Larvicidal and Oviposition Activity of Against Vector *Aedes Aegypti* and Molecular Docking Studies of Metabolites from the Crude Extract of the Endophytic Fungus *Aspergillus* Sp. Isolated from *Bertholletia* Excelsa Humn. & Bonpl.

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Research Article

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Abstract

This work showed the crude extract of the endophytic fungus *Aspergillus* sp, isolated from the almonds of *Bertholletia excelsa* Humn & Bonlp collected in the Brazilian Amazon, oviposition deterrent, and larvicidal activity of against *Aedes aegypti*. In the oviposition deterrence test was observed that females able to lay eggs preferred the control oviposition sites (46.6%), suggesting the extract also could repel the oviposition. Futhermore, the extract showed larvicidal activity with LC_{50} 26.86 µg/mL at 24 hours and 18.75 µg/mL at 48 hours. Molecular docking studies were carried out to elucidate the mechanism of action of the compounds identified against the enzyme acetylcholinesterase. The compound Aspergillol B was a potent larvicide with potential for inhibition for the acetylcholinesterase enzyme (-7.74 Kcal/mol). These unprecedented results reported indicate that the secondary metabolites obtained from crude extract of *Aspergillus* sp. present useful biological potential against vectors of public health importance and antibiotic-resistant bacteria.

1 Introduction

Aedes aegypti is the primary transmitter of human pathogens including Dengue, Chikungunya fever, and Zika virus; this species is closely associated with human habitation, and some epidemic patterns of the diseases transmitted by the species are caused by seasonal changes that affect its development, reproduction, and abundance (Kraemer et al., 2015; Pascini et al., 2012; Pliego-Pliego et al., 2020).

According to the Ministry of Health - Brazil (2020) (Ministério da Saúde, 2020), 390.684 probable cases of dengue were reported in Brazil until the 11th week of the year 2020, 11.453 cases of chikungunya, and 1.395 cases of zika virus, evidencing an increased number of these diseases in 2020.

Considering that there is no vaccine or medication available for the treatment of dengue or the other diseases transmitted by this vector, the main form of control available is controlling the vector population (Pliego-Pliego et al., 2020). The eradication of these mosquitoes is usually performed using organophosphate insecticides and pyrethroids; however, the continuous use of these compounds has selected resistant vector populations. Besides that, the low biodegradability of the current insecticides and larvicides can potentially lead to a long-term adverse effect on human health (Macari et al., 2002; Smith et al., 2016; Yang et al., 2020). Currently, there is a growing demand for new substances from natural sources, such as microorganisms (fungi and bacteria) and medicinal plants. These compounds are intensively explored in search of new bioactive metabolites (Mookherjee et al., 2020), including against the larvae of the *Ae. aegypti* (Araújo et al., 2020; Pradeep et al., 2015; Revathi et al., 2013).

The plant species *Bertholletia excelsa*, popularly known as Brazil-nut, is a species found throughout the Amazon region from countries such as Brazil, Bolivia, Peru, Colombia, Venezuela, Guyana, and it is considered a threatened species (Baldoni et al., 2020). *B. excelsa* is one of the most important species in the Amazon, and its wood and almonds are highly appreciated both in Brazil and abroad (Azevedo; and Luiz Marcelo Brum Rossi; Roberval Monteiro Bezerra de Lima, 2008).

Endophytic fungi are microorganisms capable of colonizing the interior of plant tissues without causing damage to them (Strobel, 2012); this interaction becomes favorable for the host, as it can boost their tolerance to abiotic stress (Patil et al., 2016). Fungi are found in almost all vascular plants, seaweed, mosses, and ferns studied so far, and frequently, hundred species of them can be isolated from a single plant (Chapla et al., 2013). In this sense, the study of the secondary metabolites produced by their vast biodiversity fungal is of notorious relevance for the discovery of new products for biotechnological, industrial, medicinal, and agrochemical applications (Patil et al., 2016; Rana et al., 2020).

Molecular docking is an intensive and prominent computational method in the drug discovery process. The virtual sorting followed by docking has become one of the most important methods to improve efficiency in the Search for new molecules. The benefit of docking is to identify how the ligands bind at the enzyme or receptor binding site through specific key interactions and to predict the binding affinity between protein-ligand complexes (Gupta et al., 2020).

According to (Gubiani et al., 2019), fungi belonging to the genus *Aspergillus* is well known and investigated for synthesizing secondary metabolites with diverse biological effects, considering that Amazon has the most exceptional biodiversity on the planet and because it has favorable conditions for proliferation and adaptation of new microorganism species – including temperature, humidity, and organic residues –, the search for new chemical compounds for pharmaceutical, cosmetic, and food industry in this biome has considerable potential. Hence, this work aimed to obtain and evaluate the larvicidal activity and the effects on oviposition of the crude extract from the endophytic fungus *Aspergillus sp*. isolated from the chestnut of *B. excelsa* against *Ae. Aegypti* and mechanism of action the secondary metabolites with molecular docking studies.

