Larvicidal activity of *Annona squamosa* (Atis) leaves extract on *Aedes aegypti*

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Abstract

Introduction Effective mosquito control is pivotal in the epidemiology of vector-borne diseases, but no successful preventive measures have been recorded for dengue vector control. Hence, possible alternatives to chemical larvicides have been explored, including plant alcoholic extracts. This study determined the larvicidal efficacy of *Annona squamosa* ethanolic leaf extracts against third instar larvae of *Aedes aegypti*.
Methods Three replicates of varying concentrations of *Annona squamosa* ethanolic extract (i.e., 10%, 40%, and 70%) versus positive (Novaluron) and negative controls (tap water) were used to determine larval mortality.
Results Greatest larval mortality was noted using the 70% concentration (i.e., 24% versus the observed values of 20% and 8%, respectively for the 40% and 10% ethanolic concentrations). Relative to the controls, the mean differences in the mortality rates of the *Aedes aegypti* larvae across the leaf ethanolic concentrations were statistically significant (i.e., p-value < 0.05). There was increasing trend in larval mortality over time, but 50% lethal dose was not achieved. In conclusion, the different *Annona squamosa* ethanolic leaf extracts could be used as alternative botanical larvicides against *Aedes species*.

Key words: *Annona squamosa*, *Aedes aegypti* mosquito, larvicidal activity

Vector-borne diseases (VBDs), particularly mosquito-borne diseases, pose a substantial threat to the population in the world, including countries in South and Southeast Asia, such as the Philippines. These VBDs infect over a billion people each year, contributing to more than a million deaths globally. The growing public health significance of these VBDs has created difficult challenges in over a hundred tropical and subtropical nations. In the Philippines, dengue remains to be a significant public health concern.[1] The flavivirus propagation has been facilitated by rapid urbanization, environmental degradation, lack of reliable water supply, and improper management and disposal of solid waste. Mosquito vector control measures are crucial for preventing dengue by stopping the spread of mosquito populations and enhancing environmental sanitation to improve public health services.[2-5] Mosquito vector control is believed to be one effective undertaking that can lessen the financial burden associated with dengue infection, including improvement of the dengue disability-adjusted life years (DALY).[3,6]

Although there is no single successful preventive measure recorded for mosquito vector control, addressing various links in the chain of dengue infection and transmission can significantly impact on the public health burden of dengue. Some successful strategies to prevent dengue transmission include environmental sanitation, avoiding mosquito bites, administering the dengue vaccine to the selected members of the high-risk population, and using insecticides and larvicides. The advent of conventional pesticides demonstrates a successful move to control the mosquito population. However, this success has

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been short-lived due to the development of resistance by mosquito strains to the commonly used pesticides, ecological imbalance, and potential harm of certain chemicals in manufactured pesticides to mammalian species, including humans.[7]

Thus, science and health experts have diligently worked to develop safe, biodegradable, and cost-effective larvicidal / adulticidal chemical formulations. Several studies have utilized various plant parts as potential alternatives to control the mosquito population, while maintaining balance in the ecosystem.[8-13] Environmental advocates recommend the use of plant-based preparations for mosquito control, according to the perception that these will be less harmful to the ecosystem, including non-targeted organisms. Thus, exploratory studies have been conducted, evaluating extracts from various genera of plants for their larvicidal and insecticidal properties. More than two thousand species of plants are believed to possess insecticidal and larvicidal activity. One such plant is the *Annona squamosa*, a small tree in the Annonaceae family. The various parts of the atis (sugar apple) plant also have multiple uses.[14-18]

Several previous experiments tested and screened over 3,500 species of plants, and multiple research studies demonstrated *Annona squamosa* plant parts exhibiting mosquito larvicidal activity. Hence, this study aimed to determine the efficacy of the *atis* (sugar apple) ethanolic extract, of varying concentrations, as larvicide for the *Aedes aegypti* third instar larvae. Creating an ethanolic leaf extract which could serve as an add-on or substitute to the chemical larvicides currently being used in public places (e.g., airports), if proven to be effective to it, could prove to be promising. In this way, potential adverse reactions brought by the chemical larvicides currently being used could potentially be hindered or lessened, if plant-based alternatives could be identified and mass-produced.

