Insecticidal and juvenilizing activity tests of *Euphorbia tirucalli* Latex on *Aedes aegypti* Larvae. Micropropagation and new potential for biological activity

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ABSTRACT

Euphorbia tirucalli is a succulent species and its latex is employed with several uses, amongst which vector control, as mosquitoes, at Africa and Asia. Due to latex's citotoxicity, by the presence of diterpenoids, in this study we analyzed insecticide and juvenilizing effects evoked by aqueous fraction from latex obtained from explants micropropagated with 6-Benzylaminopurin (6-BA) (1.0 mg/L) besides 6-Benzylaminopurin (6-BA) (1.0 mg/L) with Norbixin, both under white and red light fluences, during 20 days. Latex was extracted in water from stems submitted to ultrasonic method from all explants to be tested upon development of *Aedes aegypti* larvae. Neither insecticide nor juvenilizing activities were noticed to all aqueous fraction of raw latex and explants tested at 100 microlitres. These results are distinct of classic literature. After qualitative anatomic analysis of explants comparing to garden control, new micropropagations with same vegetal regulator and antioxidant will be performed, but with more during time of red light irradiance at cultive. Besides it, higher

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concentrations of those aqueous latex extracts will be performed, due to high prevalence of Dengue in Rio de Janeiro.

Keywords: Micropropagation, 6-Benzilaminopurina and light espectre, Juvenile activity, Insecticide activity.

INTRODUCTION

Euphorbia tirucalli (Aveloz) is a succulent species of cosmopolitan occurrence, whose latex has a toxic action (FURSTENBERGER & HECKER, 1986). Various scientific studies have confirmed its biological activity related to the diterpenoids present in latex in immunomodulation since the nineties (FAVERO et al., 1990).

Latex diluted in water, rich in diterpenoids and triterpenoids, is used in Popular Medicine and Northeastern ethnobotany (NEIVA, 1968) and in Traditional Medicine for the complementary treatment of several serious diseases around the world (VARRICCHIO et al., 2008).

Furthermore, there are records of insecticidal activity for E. tirucalli from India and the African continent (DAMODARAN et al., 2002). The World Health Organization (1970; 1980), in addition to larvicidal tests, has also recommended tests for steroid activity, with a juvenileizing effect, on mosquito larvae (which, without reaching the adult stage, will not be able to fly or become vectors disease transmitters).

The State of Rio de Janeiro has a high prevalence rate of Dengue, and Brazil unfortunately has a high prevalence and has been increasing the incidence with a high number of deaths. In addition to repeated efforts in health education and technological advances, every initiative to help control this serious disease corresponds to the conduct of interest in research in plant biotechnology (BENTES LOPES et al., 2024).

The tests carried out corresponded to the investigation into the existence of insecticidal and also hormonal activity for the raw latex of *E. tirucalli*, from the State of Rio de Janeiro, micropropagated in culture medium added with 6-Benzylaminopurine (BAP) and norbixin, under variations light spectrum.

MATERIALS AND METHODS

BOTANICAL CERTIFICATION

Euphorbia tirucalli exsicata was prepared from specimens of the plant grown in the garden of the Natural Products Research Institute of the Federal University of Rio de Janeiro (IPPN/UFRJ). The botanical identification was carried out by Dr. Luci de Senna Valle and the specimens of the test material were deposited in the herbarium of the National Museum of Rio de Janeiro under Botanical Registry R – 204.406. The anatomical study of the plant and explants was carried out by Natasha Conceição Gomes de Carvalho, under the supervision of Dr. Ricardo Cardoso Vieira from UFRJ.

The crude latex of *E. tirucalli* from the field and from micropropagated explants in culture medium according to Murashige & Skoog, 1962 (MS) supplemented with 1mg/L 6-BAP in addition to MS medium with 1mg/L 6-BAP associated with 1mg/L of norbixin, both cultivated under two different intensities of the light spectrum (white and red light), was obtained by ultrasonic extraction

for 40 minutes (sonication) in distilled water at a concentration of 10%. The samples were subjected to qualitative analysis regarding plant chemistry using High Performance Liquid Chromatography associated with maximum ultraviolet absorption spectrometry (HPLC/UV) (VARRICCHIO, 2005).