2 Material And Methods

2.1 Isolation and identification of the endophytic fungus *Aspergillus* sp (BIORG 9)

The almonds of *Bertholletia excelsa* were collected in the area located 1 - W 52 ° 18'20.976 " and S 0 ° 33'44.44 " and 2 - W 51 ° 57'53.338 " and S 0 ° 25'21.39 ". The endophytic fungus used in this study was isolated from the almond and stored according to the protocol described by (Holanda et al., 2019).

The initial identifications were based on direct observation through an optical microscope (OLYMPUS ® BX41), these observations had morphological aspects as a criterion (K. et al., 1972; Visagie et al., 2014). The endophytic fungus (Biorg 9 - *Aspergillus* sp.) Used in this work was identified by conventional and molecular methods at the Pluridisciplinary Chemical, Biological and Agricultural Research Center (CPQBA, https: //cbmai.cpqba. Unicamp.br) at Universidade Estadual de Campinas (UNICAMP), SP, Brazil.

2.2 Cultivation in solid and liquid medium of the endophytic fungus *Aspergillus* sp.

The strain of the endophytic fungus was grown in a solid culture medium BDA (potato-dextrose-agar), then the material was sterilized in an autoclave (Phoenix, AV50) at 121º C for 20 minutes then the sterile medium was poured into plates Petri. After 24 hours, the petri dishes containing agar extract (2%) were inoculated with spores of the fungus *Aspergillus* sp. using an inoculation needle through a central insertion point, the strain was then incubated at 28ºC (DBO, LACADEMA, model LUCA-161/03) as described by (Holanda et al., 2019) during the 7-day period.

After the activation period, the fungus was placed to grow in 200 ml of malt medium (2%) at pH 7.0 in 500 ml Erlenmeyer flasks ..-. Inoculations were performed with four circular discs (0.5 cm in diameter) from a solid culture. Each Erlenmeyer flask was incubated on an orbital shaker for 10 days (28ºC ± 2ºC, 160 rpm). The growth of the mycelial mass in liquid medium was performed in quintuplicate.

2.3 Obtaining the secondary metabolite from the mycelial mass of the fungus *Aspergillus* sp.

After the period of growth of the mycelial mass in the period of 10 days, 60 mL of ethyl acetate was added to each Erlenmeyer, kept in vigorous magnetic stirring for 30 minutes. This process is carried out so that cell lysis occurs, then the mixture was filtered. The aqueous phase was extracted with 99.5% ethyl acetate (3 x 60 mL), the organic phase being filtered and the excess solvent being removed under reduced pressure on a rotary evaporator.

2.4 Identification of metabolites produced by the endophytic fungus *Aspergillus* sp. by LC-MS / MS

The metabolites of *Aspergillus* sp. were quantified by High Performance Liquid Chromatography with mass spectrometer (LC-MS). The extract was solubilized in 1 ml of ethanol / water 1: 1 and eluted in a C18 column (500mg).

The conditions of the equipment were controlled and analyzed by LC-DAD-MS using an LC-DAD-ESI system consisting of a shimadzu HPLC 20A equipped with an LC-20AD quaternary pump, an SPD-M20A photodiode array detector, a thermostated SIL20A autosampler and a CTO-20ª column compartment, coupled to a Brucker Ion Trap, with a heated ESI source. Mass spectra were acquired in negative and positive modes in the range of 100–1000 m / z, in separate series. The operational standards were: source source 4.5 kV coating gas 9.00 L / min dry gas, 40 psi nebulizer and dry temperature 300º C. Data analysis was performed using Data Analysis software. The elution gradient was carried out with water / 0.1% formic acid and acetonitrile / 0.1% formic acid.

2.5 Larvicidal bioassay

The larvae of *Ae. aegypti*, the used Rokeffeller colony, are from the Arthropod Laboratory of the Federal University of Amapá (ARTHROLAB), all larvae used were 3 stage. They were kept in standard weather conditions with a temperature of 25 ± 2°C and relative humidity of 75 ± 5% and photoperiod of 12 hours according to the World Health Organization (WORLD HEALTH ORGANIZATION, 2005).

The extracts were prepared in different concentrations (45, 35, 20, 10 and 5 µg/mL) solubilized in DMSO (0.5%). In each bioassay was used 10 larvae in controlled conditions (25 ± 2°C). The distilled water and DMSO (0.5%) were used with negative controls. All assays were performed in quintuplicate (de Araújo et al., 2018).

