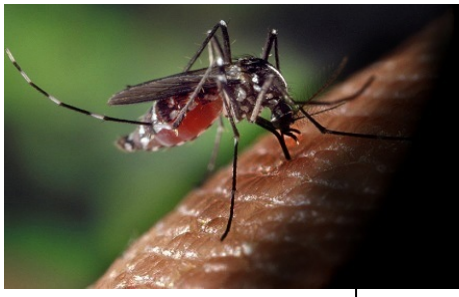

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Mortality and larvicidal activity of aqueous extract of *Stephania japonica* leaf against the larva of the mosquito *Anopheles culicifacies*

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Abstract
The *Anopheles culicifacies* mosquito is a vector for malaria found in southern India. Control of the *Anopheles culicifacies* mosquito as a vector needs to be carried out appropriately and in an environmentally responsive manner. The research aimed to examine the mortality and growth of *Anopheles culicifacies* mosquito larvae due to administration of *Stephania japonica* L leaf extract and to determine the effective concentration of *S. japonica* leaf extract in increasing mortality and reducing the growth of *Anopheles culicifacies* mosquito larvae. A bioassay for mosquito repellence and biocidal activity was performed. Mortality data was obtained by bio testing, which was then tested using probit analysis to determine the effectiveness of the test material. The results showed that larval mortality increased with increasing extract concentration. The extract's effectiveness against the test larvae was demonstrated by the toxicity level of the extract ($LC_{50\text{-}48\ hours}$) being 5.01%. Larval growth also decreased with increasing extract concentration; GI (Growth index) values ranged from 0-1, and RGI (Relative growth index) values ranged from 81% to 100%.

Keywords: *Stephania japonica*, leaf extract, *Anopheles culicifacies*, mortality, growth

1. Introduction
Public health is a critical concern in our societies. An unrecognized and uncontrolled infectious outbreak can lead to out-of-control conditions and diseases at the endemic, epidemic, or pandemic levels. Among the rising public health challenges, diseases transmitted to humans by insect vectors, known colloquially as mosquitoes, stand out [1]. The term mosquito refers to about 3000 species that have been recognized globally [2]. Females of a handful of these species also require blood from various vertebrate animals, including humans, in their diet. As a result of this circumstance, some mosquito species that feed on human blood become vectors of disease-causing viruses. *Aedes aegypti*, *Anopheles gambiae*, *Culex pipiens*, *Psorophora ferox*, *Aedes scapularis*, to name a few DENV, YFV, WNV, and CHIKV are among the viral agents spread by mosquito bites and cause dengue, yellow fever [3] and chikungunya [4]. Global concern is the demand for levels of control to limit, prevent, and even eradicate these diseases. For example, it is estimated that roughly 390 million instances of dengue occur globally each year, with 96 million of these becoming clinical cases [5]. Malaria continues to be an important public health problem in numerous countries in the central and southern region of Asia, including India, where it is considered a highly prevalent endemic disease, whose distribution of cases is in Andhra Pradesh [6].
So far, *Anopheles* mosquito control still uses chemical insecticides that are not environmentally friendly and pose a risk to mosquito resistance to insecticides. One alternative that is more environmentally friendly is to use anti-mosquito plants. Research undertaken in the districts of Warangal, Khammam, and Mahabubnagar in Andhra Pradesh, as well as the Surat district in Gujarat, has provided evidence indicating the presence of resistance to malathion in populations of *Anopheles culicifacies* sensu lato (s.l.) [7].

The use of biological controls has grown significantly in importance worldwide, and they are frequently seen as the best alternatives to insecticides. The effectiveness of natural plant components as intoxicants, development inhibitors against larvae, and repellents for adult mosquitoes is also being researched [8]. In this sense, plants are of great interest because, during their evolution, they have developed a response mechanism to biotic and abiotic stress, producing compounds known as secondary metabolites, which have been used to control insects. Such is the case of pyrethrins, produced naturally by the flowers of the genus *Pyrethrum*: *Chrysanthemum cinerariifolium* and *Chrysanthemum coccineum* [9].

In this context, species of the genus Stephania [10] are effective at controlling both agriculturally significant insects. Due to their low cost, capacity for biodegradation, and role as a rationalizing component in the use of chemical insecticides, natural insecticides must be encouraged and increased in search, with a focus on species of the Menispermaceae family, to demonstrate their usefulness in public health and provide greater experiences aimed at combating insect vectors of human diseases. In light of these factors, the current study aimed to assess *in vitro Anopheles* sp. larval mortality using an aqueous extract of *Stephania japonica* L. leaves.