In vivo tests were carried out to verify the toxic effect and the effect of juvenileization on *Aedes aegypti* larvae against the aqueous fraction of latex from explants of micropropagated *E. tirucalli* and its field control, both at a concentration of 100µl, for the observation of death within 24 hours and absence of larvae development within seven days, according to the World Health Organization protocol (WHO, 1970; OLIVEIRA FILHO, 1979; WHO, 1980; OLIVEIRA FILHO, 1994).

To determine the larvicidal activity, the test was carried out on larvae of the third growth stage of the *Aedes aegypti* mosquito (IPPN colony – resistant to any class of insecticide and the Rockefeller colony, originating in Gainsville – Florida – USA – standard lineage, susceptible to any class of insecticide), maintained in the Biology Laboratory of the Natural Products Research Center at UFRJ (NPPN) under controlled conditions of temperature (T), relative humidity (RH) and photoperiod (F) (T = 27 ± 2 ° C, RH = 70 ± 10% and F = 12:12h) using all the extracts described above.

The tests were carried out in glass beakers containing 15 mL of filtered water, where 100 µL of the sample at the appropriate concentration were added. After 30 minutes of sample homogenization, 4.9 mL of filtered (dechlorinated) water and five larvae were added to each test solution. Water controls were maintained in all tests. Bideionized water was used as a control solution, and all prepared solutions were solubilized in distilled water. The concentrations of each sample were repeated three times, with two replicates in each test. The result was observed after 24 hours, checking the number of dead larvae to determine the percentage of larval mortality (LC50) (CARVALHO et al., 2003).

RESULTS

The aqueous fraction of *E. tirucalli* latex from the garden of the Natural Products Research Institute (IPPN) of the Federal University of Rio de Janeiro (UFRJ) and micropropagated, obtained by sonication of the stem for 40 minutes (VARRICCHIO, 2008), was tested for insecticidal activity and the effect of juvenileization against *Aedes aegypti* larvae.

There was no toxic effect within 24 hours and no effect of juvenileization on the in vivo system of larvae within one week. These results are different from those observed for the activity of *E. tirucalli* latex from India, in agreement with the literature, which exerted larvicidal activity for *Aedes aegypti* as well as for the larvae of *Culex quinquefasciatus* (YADAV et al., 2002), thus regularly used in pest control. The toxic effect is explained by the increased tolerance to sodium by

this plant species (DAMODARAN et al., 2002). It is known that this halophyte varies its plant chemical production, thus culminating in fluctuations in its biological activities, which may be undesirable for one type of research model, but interesting for other models that consider complexity (GASPAR et al., 2023).

However, it was found that the species in the garden of the Natural Products Research Institute at Campus Ilha do Fundão, Ilha do Governador (RJ, Brazil) did not tolerate the introduced variations, culminating in the death of most of the explants due to oxidation, during cultivation in vitro of *E. tirucalli* under different variations of salinity, temperature and humidity conditions (GASPAR et al., 2023).

Therefore, these results are different from those obtained by Oliveira Filho (1979; 1994) when studying terpenoids chemically identified with hormonal activity that induces juvenileization in larvae of *A. aegypti*, when, due to the delay in morphological development, female mosquitoes were unable to fly and move. become contaminating vectors.

To verify whether the lack of results was the effect of micropropagation bringing morphological variations, as already noted by MALI & PANCHAL (2017), the explants cultivated with growth regulator, antioxidant and also under two different intensities of the light spectrum, white and red light, were botanical qualitative assessment requested.

QUALITATIVE ANALYSIS OF SAMPLES SUBJECTED TO DIFFERENT LIGHT CONDITIONS AND PLANT GROWTH REGULATORS

Anatomy – *Euphorbia tirucalli* of the stem of the individual from the IPPN/UFRJ garden:

The epidermis is uniseriate, being formed by cells with straight to slightly sinuous anticlinal walls, covered by a thick cuticular layer, which is projected between the anticlinal walls of the epidermal cells.

The stomata are located in recesses, below the level of the epidermal cells. The substomatal chambers are well developed, being formed by slightly braciform cells. The cortical region is formed by the chlorophyll parenchyma made up of countless layers of rounded cells.

The presence of fibrosclereids dispersed in the cortex and pericyclic fibers isolated or in small clusters is observed (2-10). Laticifers with thick walls are found close to the vascular cylinder. The vascular cylinder is at an early stage of development, with the secondary phloem formed by sieve tubes with simple sieve plates, which may be solitary or in groups of up to ten cells. The sieve tubes have one to two companion cells. The secondary xylem is formed by predominantly solitary vessel elements, and there may occasionally be groups of two to five cells. The axial parenchyma is sparse paratracheal. The medullary region is formed by parenchymatic cells with a similar shape to cortical cells that differ, however, in the absence of chloroplasts.

