsrr/V6-i6-16]

Nootropic Activity of Ethanolic Extract of Zingiber officinale and Centella asiatica on Stress Induced Rats [https://ijcsrr.org/single-view/?id=10547&pid=10500]

Ugwu Anthony Odinaka, Patrick Oliver Asogwa, Prakash Nathaniel Kumar Sarella, Asogwa Samuel Otuodi, Ravishankar Kakarparthy

DOI: 10.47191/ijcsrr/V6-i6-17
[https://doi.org/10.47191/ijcsrr/V6-i6-17]

Biodegradation of Polycyclic Aromatic Hydrocarbon Compound by Bacterial Cultures [https://ijcsrr.org/single-view/?id=10632&pid=10500]

Pravinkumar A. Domde, Hemant J. Purohit, Rajpal Singh Kashyap, Shardul S. Wagh

DOI: 10.47191/ijcsrr/V6-i6-18
[https://doi.org/10.47191/ijcsrr/V6-i6-18]

Improving Demand Management Performance using Knowledge Management Approach in an Oil and Gas Company [https://ijcsrr.org/single-view/?id=10637&pid=10500]

Randy Rahmadhiputra, Dedy Sushandoyo

DOI: 10.47191/ijcsrr/V6-i6-19
[https://doi.org/10.47191/ijcsrr/V6-i6-19]

Improving a Two-Way Interaction Customer Service Platform in Company X using AHP [https://ijcsrr.org/single-view/?id=10645&pid=10500]

Muhammad Prabu Mutawakkil, Manahan Parlindungan Saragih Siallagan
Page No. 3267-3273

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/20-12-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10645&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-20
[https://doi.org/10.47191/ijcsrr/V6-i6-20]

A Study on the Effect of Participating in Tutorial Courses on Happiness During the Summer Break of Thai High School Students in Bangkok [https://ijcsrr.org/single-view/?id=10649&pid=10500]

Kantaphon Chairungpanya, Natariny Tassanapreechachai, Napat Darasit
Page No. 3274-3279

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/21-12-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10649&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-21
[https://doi.org/10.47191/ijcsrr/V6-i6-21]

Designing Knowledge Management to Sustain Differentiation Business Strategy at CV Fel & Co International [https://ijcsrr.org/single-view/?id=10653&pid=10500]

Ade Julianto Darmawan, Dedy Sushandoyo
Page No. 3280-3295

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/22-12-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10653&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-22
[https://doi.org/10.47191/ijcsrr/V6-i6-22]

The Impact of Using Social Media on the Mental Health of Adolescents [https://ijcsrr.org/single-view/?id=10685&pid=10500]

Panupong Titisuk, Yanin Vajarapongse, Lalitpat Thongwon
Page No. 3296-3300

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/23-12-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10685&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-23
[https://doi.org/10.47191/ijcsrr/V6-i6-23]

Cultural Study of Natural Environment Exploitation: Eating Culture of the Joseon People through the Novel “Thousand-Year Kingdom” [https://ijcsrr.org/single-view/?id=10690&pid=10500]

Tam, Minh Nguyen, Thuy, Thi Tran, Kiet, Tuan Tran, Ha, Thi Thu Nguyen
Page No. 3301-3308

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/24-13-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10690&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-24
[https://doi.org/10.47191/ijcsrr/V6-i6-24]

Long-Term Effects of Taking Refractive Surgery (LASIK) on Thai People [https://ijcsrr.org/single-view/?id=10693&pid=10500]

Kawisara Kurattanasinchai, Thitanan Dumumpai, Pakkapol Booncharoen
Page No. 3309-3313

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/25-13-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10693&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-25
[https://doi.org/10.47191/ijcsrr/V6-i6-25]

Sharia External Financing Alternatives for Business Expansion Study Case: PT. XYZ [https://ijcsrr.org/single-view/?id=10701&pid=10500]

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Page No. 3314-3323

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/26-14-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10701&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-26
[https://doi.org/10.47191/ijcsrr/V6-i6-26]

The Investment Strategy of Sector Rotation over Business Cycles in the Indonesia Stock Exchange to Generate Superior Return [https://ijcsrr.org/single-view/?id=10705&pid=10500]

Rabin Sebayang, Subiakto Soekarno
Page No. 3324-3343

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/27-15-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10705&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-27
[https://doi.org/10.47191/ijcsrr/V6-i6-27]

Situation of Some Factors Affecting Case Management Activities for People with Disabilities in Da Nang [https://ijcsrr.org/single-view/?id=10709&pid=10500]