The problem is the mortality and growth of *Anopheles culicifacies* mosquito larvae after being treated with *S. japonica* leaf extract. At what extract concentration is it effectively influencing the mortality and growth of *Anopheles culicifacies* mosquito larvae? Therefore, it is necessary to research the mortality and growth of *Anopheles culicifacies* mosquito larvae due to the administration of *S. japonica* leaf extract and at what concentration of the extract is effective in increasing mortality and reducing larval growth.

The present work shows the toxic effect of aqueous extract from *Stephania japonica* L plants collected in the southern region of India on larvae of *Anopheles* spp. The purpose was to determine and compare the potential of aqueous extracts of *Stephania japonica* L. leaves as a source of compounds that may be useful to combat the population growth of mosquito vectors, which cause infectious diseases such as those described above.

2. Materials and Methods

2.1 Collection of plant material

Stephania japonica L. Leaves collected in bulk from agricultural areas in the rural vicinity of Vijayawada, located 5 km from the municipality of Vijayawada in Andhra Pradesh, India. The collecting site's geographical coordinates are located at 16º.515099' N, 80º.632095' E, as shown in Figure 1. The collected material underwent a drying procedure in a forced air circulation drying oven, which was kept at a temperature range of 40-45 °C for three to four days. The specimen was identified using established taxonomic techniques, which were duly catalogued in the Herbarium at Acharya Nagarjuna University in India. The specimen was assigned the unique serial number SJ 1962/17 for archival purposes.

2.2 Preparation of plant extracts

Large amounts of plant leaves were gathered, rinsed under a constant stream of water, forced air circulated in an oven for three hours at 60 degrees Celsius, and then ground to a granulometry of five millimetres (Figure 1). The samples were then put in plastic containers and kept in the refrigerator at a temperature of about 4 °C until the bioactive chemical extraction procedure started. According to Abubakar *et al.* (2020) [11], solid-liquid extraction methods were used to obtain the plant extracts. The solid-liquid extraction tests were carried out with water as the chosen solvent in an orbital shaker (Remi Mini Rotary Shaker -RS-12R, India). The extraction procedure was carried out in 500 ml Erlenmeyer flasks while adhering to the following operational parameters: 30ºC for the temperature, 150 rpm for the stirring speed, 18 hours for the extraction time, and 30 g of *S. japonica* leaves per litre of water. The resultant extract was centrifuged, and the solid particles were separated using Whatman No. 1 filter paper before vacuum filtering the supernatant. 350 ml of extract in total were obtained and then put in topaz-coloured glass jars to lessen the effects of photo-oxidation on the components (Figure 1).

Fig 1: The leaves and leaf powder of *Stephania japonica* L., along with its extract

2.3 Collection of Bioassays

The test animals used were *Anopheles culicifacies* mosquito larvae, instar I for the growth test and instar III for the mortality test. Larvae were obtained from the National Centre for Disease Control, Rajahmundry Branch, Andhra Pradesh, India.

2.4 Mortality test of mosquito larvae due to administration of *S. japonica* leaf extract

Testing of larval mortality and extract toxicity were tested using a bioassay in 3 stages. The first stage is a preliminary test to determine the upper threshold concentration range ($LC_{95\text{-}24\ hours}$) and lower threshold ($LC_{50\text{-}48\ hours}$). The second

stage is a toxicity test (LC50-48 hours) to determine the extract's effectiveness. The third stage is a mortality test to examine the mortality pattern of mosquito larvae due to administration of *S. japonica* leaf extract. All stages of the biotest use five levels of extract concentration and one control [12]. For each treatment concentration, 20 larvae were used and put into glasses containing 50 ml of solutions with various treatment concentrations. The calculation of concentration levels is based on the formulation of Hubert (1987) [13]. Calculation of toxicity values using Probit Analysis.

2.5 Test mosquito larvae growth due to administration of *S. japonica* leaf extract.

Testing of larval population growth was based on the method of Zhang *et al.* (1993) [14]. The extract concentrations tested were five sub-lethal concentrations below the $LC_{50\text{-}48\text{-hour}}$ value and control. Each glass contains 50 ml of test material for each treatment concentration and 20 first instar larvae. The test larval population growth parameters used are GI (Growth Index) and RGI (Relative Growth Index) [15]

$$GI = \frac{[n(I_{max}) \times I_{max}] + \sum[n'(i) \times i - l)}{N \times I_{max}} \ (Eq. 1)$$

GI = Growth Index

i = stadium number

n(I_{max}) = number of larvae that live at stage i max $n'(i)$ = number of larvae that die at stage

i I_{max}= highest stage reached by larvae N = total number of larvae in the test group

$$RGI = \frac{GI_{treatment}}{GI_{control}} \times 100\%$$

Test the secondary metabolic content of *S. japonica* leaf extract

3. Results

3.1 Mortality of mosquito larvae due to administration of *S. japonica* leaf extract

3.3.1 Preliminary Test: The results of preliminary tests (Table 1) show that the larval mortality value increases with increasing extract concentration. This *S. japonica* leaf extract is able to cause 45% mortality of *A. culicifacies* mosquito larvae at an extract concentration of 0.5% and 95% mortality of *A. culicifacies* mosquito larvae at a concentration of 8%. Thus, the lower threshold value is obtained at an extract concentration of 0.5%, and the upper threshold value is at an extract concentration of 8%.