Analysis of samples from crops:

Analysis of samples subjected to different light conditions and plant growth regulators reveals that some anatomical characters were variable when compared to the control. Among these characters, the following can be mentioned: amount of fibrosclereids and pericyclic fibers, development of the vascular cylinder (amount of phloem and xylem tissue and xylem lignification), amount of laticifers and callus formation.

1. *Sample of seedlings grown in MS + 6-BAP medium under exposure to white light for 20 days:*
   • Xylem is poorly developed and lignified;
   • Formation of callus in the cortical region and in the medullary region (medullary macula), where the cells are disorganized, with the presence of cells with lignified secondary walls.
2. *Sample of seedlings grown in MS + 6-BAP medium under exposure to red light for 20 days:*
   • In this sample, the beginning of the formation of a callus is observed, as the cells in this region are disorganized, with the presence of cells with lignified secondary walls;
   • Well-developed dairy products (larger and more abundant).
3. *Sample of seedlings grown in MS medium + Norbixin + 6-BAP under exposure to white light (20 days):*
   • Very developed and lignified xylem;
   • No changes to other characteristics.
4. *Sample of seedlings grown in MS medium + Norbixin + 6-BAP under exposure to red light for 20 days:*
   • Very lignified xylem.
   • Abundance of fibrosclereids in the cortex and pericyclic fibers;
   • Vascular cylinder well developed.

DISCUSSION

The present results with the absence of cytotoxic and juvenileizing activity may be related to the low relative concentration of the chemical substances detected through high-performance liquid chromatography in the explants of the matrix solutions (extracts obtained by latex sonication), being lower than necessary for obtaining such biological activities.

However, they may also suggest the absence or dramatic reduction in the concentration of terpenoids with steroidal hormonal activity in these extracts obtained through micropropagation with antioxidants such as Norbixin, as well as through different light intensities.

The lack of activity verified in these results is similar to that observed when testing pharmacotechnical preparations obtained from total aqueous extracts of non-sonicated aerial parts of *E. tirucalli* (VARRICCHIO et al., 2008).

Perhaps, the negative results presented here may be related to the chemical substances extracted by the ultrasonic method, which were extracted precisely due to their high polarity secondary to hydrophilicity. Terpenoids with steroidal activity may tend to be lipophilic, therefore not miscible in water (VARRICCHIO, 2008).

Also, due to the fact that the relative concentration of chemical substances present in the extracts obtained from control field plants are below the concentration necessary for insecticidal activity due to the effect of seasonal variation on the plant chemical production of the specimen located in the IPPN/UFRJ garden. This type of variation has already been verified and discussed by other researchers (DE SOUZA et al., 2023).

However, the present observations differ from the result described for field and micropropagated *Euphorbia pulcherrima*, which has diterpenoids that exert steroidal activity (BIESBOER et al., 1982). However, they may also suggest the absence of terpenoids with steroidal hormonal activity in these aqueous fractions of micropropagated explants, a common fact reported in the literature (VARRICCHIO, 2005).

Although the analysis of plant chemical production and plant development were not focused on in this article, the study of the anatomical variation of explants cultured in MS medium plus somatogenic mitosis-inducing growth regulator (6-BAP) and MS with 6-BAP associated with the anti-oxidant Norbixin, both cultivated under different fluences of the light spectrum (effects of red light compared to white light) suggested bringing relevant contributions to new experiments in plant biotechnology (PINTO, 2019).

The qualitative anatomical description of cultivation with 6-BAP associated with norbixin under white light suggested reducing plant stress and reducing callus formation, when compared to the description of cultivation with 6-BAP under the effect of white light, which tended to disorganize the tissue and to initiate callus formation with lignification of the xylem, while inducing somatic mitosis.

Of note, micropropagation in MS + 6-BAP culture medium under red light was suggested to be capable of inducing the formation of laticiferous ducts. This observation is similar to that verified by VARRICCHIO and collaborators (2022, a) in *in vitro* cultures under red light fluence in MS0 medium. There are various applications for using the non-latex-producing stem as forage. Also due to the richness of antioxidants in this stem (VARRICCHIO, 2008).