Do Nghiem Thanh Phuong, Bui Thi Bao Ngoc
Page No. 3344-3348

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/28-15-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10709&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-28
[https://doi.org/10.47191/ijcsrr/V6-i6-28]

Determining Marketing Mix of CV Nutri Pro by Using Big Data Analytics [https://ijcsrr.org/single-view/?id=10713&pid=10500]

Vira Maulina Putri, Manahan Siallagan
Page No. 3349-3364

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/29-15-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10713&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-29
[https://doi.org/10.47191/ijcsrr/V6-i6-29]

Four New Techniques of Addition and Subtraction of any Two Numbers [https://ijcsrr.org/single-view/?id=10730&pid=10500]

Supratiningsih ., Suparti ., Dafik ., Heri Murtomo, O A Safiati
Page No. 3365-3376

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/30-16-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10730&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-30
[https://doi.org/10.47191/ijcsrr/V6-i6-30]

Developing a Business Model Canvas for Online Classified Ads Platform: A Startup Case Study [https://ijcsrr.org/single-view/?id=10741&pid=10500]

Reynara Napitupulu, Wulan Asti, Pri Hermawan
Page No. 3377-3399

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/31-17-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10741&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-31
[https://doi.org/10.47191/ijcsrr/V6-i6-31]

Relationship between Pesticide Exposure and Type 2 Diabetes Mellitus in Farmers: Systematic Review [https://ijcsrr.org/single-view/?id=10752&pid=10500]

Merris Hartati Sormin, Fitria Saftarina, Sudjarwo .
Page No. 3400-3407

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Full text [https://ijcsrr.org/single-view/?id=10752&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-32
[https://doi.org/10.47191/ijcsrr/V6-i6-32]

Development of Ethnomatematics-Based E-Book Teaching Materials to Train Numeracy Skills in Geometry Materials in Elementary Schools [https://ijcsrr.org/single-view/?id=10756&pid=10500]

Rizky Amalia Dhiaul Zaini, Wiryanto ., Rooselyna Ekawati
Page No. 3408-3415

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/33-19-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10756&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-33
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Proposed Business Strategy for Law firm (Case Study of RP) [https://ijcsrr.org/single-view/?id=10759&pid=10500]

Grace Novia
Page No. 3416-3433

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/34-19-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10759&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-34
[https://doi.org/10.47191/ijcsrr/V6-i6-34]

Propose New Marketing Strategy for the New Branch of Café Kopi Madi [https://ijcsrr.org/single-view/?id=10767&pid=10500]

Fauzan Haeqal Arifin, Isti Raafaldini Mirzanti, Satya Aditya Wibowo
Page No. 3434-3449

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Full text [https://ijcsrr.org/single-view/?id=10767&pid=10500]

DOI: 10.47191/ijcsrr/V6-i6-35
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The Portrait of the Tirta Agung Tourism Village as a Geological Tourist Destination: The Case of Ijen Geopark in Bondowoso, Indonesia [https://ijcsrr.org/single-view/?id=10771&pid=10500]

I Ketut Mastika, Pandu Satriya Hutama, Didik Eko Julianto, Dwi Windradini, B.P., Pramesi Lokaprasida, Panca Oktawirani, Djoko Mulyono, Elly Suhartini, Jati Arifiyanti, Lukman Wijaya Baratha
Page No. 3450-3459

PDF [https://ijcsrr.org/wp-content/uploads/2023/06/36-19-2023.pdf]

Full text [https://ijcsrr.org/single-view/?id=10771&pid=10500]

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The Differences in Toxicity of Pletekan Leaf Extracts (*Ruellia tuberosa L.*) and Lime (*Citrus auraiflia*) on Mortality of Culex sp. Mosquito Larvae

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ABSTRACT: Mosquitoes Culex sp. is the main vector of filariasis. Population control using chemical larvicides continuously can cause resistance. The leaves of the pletekan plant (Ruellia tuberosa L.) and lime fruit (Citrus aurantifolia) have the potential as natural larvicides. This study aims to determine the differences in high toxicity (LC50) of pletekan leaf extract and lime fruit on the mortality of Culex sp. mosquito larvae with an exposure time of 24 hours. Pletekan leaf extract and lime fruit were extracted by maceration using 97% ethanol. The LC50 can use probit analysis with SPSS applications. The results showed that pletekan leaves were toxic to Culex sp. mosquito larvae with an LC50 value of 710.169 ppm with the lowest concentration of 615.231 ppm and the highest concentration of 796.157 ppm. The LC50 value of lime fruit extract was 1026.749 ppm, with a lower limit of 846.406 ppm and an upper limit of 1294.531 ppm.