Table 1: Mortality of *Anopheles culicifacies* larvae after treatment with various concentration levels of *S. japonica* leaf extract for 48 hours.

Concentration of the test extract	Number of test larvae	Total mortality of test larvae	Mortality percentage of test larvae (%)
0.00%	20	1	5
0.50%	20	9	45
1.00%	20	11	55
2.00%	20	11	55
4.00%	20	14	70
8.00%	20	19	95

The actual test results (Table 2 & Figure 2) show that *S. japonica* leaf extract significantly affects the mortality of *A. culicifacies* mosquito larvae. It was shown that the lowest test concentration of 0.87% significantly affected the mortality of *A. culicifacies* mosquito larvae. Based on the Mann-Whitney Test, the results showed that all concentrations of *S. japonica* leaf extract tested had a real effect on *A. culicifacies* mosquito larvae.

Table 2: Mortality of *Anopheles culicifacies* larvae after treatment with various concentration levels of *Stephania japonica* leaf extract for 72 hours

Test extract concentration (% v/v)	Number of test larvae	Total mortality of test larvae	Average
0.00%	50	0	0^{a}
0.91%	50	13	4.1^{b}
1.59%	50	13	4.1^{b}
2.76%	50	19	6.39^{bc}
4.81%	50	23	7.5^{c}
8.37%	50	31	10.27^{cd}

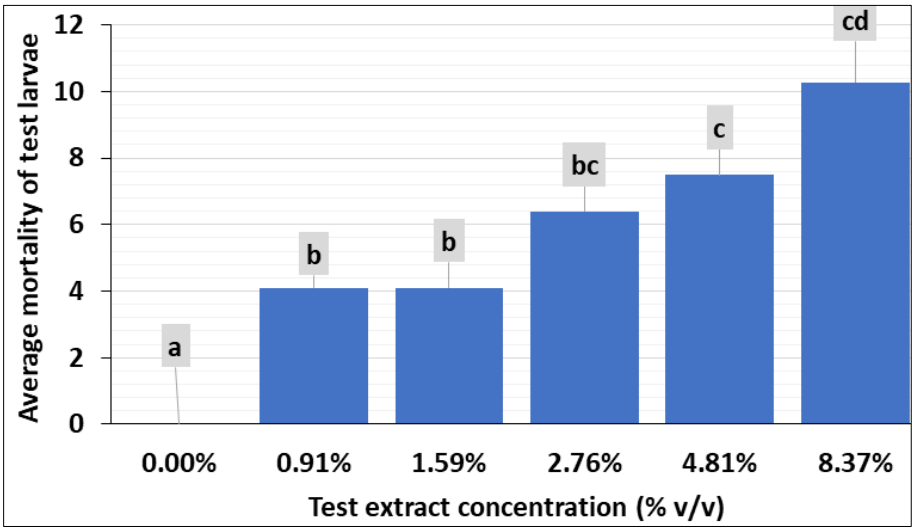

Fig 2: Average Mortality of *Anopheles culicifacies* larvae after treatment with leaf extract for 72 hours

The bioactive compounds contained in *S. japonica* leaf extract work simultaneously to kill *A. culicifacies* mosquito larvae, so it is not yet known exactly what type of compound has a specific effect on a kind of poison, whether as a stomach poison, contact poison or fumigant. In accordance with what Chaudhary *et al*. (2017) [16] stated, the use of crude extracts is one of the advantages of using botanical insecticides because bioactive compounds whose types and benefits are unknown or unknown can work together to increase the stability and potential of *S. japonica* leaf extract as a botanical insecticide.

The results of testing the effectiveness or toxicity of *S. japonica* leaf extract against *A. culicifacies* mosquito larvae (Table 3) are shown by the LC50-48-hour value, namely at a concentration of 5.01%, which means that at a concentration of 5.01% *S. japonica* leaf extract it is capable of causing larval death. *A. culicifacies* mosquitoes tested were 50%.