The samples from the culture with 6-BAP associated with Norbixin under red light showed a well-developed vascular cylinder, evidence of interest when there is interest in the biological activities of raw latex as an antimicrobial (DE SOUZA, 2021).

These qualitative anatomical notes were made from 3 samples of each culture, occurring for 20 days of cultivation, when the aqueous latex extracts were prepared using the ultrasonic method for larvicidal and juvenileizing assays.

Therefore, based on these preliminary results, new protocols will be developed with 6-BAP associated with Norbixin under red light fluence over 30 days (avoiding exceeding this time to prevent oxidation of the explants), once again it will be marked the plant chemical production of interest, already established in accordance with previous studies and, then, new biological tests will be carried out to investigate steroidal juvenileizing and insecticidal activity.

Biolarvicidal extracts, that is, extracts that exhibit hydrophilic affinity potential, are relevant for combating A. aegypti and other effective actions against vectors in these joint, multi and interdisciplinary efforts, for the promotion of environmental health (RIBEIRO, 2004; VARRICCHIO et al., 2022; BENTES LOPES et al., 2024).

Furthermore, regarding geographic variations, for latex from the Andean continent, its mechanism of action via mitochondrial uncoupling was verified (BETANCUR-GALVIS et al., 2002; 2003). While the best chemical yield from the extraction at 37ºC of E. tirucalli from the IPPN/UFRJ garden confirmed the black historical report, which brought this species considered exogenous on slave ships, when it was used as food (soup) (VARRICCHIO et al., 2008).

In a previous micropropagation test with blue light fluence, the presence of receptors for blue light had already been suggested, due to better stomatal conductance and increased mitochondrial respiration, resulting in an increase in the final net yield of the photosynthetic profile, with a gain in biomass and bioenergy. “petroleum plant” specimen (MCGARVEY & CROTEAU, 1995; VARRICCHIO, 2005; VARRICCHIO et al., 2008a). Furthermore, in the present observation of field specimens and explants obtained in experiments, the Kranz anatomy, described for *E. tirucalli* from the African continent by MWYNE & VAN DAMME (2011), was not verified.

RAHUMAN et al. (2008) reaffirmed the insecticidal activity of *E. tirucalli* from India on *Aedes aegypti*. While ethanolic extract of the stem of *E. tirucalli* L. from Jakarta (Indonesia) were effective as larvicides with LC50 171,480 ppm (0.0171%) and relatively safer to use than synthetic larvicides (Temephos) by the group of Yusuf et al. (2020).

Furthermore, the variations obtained under cultivation with red light for all media drew our attention to the potential of phytoremediation through phytoextraction, as it is a halophyte that reacted positively with the formation of structures that can collaborate in bioaccumulation (KEHRIG et al., 2011). Perhaps, this species will be able to participate in stages of biotransformation of water

and soil pollutants, the current focus of our studies (GASPAR et al., 2023; KATHAR et al., 2023) associating them with a biotechnological measure in environmental health, as recommended by professor Helena Ribeiro since 2004 (VARRICCHIO et al., 2022, a; GASPAR et al., 2023; BENTES LOPES et al., 2024).

Finally, Dengue (Dengue Hemorrhagic Fever) is an acute febrile infectious disease caused by a virus belonging to the Flaviviridae family, of the Flavivirus genus. The dengue virus has four serotypes, generally called DENV-1, DENV-2, DENV-3 and DENV-4. These are also classified as arboviruses, meaning they are normally transmitted by mosquitoes. The main method of transmission of this disease is through the bite of the female *Aedes aegypti* mosquito when it is infected with the virus. Once infected, the individual may experience fever, headache, prostration, nausea, muscle and/or joint pain and pain behind the eyes (AGÊNCIA FIOCRUZ DE NOTÍCIAS, 2013; MINISTÉRIO DA SAÚDE, 2024).

The ways to combat this disease revolve mainly around awareness campaigns so that the population is alert and eliminates possible breeding grounds for the transmitting mosquito, such as environments where water can accumulate, such as tires, plastic bottles and dishes. of potted plants. In recent years, however, due to advances in technology, different methods of prophylaxis and combating the dengue mosquito have been researched and developed (AGÊNCIA FIOCRUZ DE NOTÍCIAS, 2013; MINISTÉRIO DA SAÚDE, 2024).