KEYWORDS: Citrus aurantifolia, Culex sp., Ruellia tuberosa L, Toxicity.

INTRODUCTION

Culex sp. mosquitoes can potentially carry vectors for several serious diseases, such as bancroftian filariasis, West Nile virus, and encephalitis (Andrianto, 2020). Filariasis is an infectious disease caused by filarial worms Brugia timori, Brugia malayi, and Wucheria bancrofti. Filariasis is not lethal, but it can cause chronic physical pain and even permanent disability, resulting in decreased patient productivity and activity (Portunasari et al., 2016). Indonesia is the second most endemic country for filariasis after India (Okona in Portunasari, 2016). According to the East Java Health Office (2021), in East Java, 329 cases of elephantiasis or chronic clinical filariasis were recorded until 2020, occurring in 34 districts/cities. Unhealthy environments, such as stagnant dirty water, rivers full of garbage, and the presence of bushes, can contribute to the increased population density of Culex sp. mosquitoes (Juariah and Irawan, 2017; Milati and Siwiendrayanti, 2021).

The community utilizes chemical methods, such as synthetic insecticide abate, to control the population of Culex sp. mosquitoes. Continuous and long-term use of chemical insecticides or larvicides can lead to the contamination of pesticide residues in water and result in resistance among Culex sp. mosquito larvae (Santoso and Haminudin, 2018). The use of natural larvicides is expected to reduce the negative impacts associated with the use of chemical insecticides. One potential natural insecticide is the pleketan or purple queen plant (Ruellia tuberosa L.) and lime (Citrus aurantifolia).

The pleketan plant (Ruellia tuberosa L.) typically grows as a weed or wild grass that can be found around houses. According to the research by Vitalia et al. (2016), it is known that the pleketan plant contains relatively high levels of secondary metabolite compounds, which are used to kill A. salina Leach larvae. The secondary metabolite compounds in the pleketan plant can inhibit feeding and oviposition. Some secondary metabolite compounds in pleketan leaves include flavonoids, alkaloids, polyphenols, triterpenoids, and steroids (Khachitpongpanit et al., 2016). Secondary metabolite compounds in plants, such as alkaloids, flavonoids, and saponins, have the potential to act as larvicides (Cahyati et al., 2017). It is necessary to conduct experiments to determine the toxicity of pleketan leaf extract on the mortality of Culex sp. mosquito larvae.

Lime, scientifically known as Citrus aurantiifolia, is a perennial plant that produces round-shaped fruits resembling ping-pong balls and can grow up to 3 meters tall. Its compound leaves have elliptical shapes with rounded bases, blunt tips, and serrated edges, measuring approximately 3-10 cm in length and 2-5 cm in width, with winged petioles (Kurniawati, 2010). Lime fruit (Citrus aurantifolia) is highly suitable for development as a larvicide. The fruit contains compounds such as flavonoids that can act as antimicrobials against microorganisms. Lime also contains various chemical compounds such as citric acid, amino acids (tryptophan and lysine), flavonoids, essential oils (limonene, linalyl acetate, geranyl acetate, phellandrene, citral, camphor, kadine, acetaldehyde, and anisaldehyde), as well as vitamins A, B1, and C (Haq et al., 2010). Therefore, research is needed to investigate the differences in

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toxicity between pleketan leaf extract (Ruellia tuberosa L.) and lime fruit (Citrus aurantifolia) on the mortality of Culex sp. mosquito larvae.

METHODS

The research on the differences in toxicity between pleketan leaf extract (Ruellia tuberosa L.) and lime fruit (Citrus aurantifolia) on the mortality of Culex sp. mosquito larvae was conducted as a laboratory experiment using a Completely Randomized Design (CRD). The study was conducted at the Laboratory of Biology Education Program, Faculty of Education and Educational Sciences, University of Jember.

A. Preparation

The preparation stage is a phase or step before the research, including equipment preparation, test larvae preparation, and extract preparation. This stage optimizes the working procedures to ensure the required equipment is available. The larvae preparation stage involves identifying the larvae to determine if the selected larvae species is suitable for the desired purpose. Additionally, the selection of late-third instar to early-fourth instar larvae is conducted.