Methods

A. Identification and Collection of Plant Material

The plant of interest, *Annona squamosa* (i.e., local name *atis* or sugar apple), belonging to the Family Annonaceae, was identified and authenticated by the Bureau of Plant Industry (BPI). After certification was granted for authenticity, mature leaves of *Annona squamosa* were collected. The bulk samples were collected, washed with tap water, and shade-dried at room temperature for 7 to 14 days. The dried leaves were ground into fine powder using an electric blender, from which the extract was prepared. Blended leaves then underwent maceration at the University of the Philippines Pharmacy – National Institutes of Health. Maceration was performed by placing the powdered leaves in a beaker and soaking them in 10%, 40%, and 70% ethanol. The mixtures were then allowed to stand at room temperature for three (3) days with frequent agitation. The resulting extracts were stored in the refrigerator until analysis.

B. Test Organism

The larvae of *Aedes aegypti* were reared by placing an improvised black pail trap containing 1,000 ml of water and a submerged piece of black board in various designated areas across Terminals 1 to 4 of Ninoy Aquino International Airport (NAIA) in Manila, Philippines. The traps were left in place for seven (7) days to facilitate larval development. To synchronize and promote hatching, one (1) tablespoon of yeast was dissolved and added to the 1,000 ml of water after 24 hours. The observation of the mosquito life cycle then followed. On the fifth day, larvae that were noted to be 4-5 mm in length were readied for harvest. Twenty-five (25) pieces of third instar larvae were carefully transferred to the testing cup filled with 200 ml of dechlorinated water using a pipette and covered with mesh. A designated entomologist and a vector control technician of the Department of Health (DOH) supervised the rearing and harvesting of the experimental organisms. Laboratory-reared *Aedes aegypti* larvae, specifically third instar, were then subjected to different concentrations of the *Annona squamosa* ethanolic leaf extracts.

C. Larvicidal Activity

Twenty-five (25) samples of third instar *Aedes aegypti* larvae were placed in a testing cup, which was then covered with a safety net or mesh to prevent any accidental emergence of the larvae as adult mosquitoes. For each concentration, three replicates were established alongside corresponding controls. These included Novaluron as the positive control, tap water as the negative control, and ethanol at concentrations of 10%, 40%, and 70%. Two (2) ml of ethanolic leaf extract was added to the experimental larvae, as well as to the non-experimental arm.

Every two (2) hours, larval mortality was counted for a period of twenty-four (24) hours. The number of dead larvae was recorded, with dead larvae being recognized as non-movement of the larvae despite physical stimulation and probing. The test runs were performed thrice conducted on different days.

Results

Table 1 shows the mortality rate of *Aedes aegypti* larvae, observed after 24 hours of exposure to different concentrations of *Annona squamosa* leaf extract. The higher the extract concentration, the higher the mortality rate of *Aedes aegypti* larvae --- i.e., more larvae died at a 70% ethanolic leaf extract concentration compared to the 40% and 10% concentrations. The positive control, Novaluron, had a 68% mortality rate, while the negative control, tap water, yielded a 0% mortality rate.

Table 2 reveals the mean differences in the mortality rate of *Aedes aegypti* larvae across the various ethanolic leaf extract concentrations, compared to the positive and negative controls. The mean difference was derived using the standard deviation formula. The results indicated a significant difference between the experimental intervention and control arms (i.e., all with p-values < 0.05).

Table 3 demonstrates the proportion of *Aedes aegypti* larvae that died over 24 hours at two-hour intervals. It showed that at a 70% concentration, there was a higher mortality rate, compared to the 40% and 10% concentrations, and the mortality rate of the larvae increased over time. The mortality of the larvae was first observed after four hours of observation and progressed after that. However, from 18 to 24 hours, less mortality was observed at 10% and 40% concentrations.