The content of mortality count was carried out in periods of 24 and 48 hours after exposure to the larvae solutions. They were considered dead larvae were not able to reach the water surface (Rodrigues et al., 2014) and using the readings was possible to establish the lethal concentrations (LC_{50} and LC_{90}) by probit analysis. All bioassay experiments were conducted according to standard (World Health Organization, 2005).

2.6 Oviposition-deterrence test

The oviposition-deterrence effect of the acetyl extract of the endophytic fungus *Aspergillus* sp. in the laying of eggs. *Ae. aegypti* has been studied in 50 pregnant females (fed with blood) in 40 x 40 x 40 cm opposition cages under conditions of 25 ± 2 ° C and relative humidity of 75 ± 5% and a 12-hour photoperiod according to WHO. The cages contained dark ovitraps with extract at concentrations of 45, 35 and 20 µg / mL and control with distilled water and DMSO (0.5%). The test was done in quadruplicate and the number of eggs was rated with 48 hours.

2.7 Statistical analysis

For larvicidal activity the lethal concentrations (LC_{50} and LC_{90}) were determined after 24 and 48 h of incubation and calculated using Probit analysis with StatGraphics Centurion. To evaluate the oviposition-deterrence test, an analysis of variance (ANOVA) was performed with Turkey to determine if there is a significant difference between concentrations and control with GraphPad Prism 8.0.

2.8 Molecular docking simulations

In this stage, the molecules were optimized by computational method DFT B3LYP 6-31G** (Braga and Valle, 2007; Lo et al., 2017; Siegel et al., 2017; Warren et al., 2016) and used as an input file for molecular docking simulations, in order to evaluate the score of the energy function through the free-energy (ΔG) of molecule interaction from virtual ligand-based screening as well as the analysis of conformations, mode of interaction and binding affinity with the selected receptors.

2.9 Selection of enzymes and inhibitors

Crystallographic structure of recombination Human acetylcholinesterase (AChE) complexed with -(-) galantamine (GNT), elucidated by X-ray diffraction was downloaded from the Protein Data Bank (PBD) with code 4EY6 and a solution of 2,4 Å (Cheung et al., 2012). The crystallographic structure of *Drosophila melanogaster* acetylcholinesterase (AChE) in complex with the tacrine derivative, 9- (3-iodobenzylamino) -1,2,3,4-tetrahydroacridine (I40) was downloaded from the Protein Data Bank (PDB), with PDB ID 1QON and resolution of 2.7 Å (Harel et al., 2000). The crystallographic structure of juvenile hormone complexed with methyl – (2E, 6E) -9 - [(2R) -3,3-dimethyloxiran-2-yl] -3,7-dimethylnona-2,6-dienoate, (JH3), was

downloaded with the code PDB 5V13, with a resolution of 1.87 Å (Kim et al., 2017). The crystallographic structure of juvenile hormone complexed with methyl – (2E, 6E) -9 - [(2R) -3,3-dimethyloxiran-2-yl] -3,7-dimethylnona-2,6-dienoate, (JH3), was downloaded with the code PDB 5V13 with a resolution of 1.87 Å (Kim et al., 2017).

2.10 Virtual high-throughput screening (vHTS)

Heteroatoms, co-crystallized ligand and water molecules were removed using the Discovery Studio 5.0 software. The validation of molecular docking protocols was performed by overlapping the elucidated crystallographic structure (experimental) with the generated model (theoretical), that is, comparison between the crystallographic ligand and the best conformation obtained with molecular docking based on the value of the Root Mean Square Deviation (RMSD).

The X, Y and Z coordinates of the receptors were determined according to the average region of the active site. The coordinates used for the center of the grid can be seen in Table 1.

2.11 Biological Activity Predictions of the molecules

The Prediction of Activity Spectra for Substances (PASS) were performed while using the webserver http://www.pharmaexpert.ru/passonline. PASS makes it possible to relate effects of the molecule based entirely on the molecular formula using Multilevel Neighbors of Atoms (MNA) descriptors, suggesting that biological activity is in function of its chemical structure (Ferreira et al., 2017; Kirchmair et al., 2015). Only molecules with insecticidal and anticholinesterase activities were selected at this stage.

3 Results And Discussion

3.1 Endophyte species identification

The ITS fragments and the beta-tubulin gene were successfully amplified and sequenced from the samples' genomic DNA. The fungus sample Biorg 09 (Fig. 1) named CPQBA 1929/19 DRM 03 was sequenced and identified as *Aspergillus* sp. as the partial sequence of the beta-tubulin gene had 100% similarity with the corresponding sequences from the *Aspergillus* genus deposited in GenBank and CBS-KNAW databases. The genetic distance analysis retrieved the CPQBS 1929/19 DMR 03 sample in a low resolution cluster (68%) with the type strain of the species *A. violaceofucus* CBS 123 and *A. japonicus* CBS 11451, the latter being a synonym for the first, thus, the results of the analyzes carried out in the databases and phylogeny suggest the final identification as *Aspergillus* sp., within the nigri section (Fig. 2).