B. Tools and materials

The equipment used in this research includes scissors, a blender, a stirrer, a spatula, jars, plastic cups, beaker glass, a digital scale, Erlenmeyer, a dropper pipette, measuring glass, rotary evaporator, filter paper, 250 ml glass jars, timer, sterilized toothpicks, microscope, cover glass, filter paper, and oven. Meanwhile, the materials required for this research include pleketan leaves (Ruellia tuberosa L.), lime (Citrus aurantifolia), Culex sp. mosquito larvae late-third instar to early-fourth instar, distilled water, 97% ethanol, and 1 g Abate brand.

C. Preparation of Pletekan Leaf Extract and Lime Fruit

The process of making pleketan leaf extract in this research is based on Wahyuni et al. (2016), which involves selecting high-quality pleketan leaves without pests or diseases. Next, the leaves are cleaned and chopped into small pieces until clean. The next step is to dry the pleketan leaves under sunlight until they reach a dry weight. Once dry, the pleketan leaves are ground into powder and weighed 250 grams into a container. The extraction process is conducted through maceration by adding 97% ethanol in a ratio of 1:3 (750 ml ethanol) to the container. The mixture is stirred until homogenous and covered. Stirring is performed every hour to ensure homogeneous mixing. This maceration process is carried out for 3x24 hours. The mixture is then filtered using filter paper to separate the sediment from the liquid. The 97% ethanol solvent is separated from the extract using a rotary evaporator at a temperature of 50°C for 2 hours. The result from the rotary evaporator is oven-dried at 40°C until a thick extract is obtained. The next step is to store the extract in a container and place it in a refrigerator.

The process of making lime fruit extract in this research begins with drying thinly sliced lime fruit for approximately one week. Then, it is ground into powder using a blender. Next, 200 grams of lime fruit powder is subjected to maceration. The maceration process involves dissolving the powder in 600 ml of 97% ethanol at a ratio of 1:3, stirring it with a spatula until homogenous, and tightly sealing the container. The maceration process takes place for 3x24 hours, requiring periodic stirring. The filtered maceration result is then processed using a rotary evaporator at 50°C for 3 hours. If the extract is still slightly diluted, it must be left in the oven at 50°C until a thick lime fruit extract is obtained.

D. Toxicity Test of Pletekan Leaf Extract (Ruellia tuberosa L.) and Lime Fruit (Citrus aurantifolia).

The pleketan leaf extract toxicity test (Ruellia tuberosa L.) was conducted using a serial concentration method. The test was carried out by preparing eight plastic cups filled with 100 ml of distilled water. Next, the pleketan leaf extract was dissolved in 100 ml of distilled water to obtain concentrations of 50 ppm, 340 ppm, 630 ppm, 920 ppm, 1210 ppm, and 1500 ppm, as well as a positive control (abate) and a negative control (distilled water). The next step was to introduce 20 larvae of Culex sp. mosquitoes into each of the 8 plastic cups containing the pleketan leaf extract solutions. Then, observations were made, and the larvae's mortality (number of deaths) was recorded after 24 hours.

The toxicity test of lime fruit extract (Citrus aurantifolia) consisted of 8 treatments with four replications. The treatments involved mixing distilled water with lime extract, with six serial concentrations including 50 ppm, 360 ppm, 670 ppm, 980 ppm, 1290 ppm, and 1600 ppm. The other two treatments were the negative control (distilled water) and the positive control (distilled water and abate). The next step was to introduce 20 larvae of Culex sp. mosquitoes into 8 plastic cups containing the lime extract solutions. Then,

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observations were made, and the larvae's mortality (number of deaths) was recorded after 24 hours. The mortality data were analyzed using probit analysis with SPSS version 25 software to determine the LC50 toxicity.

RESULT AND DISCUSSION

A. Result

Based on the research that has been conducted, the number of deaths (mortality) and the average mortality of Culex sp. mosquitoes due to the administration of pletekan leaf extract (Ruellia tuberosa L.) are shown in Table 1.

Table 1. Final test results of pletekan leaf extract (Ruellia tuberosa L.) on the mortality of Culex sp. mosquito larvae.

Concentration	Total mortality of *Culex* sp. mosquito larvae.				Average	Mortality (%)
	1	**2**	**3**	**4**		
50 ppm	0	1	1	1	1	5
340 ppm	5	4	4	5	4	20
630 ppm	8	9	8	8	8	40
920 ppm	13	12	11	13	12	60
1210 ppm	15	15	16	16	15	75
1500 ppm	19	18	18	19	18	90
Control (-)	0	0	0	0	0	0
Control (+)	20	20	20	20	20	100

Based on the research that has been conducted, the number of deaths (mortality) and the average mortality of Culex sp. mosquito larvae due to the administration of lime fruit extract (Citrus aurantifolia) are shown in Table 2.