This study did not achieve the lethal dose 50 (LD50) within 24 hours of exposure and observation. The ethanolic leaf extract of *Annona squamosa* resulted in 24% mortality at a 70% concentration. Nonetheless, the experiment indicated that increasing the ethanol solvent concentration for extracting *Annona squamosa* leaves corresponded with higher mortality rates in *Aedes aegypti* larvae.

Table 1. Mortality rates of *Aedes aegypti* larvae after 24 hour-exposure to different *Annona squamosa* ethanolic leaf extract concentrations.

Percentage (%) of Extract	Dead Larvae Mean After 24 Hours (N=25)	± Standard Deviation	Mean Mortality Rate	± Standard Deviation
10%	2	0.25	8%	0.02
40%	5	1.92	20%	0.38
70%	6	1.96	24%	0.47
Novaluron	17	0.41	68%	1.90
Water	0	0	0%	0

Table 2. Mean difference in mortality rates of the *Aedes aegypti* larvae across several *Annona squamosa* ethanolic leaf extract concentrations versus the positive (Novaluron) and negative (tap water) controls.

	Annona Ethanolic Extract	Positive Control (Novaluron)	Negative Control (Water)
***Annona* Ethanolic Extract**		20.556* Sig < 0.001	-4.111* Sig 0.007
Positive Control (Novaluron)	-20.556* Sig < 0.001		-24.667* Sig < 0.001
Negative Control (Tap Water)	4.111* Sig 0.007	24.667* Sig < 0.001	

Table 3. The proportion of dead *Aedes aegypti* larvae at two-hour intervals over a 24-hour observation period and exposure to varying concentrations of *Annona squamosa* leaf ethanolic extract.

Percentage of Extract	Number of Replicates	Proportion of Dead Larvae at 2-Hour Intervals											
		2 hours	4 hours	6 hours	8 hours	10 hours	12 hours	14 hours	16 hours	18 hours	20 hours	22 hours	24 hours
0.1 (10%)	1st	0%	0%	4%	0%	0%	4%	0%	0%	0%	0%	0%	0%
	2nd	0%	0%	0%	4%	0%	0%	0%	4%	0%	0%	0%	0%
	3rd	0%	0%	4%	0%	0%	4%	0%	0%	0%	0%	0%	0%
0.4 (40%)	1st	0%	0%	4%	0%	4%	0%	8%	0%	0%	8%	0%	0%
	2nd	0%	0%	4%	0%	4%	0%	8%	0%	8%	0%	8%	0%
	3rd	0%	0%	4%	0%	4%	0%	8%	0%	8%	0%	8%	0%
0.7 (70%)	1st	0%	0%	4%	0%	4%	0%	0%	0%	0%	0%	0%	0%
	2nd	0%	0%	4%	0%	0%	4%	0%	0%	8%	0%	8%	8%
	3rd	0%	4%	0%	9%	0%	8%	11%	0%	12%	0%	13%	0%

Discussion

This study investigated the efficacy of *Annona squamosa* ethanolic leaf extract in eliminating third-instar *Aedes aegypti* larvae. The findings indicated that while the ethanolic leaf extract of *atis* was able to kill mosquito larvae, it did so at a slower rate compared to the positive control (Novaluron), which led to a quicker larval mortality rate.

A similar outcome was observed in another experimental study wherein thirteen different plant species were tested with different concentrations of a positive control (temephos) and a negative control (water), resulting in mortality rates of 100% and 0%, respectively. The investigation of the Ipomoea cairica plant extract indicated larval mortality rates of 40% for the leaves and 100% for the stem.[19]

Another epidemiologic experiment evaluated the larvicidal activity of fifty plant species from the Columbian Caribbean Region using 95% ethanol with maceration technique, employing temephos as positive control and dimethyl sulfoxide (DMSO) 1% as negative control. Seeds of the following plants stood out for their larvicidal activity, namely *Annona squamosa*, *Annona cherimolia*, *Annona muricata*, *Tabernaemontana cymose* Jacq, and *Mammae americana* L. which determined lethal dose fifty (LD50) values of 58.44ppm, 65.10ppm, 84.82 ppm, 25.02 ppm, and 38.58 ppm, respectively.[20]