Currently, research appears to be mainly focused on the use of drones both for mapping mosquito breeding sites and for their elimination, which, as it is done remotely, considerably reduces the risks of contagion to humans (AGÊNCIA FIOCRUZ DE NOTÍCIAS , 2013; MINISTRY OF HEALTH, 2024).

Civil and environmental engineer Ricardo Machado, for example, proposed a technology that consists of a modular release and packaging system, integrated with drones, which performs the controlled release of *Aedes Aegypti* mosquitoes in demarcated regions. The solution allows sterile male mosquitoes to be released to mate with wild females, who mate once in a lifetime. Consequently, no offspring will be produced and the insect population will tend to decrease over time until its eradication. “The release by drones of sterile mosquitoes produced using this technique is more efficient than that carried out using the traditional terrestrial method, in which sterile males, stored in drums, are released weekly in open cars”, says engineer Machado (CONFEA, 2024).

Drones release males homogeneously, unlike terrestrial means, which are restricted to public roads. Furthermore, they are low-cost, easy to maintain and make it possible to launch 17,000 insects in 10 minutes of flight, covering an area of 100,000 square meters. According to Ricardo Machado, this method should be able to reduce 90% of the *Aedes aegypti* population and new cases of the disease within three to four weeks. The project, however, is in an experimental phase. In this way, the

company seeks partnerships with sterile insect breeders, who will bear the costs of the service (CONFEA, 2024).

In turn, TechDengue, created by the company Aero Engenharia, has already been contracted by more than 50 municipalities in Minas Gerais. Its operating model consists of three stages, planning, execution and geoanalytics. During planning, an analysis of the areas is carried out in conjunction with city halls and municipal health departments, in order to understand their particularities and structure planning with specific actions according to the interests and needs of each municipality. During execution, drones fly over pre-determined areas in the planning phase, aiming to capture georeferenced images that will be processed, with the help of artificial intelligence, with the aim of finding possible mosquito breeding sites (TECHDENGUE, 2023).

Once the locations have been mapped, the drone disperses larvicide to eliminate vectors and only lands when it is completely empty. In this way, TechDengue allows the treatment of 26 *Aedes aegypti* breeding points in a single 30-minute flight. In the geoanalytical phase, then, data from all areas that received the service are captured, stored, processed and analyzed, generating 100% digital and interactive reports so that municipal bodies can monitor the evolution of actions, facilitating decision-making, since they are based on real data (TECHDENGUE, 2023).

TechDengue has already been carried out in municipalities such as Belo Horizonte, Brumadinho and Cotagem, in the state of Minas Gerais, as well as in the Municipality of Itajaí, Santa Catarina, and Anápolis, Goiás. In total, more than 16 thousand hectares were mapped, 44 thousand possible outbreaks of mosquito reproduction (POI's) identified, 6 thousand outbreaks treated and 128 terabytes of information generated. It is worth mentioning that this technology presents more than 90% assertiveness due to the laser targeting system, which allows reaching not only long distances but also difficult-to-access areas (TECHDENGUE, 2023; DIÁRIO DO COMÉRCIO, 2024).

Therefore, in a still incipient but promising way, technology is being used to combat Dengue, presenting significant solutions given the speed in discovering and eliminating mosquito outbreaks, with the use of drones and other technologies, such as artificial intelligence, reducing the incidence of the disease in the population and preserving the health of agents, who will no longer be exposed to risky environments (TECHDENGUE, 2023; DIÁRIO DO COMÉRCIO, 2024).

CONCLUSION

Unlike the latex of *Euphorbia tirucalli* from the African and Asian continent, the crude latex of *E. tirucalli* from the field, from the IPPN/UFRJ garden (Campus Ilha do Fundão, Ilha do Governador – Rio de Janeiro - Brazil) and the latexes from explants micropropagated in medium MS supplemented with 1mg/L 6-BAP and 1mg/L norbixin, under white and red light fluences, at the concentration tested, were neither larvicidal nor juvenileizing for the *Aedes aegypti* mosquito.

New preparations and higher concentrations of *E. tirucalli* will continue to be tested to verify the toxic and juvenileizing effect against models of *A. aegypti* larvae in an attempt to contribute to controlling the rate of Dengue transmission vectors (MS, ANVISA, 2004).

Based on a qualitative analysis of plant anatomy, red creep aroused our interest due to its effect on plant anatomy, thinking, based on this preliminary analysis, about future trials in phytoremediation, perhaps using plant biotechnology combined with new features brought by advanced technology.

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