Table 2: Final Test Results after Administration of Concentration of Lime Fruit Extract (Citrus aurantifolia) on Mortality of Culex sp. Mosquito Larvae. within 24 hours of discharge.

Concentration (ppm)	Number of larvae deaths (Repeat)				Average	Mortality %
	1	2	3	4		
Kontrol (-)	0	0	0	0	0	0
Kontrol (+)	20	20	20	20	20	100
50	0	1	2	2	1	5
360	4	5	5	5	5	25
670	7	6	7	6	6	30
980	11	10	8	10	10	50
1290	15	13	12	13	13	65
1600	18	18	17	19	18	90

Tables 1 and 2 above show that as the concentration of lime fruit extract increases, the average percentage of Culex sp. mosquito larvae mortality also increases. In the final test results, the negative control variable (aquades) did not result in larval mortality after 24 hours of observation. Furthermore, the positive control treatment using abate at a concentration of 50 ppm killed 20 larvae or 100% of the test larvae in each repetition with a 24-hour exposure period. The analysis of the probit LC50 for pletekan leaf extract can be seen in Table 3, while the results of the probit analysis can be seen in Table 4.

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IJCSRR @ 2023
www.ijcsrr.org

Table 3. Results of Probit LC50 Analysis Toxicity of Pletekan Leaf Extract (Ruellia tuberosa L.) on Mortality of Culex sp. Mosquito Larvae within 24 hours of discharge

LC50	Lower bound (ppm)	Upper bound (ppm)
710,169	615,231	796,157

Table 4. Analysis of Probit LC50 Toxicity of Lime Extract (Citrus aurantifolia) on Mortality of Culex sp. Mosquito Larvae. within 24 hours of discharge.

Lethal (LC_{50})	*Concentration*	LC_{50} (ppm)	The lower bound (ppm)	Upper bound (ppm)
Lime fruit extract		1026,749	846,406	1294,531

According to the analysis conducted, it is known that the concentration of pletekan leaf extract required to kill 50% of Culex sp. mosquito larvae is 710,169 ppm, with the lowest concentration being 615,231 ppm and the highest concentration being 796,157 ppm. Meanwhile, the probit analysis results to determine the Lethal Concentration of 50% (LC50) of lime extract (Citrus aurantifolia) on Culex sp. mosquito larvae mortality within a 24-hour exposure period. Based on the results of the probit analysis, the data obtained in Table 4 shows that the concentration of lime extract (Citrus aurantifolia) required to kill 50% of the test larvae during the 24-hour exposure period is 1026.749 ppm, with a lower limit value of 846.406 ppm and an upper limit of 1294.531 ppm.

B. Discussion

The toxicity test of pletekan leaf extract on Culex sp. mosquito larvae (Table 3) shows that the concentration required to kill 50% of the larvae (LC50) is 710.169 ppm. If the concentration of pletekan leaf extract is below 615.231 ppm, the mortality rate of the test larvae would be less than 50%. On the other hand, if the concentration of pletekan leaf extract is above 796.157 ppm, the mortality rate of the test larvae would be higher than 50%. Meanwhile, the toxicity test of lime extract (Citrus aurantifolia) on Culex sp. mosquito larvae, which is required to kill 50% of the test larvae during a 24-hour exposure period, is 1026.749 ppm, with a lower limit value of 846.406 ppm and an upper limit of 1294.531 ppm. An extract can be considered toxic if its LC50 value is ≤1000 ppm (Meyer et al., 1982). It is considered non-toxic if the extract has an LC50 concentration above 1000 ppm (Bara et al., 2022). Therefore, it can be concluded that pletekan leaf extract (Ruellia tuberosa L.) and lime fruit extract (Citrus aurantifolia) are toxic to Culex sp. mosquito larvae.