Table 3: Effectiveness (toxicity) of *S. japonica* leaf extract against *Anopheles culicifacies* larvae

No	Extract concentration (%)	Number of test animals	Larval Mortality Percentage (%)	
1	0.00%	50	0	LC value 50-72 hours 5. 01%
2	0.91%	50	13	
3	1.59%	50	13	
4	2.76%	50	19	
5	4.81%	50	23	
6	8.37%	50	31	

3.2 Growth test of *Anopheles culicifacies* mosquito larvae at sub-lethal concentrations of leaf extract

Table 4: GI and RGI values from growth test results of *Anopheles culicifacies* mosquito larvae when given *S. japonica* leaf extract

	L1		L2		L3		L4					
Cones Extract (%)	H	M	H	M	H	M	H	M	Number of live larvae	Number of dead larvae	GI	% RGI
0.16	20	0	20	0	15	5	15	0	15	5	0.88	97
0.31	20	0	19	1	12	7	12	0	12	8	0.79	88
0.93	20	0	16	4	11	5	11	0	11	9	0.73	81
1.29	20	0	16	4	11	5	11	0	11	9	0.73	81
1.53	20	0	17	3	11	6	11	0	11	9	0.73	81

Based on Table 4, the GI value and RGI value decrease as the concentration of the extract tested increases. A high extract concentration will cause a low GI value because the larvae die in the early instars, so they cannot move to the next instar stage. Meanwhile, at low extract concentrations, the GI value was high because the test larvae remained alive in the early instars, so they could moult and continue to the next instar stage.

3.3 Biocidal activity

To evaluate the lethality of the *S. japonica* extract on larvae of *Anopheles* spp., the extract presented a repellence greater than 50%. Mortality was observed when the pupae were exposed to *S. japonica* extract, as shown in Figure 3 a and b.

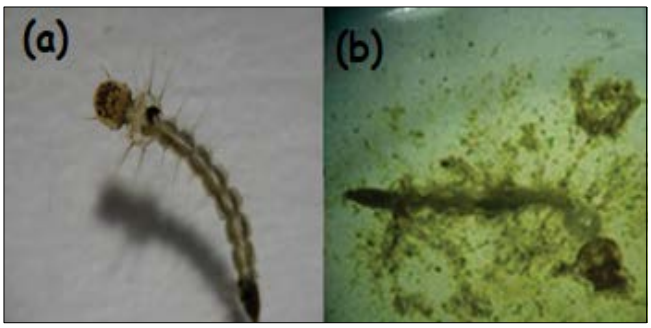

Fig 3: Observation of the biocidal effect of the leaf extracts: A). Larvae before treatment. B). after treatment.

4. Discussion

The investigated *S. japonica* leaf extract not only has stomach and contact toxic qualities but also has an indirect fumigant impact, likely due to the release of bioactive compounds that volatilize as gas. According to Stejskal *et al* (2021) [17], fumigants are a type of pesticide distinguished by their high volatility, allowing them to evaporate easily into gaseous form. Once in this form, they can enter the insect's body via the respiratory or tracheal systems before spreading throughout the organism. Pesticides that affect the respiratory system of insects, according to Ware (1994) [18], have the ability to disrupt respiratory enzymes by inhibiting both the electron transport system and oxidative phosphorylation. According to Gureev *et al* (2022) [19], an electron transport system blockage causes paralysis and, eventually, death. The action of bioactive compounds on the NPNH and NADH electron transport pathways causes this behaviour.

The death of mosquito larvae is caused by the bioactive compounds in *S. japonica* leaf extract, which act as toxicants. The bioactive compounds contained in *Stephania* genus leaf extract include alkaloids and terpenoids [20-21]. Toxic compounds that enter the insect's body can cause a decrease in the insect's growth rate. It is thought to result from the insect's failure to respond to food requirements because the food contains allelochemical compounds. The allelochemical compounds (secondary metabolites) contained in *S. japonica* leaf extract are terpenoids, and according to Ninkuu (2021) [22], terpenoid compounds have great potential as feeding inhibitors in several insects.

From an ecological point of view, biocides of natural origin can constitute an alternative to synthetic ones. This is why it is necessary to have a wide range of compounds with biocidal properties that contribute to the sustainability of current integrated pest control strategies. Consequently, it is necessary to carry out further *in vitro* studies using different parts of the plant and carry out phytochemical screening for the different species of plants and insects.

Given the difficulty of comparing the insecticidal potential of these extracts, it is necessary to standardize and finally optimize the extraction methods to obtain the highest possible lethality at the lowest concentration. Subsequent studies will require chemically identifying the active components of the extracts and their possible scaling up to an industrial level.

5. Conclusion

S. japonica leaf extract can increase mortality and reduce the growth of *A. culicifacies* larvae. The effective concentration (LC50-48 hours) of *S. japonica* leaf extract for mortality is 5.01% (v/v), and a concentration of 0.93% (v/v) is effective in reducing the growth of *A. culicifacies* mosquito larvae.

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7. Conflict of Interest

The authors declare no conflicts of interest with respect to this work.

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