Another study investigated the larvicidal activity of the ethanolic extract of *Inula racemosa* Hook (Family Compositae) roots against *Aedes alpobictus* using 95% ethanol. This experimental investigation showed the ethanol extract's lesser toxicity (i.e., 14 times less toxic), compared to the positive control chlorpyrifos.[21]

The lethal activity of *Annona squamosa* leaf extract may be due to various phytochemical compounds it contains such as alkaloids, flavonoids saponins, tannins, glycosides and triterpenoids. These work in different ways to cause mortality in the *Aedes aegypti* larvae. In the phytochemical analysis of *Annona squamosa* Linn, alkaloids, tannins, flavonoids, phenols, and saponins were detected in the leaves.[22]

This is further supported by findings in another epidemiologic investigation which evaluated the in vitro larvicidal activity of *Annona squamosa* leaves extract against *Culex* mosquito. This study revealed that *Annona squamosa* had phytochemical components, such as tannins, glycosides, alkaloids, and terpenoids, which were all theorized to contribute to the plant's larvicidal activity.[23]

Another review on the different larvicidal compounds isolated from plant extracts against *Aedes aegypti* demonstrated that acetogenins had the highest larvicidal potential, killing 50% of *Aedes aegypti* larvae at a concentration of 0.01 ug/ml.[24]

While all *Annona squamosa* leaf extract concentrations have a mortality impact on *Aedes* species, their effects are slower than those of Novaluron, which has a faster effect on the death of *Aedes* larvae. The lower efficacy of *Annona squamosa* leaf extract could be attributed to plant-based factors. These include the plant's geographical origin, plant age, time of harvest or collection, part of the plant to be used, the storage condition of the plant material, type and quantity of active chemicals the plant contains, extraction technique, plant species selected, and the solvent used for isolation and its potentials.[25-27] Furthermore, other factors, including temperature, air humidity, and water pH, may affect the body resistance of each larva.[9]

The use of suitable solvents affects the efficacy of larvicides in *Annona squamosa*. Ethanol is a universal solvent that can bind all chemical components in natural plants. It has been stated that the metabolites found in the cytoplasm of plants may be dissolved in the solvent and eventually extracted.[28]

Another plausible explanation for the lower mortality of using plant extracts to kill *Aedes aegypti* is brought about by the extraction procedure done, which is the maceration process. Maceration is one of the bioactive natural product extraction methods that use water, aqueous, and non-aqueous solvents that have been conducted at room temperature. Maceration is the simplest, most practical method with the lowest energy consumption or expenditure. It is compatible with ionic liquid at room temperature, which is extremely important for obtaining secondary metabolites since ionic liquid has been shown to have a high extraction capacity, not requiring the external supply of energy.[29]

Conclusion

In conclusion, the different *Annona squamosa* ethanolic leaf extracts could be used as alternative botanical larvicides against *Aedes species*. This epidemiologic experiment documented varying mortality rates of *Aedes aegypti* larvae, exposed to 70%, 40%, and 10% ethanolic leaf extract concentration. It also showed a significant difference using the experimental intervention and controls, as it displayed the mean difference in mortality across the various ethanolic leaf extract concentrations, compared to positive and negative controls. The proportion of *Aedes aegypti* larval death at two-hour intervals over 24 hours showed that mortality rate of larvae generally increased over time. Although the study did not reach the lethal dose at 50 (LD50) due to the short period of observation (i.e., 24 hours only), *Annona squamosa* leaf extract still had 24% larval death in the study.

Recommendations

However, additional research with various mosquito genera and extraction techniques is necessary to firmly establish the use of plant extracts as viable alternatives for controlling mosquito populations and reducing the incidence of dengue and other vector-borne diseases (VBDs). Moreover, conducting detailed phytochemical analyses of these plant species and determining their lethal dose 50 could effectively clarify the larvicidal properties of these plant-based solutions.