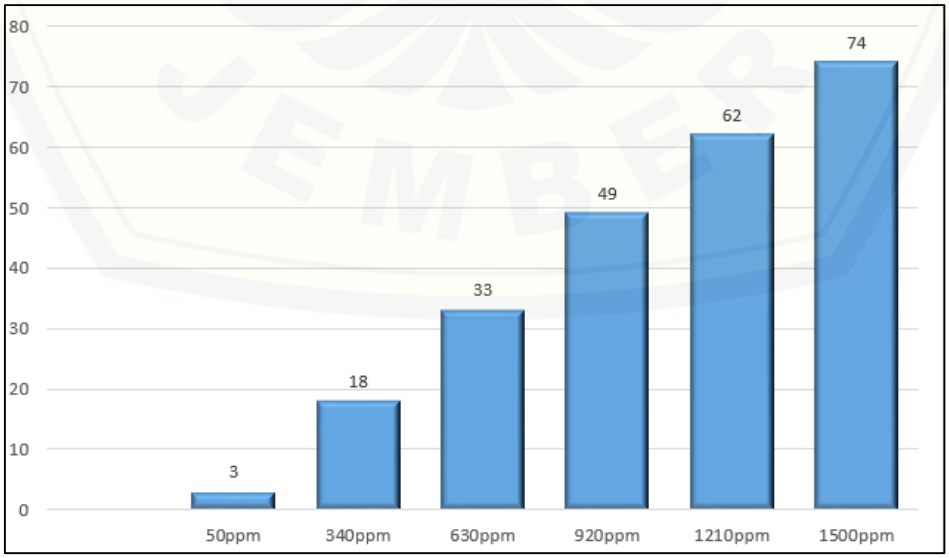

Figure 1. Histogram of Culex sp. Mosquito Larvae Mortality. on Pletekan Leaf Extract (Ruellia tuberosa L.) in 24 Hours Exposure Time

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IJCSRR @ 2023
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Figure 1 shows the graph depicting the relationship between the mortality of Culex sp. mosquito larvae and the concentration of pletekan leaf extract over a 24-hour exposure period. The graph illustrates that as the concentration of pletekan leaf extract (Ruellia tuberosa L.) increases, the average percentage of Culex sp. mosquito larvae mortality increases within the 24-hour exposure period.

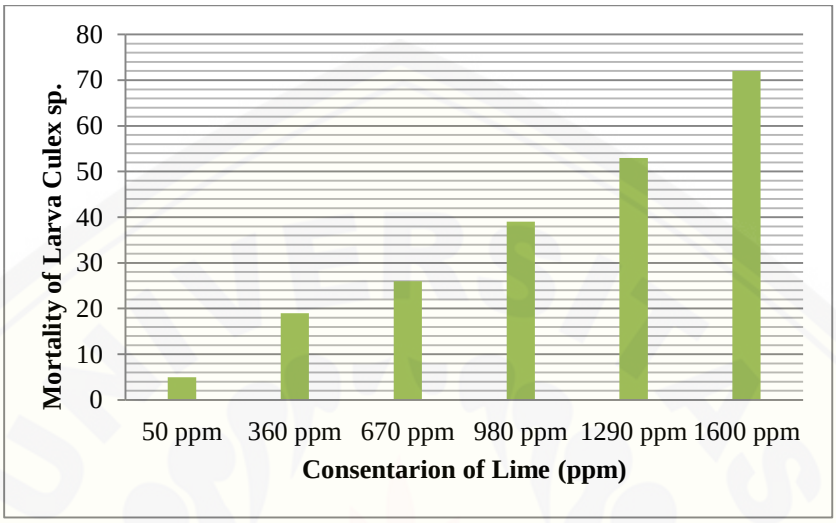

Figure 2. Histogram of Mortality of Culex sp. Mosquito Larvae in the Final Test with Lime Fruit Extract (Citrus aurantifolia) in 24 Hours Exposure Time

Figure 2 demonstrates that as the concentration of lime fruit extract (Citrus aurantifolia) used increases, the average percentage of Culex sp. mosquito larvae mortality also increases.

After the final test, the next step is to perform observations with the assistance of a microscope. The differences in Culex sp. mosquito larvae before and after treatment with pletekan leaf extract can be seen in Figure 3. The differences in Culex sp. mosquito larvae before and after treatment with lime fruit extract can be seen in Figure 4.

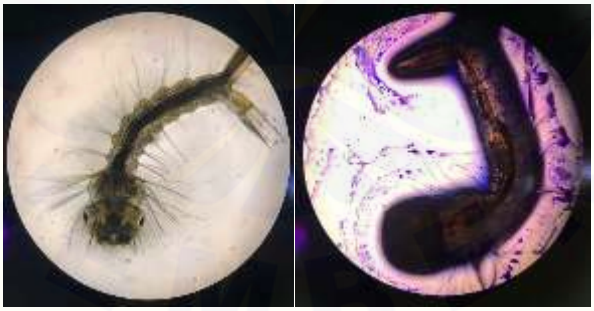

Figure 3. Morphology of Culex sp. Mosquitoes (a) before administration of the extract (magnification 40x10); (b) after administration of the extract (magnification 40x10)

Figure 4. Morphology of Culex sp. Mosquitoes (a) before administration of the lime extract (magnification 40x10); (b) after administration of the lime extract (magnification 40x10)

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IJCSRR @ 2023
www.ijcsrr.org

Based on the observations under the microscope in Figures 3 and 4, (a) the morphology of Culex sp. mosquito larvae when they are still alive shows that their body cells are still colored and there is no damage. On the other hand, the morphology of Culex sp. mosquito larvae when they are dead due to treatment shows transparent larval body cells and many damaged organs. Observations are carried out by adding drops of methylene blue to the dead larvae to confirm whether the larvae are truly dead. The dead cells will immediately change to blue, as seen in Figures 3 and 4 (b).

Before treatment with serial extract concentrations, mosquito larvae moved agilely when touched with a sterile stick. After being treated with the extract for 24 hours, the larvae moved slowly and showed no movement when touched with a sterile stick. The dead mosquito larvae also settled at the bottom of the container. Based on the observations (Figure 3), it can be seen that the pletekan leaf extract can damage the abdomen of Culex sp. mosquito larvae. The abdomen of the mosquito larvae is severed and damaged, as seen in Figures 3 and 4 (b). The damage to the abdomen of the mosquito larvae may be caused by the compounds present in the pletekan leaf extract.

The process of larval death due to exposure to plant compounds can occur through four phases: stimulation, convulsions, paralysis, and, ultimately, larval death. The stimulation phase is characterized by changes in the animal's behavior from its normal state, spreading to the level of the antennae and mouthparts. The symptoms continue until the paralysis stage and then affect the respiratory organs, ultimately leading to larval death (Dinata, 2018). Administration of pletekan leaf extract to Culex sp. mosquito larvae results in larval death or mortality and changes in the larval body.

Based on the research conducted by Kadir and Anggraeni (2020), it is known that the active compounds extracted from pletekan leaves (Ruellia tuberosa L.) using ethanol solvent include tannins, flavonoids, and triterpenoids. According to a study by Vitalia et al. (2016), ethanol-extracted pletekan leaf extract contains compounds such as saponins, phenols, alkaloids, and flavonoids. Compounds like saponins and flavonoids are present in pletekan leaves and act as stomach poisons. Compounds such as alkaloids, flavonoids, and saponins can cause damage to the midgut area, a specialized region for digestion and secretion (Dhanasekaran et al., 2020).

Saponins can enter the larva's body through the mouth. As stomach poisons, saponins work by reducing the activity of protease enzymes and food absorption. As a result, larval growth is inhibited, eventually leading to larval death (Wahyudi et al., 2021). Saponins are substances that, when mixed with water, produce foam which, when hydrolyzed, yield sugars and sapogenins. Sapogenins can bind to cholesterol and insect toxins and cause hemolysis of blood. Saponins can irritate the mucous membranes of the digestive tract and have a bitter taste, thus reducing the larva's appetite (Moniharapon et al., 2020). As a nerve poison, saponins can affect the cholinesterase enzyme system. Cholinesterase enzymes are phosphorylated and deactivated by anti-cholinesterases, leading to the accumulation of acetylcholine in the synaptic cleft, causing muscle spasms, paralysis, and death in larvae (Adnyana et al., 2022). As a contact poison, saponins work by removing the protective waxy layer of the body, resulting in the loss of body fluids and eventually leading to the death of the larvae over time (Ahmad and Adriyanto, 2019).

Flavonoid compounds in larvae act as respiratory toxins and nerve toxins. Flavonoids can inhibit respiration by weakening the nerves, preventing larvae from breathing. Flavonoid compounds enter through the siphon, causing damage to the siphon. The siphon appears abnormal and curved, disrupting larval activities (Ristiati et al., 2017). According to Rattan, cited in Nindiastuti et al. (2022), flavonoid compounds also act as nerve toxins by interfering with the activity of acetylcholinesterase enzymes, leading to an accumulation of acetylcholine that disrupts the transmission of impulses from nerve cells to muscle cells, causing muscle spasms, paralysis, and death.

Alkaloids are compounds that can act as contact toxins for larvae. Alkaloid compounds enter the larval body through the skin via absorption. Skin cells are degraded, causing cell damage and disrupting nerve function (Huljani and Ahsanunnisa, 2019). Alkaloids can also act as stomach toxins for larvae. They can inhibit insect growth by affecting three hormones: brain, ecdysis, and growth. If the development of these hormones is disrupted, it can lead to metamorphosis failure (Lubis et al., 2018).