References

1. George AM, Ansumana R, de Souza DK, Niyas VKM, Zumla A, Bockarie MJ. Climate change and the rising incidence of vector-borne diseases globally. Int J Infect Dis 2024 Feb;139:143-5. doi: 10.1016/j.ijid.2023.12.004. Epub 2023 Dec 13.
2. Rocklöv J, Dubrow R. Climate change: an enduring challenge for vector-borne disease prevention and control. Nat Immunol 2020 May;21(5):479-83. doi: 10.1038/s41590-020-0648-y. Erratum in: Nat Immunol 2020 Jun;21(6):695. doi: 10.1038/s41590-020-0692-7.
3. Chala B, Hamde F. Emerging and re-emerging vector-borne infectious diseases and the challenges for control: A review. Front Public Health 2021 Oct 5;9:715759. doi: 10.3389/fpubh.2021.715759.
4. Torto B, Tchouassi D. Grand challenges in vector-borne disease control targeting vectors. Front Trop Dis 2021;1:1.
5. Dey P, Goyary D, Mandal S, Dey BK, Verma A. Vector-borne diseases: prevalence, impacts, and strategies to address disease burden and threats. Int J Mosq Res 2023;10(1):29–38. doi:10.22271/23487941.2023.v10.i1a.659
6. Sulistiawati, Mas'ulun MJ, Ramadhany AK, Hanafie AN, Alfiani RF, Husnah SE et al. Effectiveness of the Aedes aegyptimosquito vector control program in Southeast Asia – a systematic review. Pharmacogn J 2023;15(5):969–75.
7. Onen H, Luzala MM, Kigozi S, Sikumbili RM, Muanga CK, Zola EN, Wendji SN, Buya AB, Balciunaitiene A, Viškelis J, Kaddumukasa MA, Memvanga PB. Mosquito-borne diseases and their control strategies: An overview focused on green synthesized plant-based metallic nanoparticles. Insects 2023 Feb 23;14(3):221. doi: 10.3390/insects14030221.