Tannins can act as stomach toxins for larvae. Tannin compounds disrupt the process of protein utilization in the digestive tract. Tannins also disrupt larval growth (Hasanah et al., 2019). Tannins have a bitter taste, which causes a loss of appetite in larvae, resulting in decreased energy requirements and, ultimately, larval death (Poerwanto et al., 2020). Triterpenoid compounds can decrease digestive enzyme activity and affect food absorption (Ilham et al., 2019). Phenolic compounds act as dehydrating toxins, leading to fluid deficiency and death (Rahmah et al., 2022).

Ruellia tuberosa leaves can be used as a natural larvicide alternative to chemical larvicides. Research results indicate that the

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IJCSRR @ 2023
www.ijcsrr.org

extract from Ruellia tuberosa leaves is toxic to Culex sp. mosquito larvae within a 24-hour exposure period. Larval mortality can be attributed to the compounds present in Ruellia tuberosa leaves. The toxic constituents of Ruellia tuberosa leaves include alkaloids, saponins, flavonoids, triterpenoids, and phenols.

The toxicity test research on Citrus aurantifolia (lime) fruit extract against Culex sp. mosquito larvae showed larval mortality. This is due to the presence of active toxic compounds in the extract. The compounds present in each Citrus aurantifolia fruit extract can attack the nervous system of insects, one example being alkaloids found in Citrus aurantifolia fruit. Alkaloids are inherently toxic, disrupting the nervous system and damaging cell membranes. This class of compounds usually inhibits the enzyme acetylcholinesterase, leading to the accumulation of acetylcholine in the synapses. The induced effect can hinder the neurotransmitter process (Soemirat, 2003). The nerve degeneration caused by the accumulation of acetylcholine reduces the larvae's sensitivity to food impulses and deadly predators (Lestari et al., 2014).

Flavonoids are also present in Citrus aurantifolia (lime). The mechanism of action of flavonoids is a respiratory inhibitor. Flavonoids work by entering the body of the test larvae through the respiratory tract, causing nerve and respiratory damage, ultimately leading to larval lethargy. Enrolling flavonoid compounds through the siphon spirals will damage the siphon, forcing the larvae to position themselves at the water surface to facilitate oxygen absorption (Zahroh et al., 2022). Flavonoids have a derivative called rotenone, which acts as a contact and stomach poison. Rotenone is a natural insecticide that belongs to the flavonoid derivative. It can also be used as a fish poison. Rotenone works by inhibiting respiratory enzymes such as NAD+ (a coenzyme involved in oxidation and metabolism) and Coenzyme Q (a respiratory coenzyme responsible for electron transport in the chain), leading to respiratory failure (Wahyuni et al., 2015).

Lime (Citrus aurantifolia) also contains saponin compounds. Saponins act as stomach poisons that can enter the digestive tract (mouth) and poison the larvae. Saponins also act as contact poisons, which can be externally perceived when larvae experience physical disturbances (mite molting), causing the protective layer to peel off from the larval body and resulting in death due to significant fluid loss. The mechanism of action of saponin compounds is the denaturation of proteins and outer membrane enzymes, and vulnerable cell walls, which can then bind to the cytoplasmic membrane and disrupt and weaken cell membrane stability. This can force cytoplasmic fluid out of the cell and cause cell death (Putri et al., 2022).

The toxic effects a substance induces depend on the concentration of the substance used in this study. It also depends on the toxic compounds contained in the test material when the exposure time is the same. Lime (Citrus aurantifolia) contains bioactive compounds such as flavonoids, saponins, d-limonene, and alkaloids, which have been proven to act as contact poisons on mosquitoes (Naria, 2015).

CONCLUSION

The toxicity value (LC50) of Ruellia tuberosa L. leaf extract on Culex sp. mosquito larvae mortality within 24 hours is 710.169 ppm. The LC50 value can be considered toxic as it has an LC50 value ≤ 1000 ppm. Meanwhile, the toxicity value (LC50) of Citrus aurantifolia (lime) fruit extract on Culex sp. mosquito larvae mortality within 24 hours is 1026.749 ppm.

SUGGESTION

Further research is needed to detect the active compounds present in Ruellia tuberosa L. leaves extract using TLC (Thin Layer Chromatography) analysis. Additionally, experiments should be conducted by combining the extract with other tested toxic substances. Furthermore, mosquito houses or mosquito colonies should be established using mosquito cages. In addition, further research should be conducted using different extraction methods.

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