8. Poerwanto SH, Harjanti DW, Prasetyorini T. Efficacy of ethanol and methanol extracts of white weed (*Ageratum conyzoides* L.) leaves on the mortality and development of mosquito *Aedes aegypti* L. larvae. AIP Conf Proc 2020;2260(1):040027. doi:10.1063/5.0016016
9. Ilham R, Lelo A, Harahap U, Widyawati T, Siahaan L. The effectivity of ethanolic extract from papaya leaves (*Carica papaya* L.) as an alternative larvacide to *Aedes* spp. Open Access Maced J Med Sci 2019 Oct 14;7(20):3395-9. doi: 10.3889/oamjms.2019.432.
10. Hikmawanti NPE, Fatmawati S, Asri AW. The effect of ethanol concentrations as the extraction solvent on antioxidant activity of Katuk (*Sauropus androgynus* (L.) Merr.) leaves extracts. IOP Conf Ser Earth Environ Sci 2021; 755: 012060. doi:10.1088/1755-1315/755/1/012060
11. Mohanraj SPRS. Insecticidal effects of ethanolic leaf extract of *Millingtonia hortensis* L.F. (Bignoniaceae) against *Aedes aegypti* (Diptera: Culicidae). J Adv Sci Res 2023; 14(2):71–6.
12. Dastiana SN, Rosa E, Setyaningrum E, Mumtazah DF. Effectivity test of ethanol extract of black pepper (*Piper nigrum*) against mortality of *Aedes aegypti* and its effect on midgut morphological changes. World J Biol Pharm Health Sci 2023;15(3):51–8. doi:10.30574/wjbphs.2023.15.3.0379
13. Susanti DA, Milyunier FMP, Nufus GM, Pramesti LA, Khamimi NLN, Anggitasari W, et al. Ethanolic extract of red dragon fruit inhibits growth of mosquito larvae. Healthc Low Resour Settings 2024;12:11772. doi:10.4081/hls.2023.11772.
14. Singh Y, Bhatnagar P, Thakur N. A review on insight of immense nutraceutical and medicinal potential of custard apple (*Annona squamosa* Linn). Int J Chem Stud 2019;7:1237–45.
15. Kumar B. A comprehensive review of *Annona squamosa*: nature's gift. Int J Pharm Prog Health Sci 2021;1(2):1-11.
16. Safira A, Widayani P, An-Najaaty D, Rani CAM, Septiani M, Putra YAS, Solikhah TI, Khairullah AR, Raharjo HM. A review of an important plant: *Annona squamosa* leaf. Pharmacogn J 2022;14(2):456–63. doi:10.5530/pj.2022.14.58.
17. Reddy DRB, Malleswari K, Kumar EN. Phytochemicals and pharmacological activities of *Annona squamosa*. Int J Multidiscip Res 2023;5(1):1.
18. Dhakar S, Jain SK, Tare H. Exploring the multifaceted potential of *Annona squamosa*: a natural treasure for health and wellness. Int J Pharm Qual Assur 2023;14(4):1279–82. doi:10.25258/ijpqa.14.4.69
19. Álvarez-Valverde V, Rodríguez G, Argüello-Vargas S. Insecticidal activity of ethanolic plant extracts on *Aedes aegyptilarvae*. Uniciencia 2023;37(1):1–11. doi:10.15359/ru.37-1.5
20. Oliveros-Díaz AF, Pájaro-González Y, Cabrera-Barraza J, Hill C, Quiñones-Fletcher W, Olivero-Verbel J, et al. Larvicidal activity of plant extracts from Colombian North Coast against *Aedes aegypti* L. mosquito larvae. Arab J Chem 2022;15(12):104365. doi:10.1016/j.arabjc.2022.104365.
21. He Q, Liu XC, Sun RQ, Deng ZW, Du SS, Liu ZL. Mosquito larvicidal constituents from the ethanol extract of *Inula racemosa* Hook. f. roots against *Aedes albopictus*. Biomed Res Int 2014;2014:738796. doi:10.1155/2014/738796.
22. Aswathi T, Sreekumar SH. A review on phytochemical analysis and pharmacological. Int Res J Mod Eng Technol Sci. 2023 Oct;5(10):3296. Available from: https://www.irjmets.com
23. Akhila S, Anu V, Smriti S. In vitro larvicidal activity of extracts of leaves of *Annona squamosa*. World J Pharm Pharm Sci 2021 Jan;10(6):1368–73. doi:10.20959/wjpps20216-19007.
24. De Souza Wuillda ACJ, Martins RCC, Costa FDN. Larvicidal activity of secondary plant metabolites in *Aedes aegypti* control: an overview of the previous 6 years. Nat Prod Commun 2019 Jul;14(7):1–11. doi:10.1177/1934578X19862893
25. Marcellia S, Margareta TI, Retnaningsih A. Effectiveness of the larvacide ethanol extract of soursop (*Annona muricata* L.) leaves against *Aedes aegypti* larva. MBIOMJ 2024 Jan;34(1):45. Available from: https://e-journal.unair.ac.id/MBIO/
26. Hillary EV, Ceasar SA, Ignacimuthu S. Efficacy of plant products in controlling disease vector mosquitoes, a review. Entomol Exp Appl 2024 Jan;172(3). doi:10.1111/eea.13401.
27. Ahmed N, Alam M, Saeed M, Ullah H, Iqbal T, Al-Mutairi KA, Shahjeer K, Ullah R, Ahmed S, Ahmed NAAH, Khater HF, Salman M. Botanical insecticides are a non-toxic alternative to conventional pesticides in the control of insects and pests. IntechOpen 2021 Oct 25. doi: 10.5772/intechopen.100416.
28. Aditama W, Junaidi, Sitepu FY. Optimizing maceration with ethanol and water solvents against the toxicity of extract of wuluh starfruit (*Averrhoa bilimbi* L.) in controlling larvae of Aedes aegypti. Int J Mosq Res. 2019;6(1):109-13.
29. Ternelli M, Brighenti V, Anceschi L, Poto M, Bertelli D, Licata M, Pellati F. Innovative methods for the preparation of medical Cannabis oils with a high content of both cannabinoids and terpenes. J Pharm Biomed Anal 2020 Jul 15;186:113296. doi: 10.1016/j.jpba.2020.113296. Epub 2020 Apr 6.