The *Aspergillus* genus is found in all areas throughout the world and has a high economic and social impact. In this genus are found species used in biotechnology for the production of metabolites with

useful applications, such as antibiotics, organic acids, medicines or enzymes, and food fermentation agents (Samson et al., 2014).

The nigri section of the *Aspergillus* genus, commonly known as black *Aspergillus*, was formerly identified and classified based on morphology, according to Yokoyama et al. (Yokoyama et al., 2001). However, this section is one of the most challenging groups for identification, and several taxonomic schemes have already been proposed. Currently, molecular methods are used for this purpose (Varga et al., 2011).

Ferranti et al. (Ferranti et al., 2018), studying the fungal diversity on the surface of *Vitis labrusca* and hybrid grapes, reported 13.9% of the *Aspergillus* genus, and species of the nigri section were found in most samples. The molecular characterization of *Aspergillus* in grapes showed a genetic divergence between strains of this genus and reported an ochratoxigenic strain, often isolated from grapes, called *A. tubingensis* (Martínez-Culebras et al., 2009). It is observed that the nigri section presents a high diversity, as was also observed in this study.

3.2 LC-MS/MS analysis

The LC-MS/MS is used for the obtention of spectra from the products' ions to assess the chemical structure of its compounds. It can analyze neutral losses and simultaneous product ions from a single dataset (Renaud et al., 2017). Employing this technique, we identified several metabolites produced by *Aspergillus sp.* isolated from *Bertholletia excelsa*, as shown in Table 2.

More than 330 species of fungi are known in the *Aspergillus* genus, found in different ecosystems; this genus has a high impact on the health of humans, animals, and plants. The species of this genus produce a remarkable number of secondary metabolites with the most varied biological activities reported (Soltani, 2016).

The crude acetyl extract of *Aspergillus* sp. (Biorg 09) isolated from *B. excelsa* presented several molecules, some of them already reported to have biological activities with potential applications in the treatment of infectious diseases, diabetes, Alzheimer, physiological disorder and are a source of antioxidant agents (Mikawlrawng, 2016). Some of the metabolites found, like enniatins, are characteristic of *Fusarium* species (Jonsson et al., 2016).

Cyclodepsipeptides (enniatin) were described more than 60 years ago, and also have broad biological activities like antitumor, anthelmintic, insecticide, antibiotic, antifungal, immunosuppressant, anti-inflammatory and antimalarial (Sivanathan and Scherkenbeck, 2014). It is important to note that this class of metabolite is identified mainly in fungi of the genus *Fusarium* (Renaud et al., 2017). Some of their biological activities are attributed to the capacity to increase the flow of alkali metal ions through biological membranes (Ivanova et al., 2006).

Aspernolide, an aromatic butenolide compound, is a metabolite found in some endophytic species, such as in the marine endophytic fungus *A. terreus* (Parvatkar et al., 2009). Ibrahim et al., 2015 evaluated the antimicrobial activity of butyrolactones isolated from this endophytic fungus against *S. aureus*, and the

and anti-leishmanial activity against the promastigote phase of *Leishmania donavi*. Another relevant substance identified in *Aspergillius sp.* was aspulvinone, already reported to be an inhibitor of the viral neuraminidase H1N1 (Gao et al., 2013), and a potent antioxidant agent (Zhang et al., 2015).

The compound paclitaxel, known as taxol, is a well-known and effective drug used in the chemotherapy of cancers in the head, neck, breast, lung, bladder, ovary, and cervix. It is a substance produced originally by plants from the genus *Taxus*. However, the symbiosis between plant and fungi made some endophytic fungi also produce this molecule (Kumar et al., 2019).

According to Zhu (Zhu and Chen, 2019), the extraction of paclitaxel from endophytic fungi has proven to be an effective alternative way to get the drug. Among endophytic fungi, several species, including *Aspergillus fumigatus*, *Pestalotiopsis microspora*, *Alternaria brassicicola*, are reported to produce this compound (Gill and Vasundhara, 2019; Kumar et al., 2019; Subban et al., 2019; Zhu and Chen, 2019), and our results indicate that *Aspergillus sp*. isolated from Brazil-nut tree also can.

3.3 Larvicidal effect of the extract and oviposition deterrence test against *Aedes aegypti*

In the larvicidal test against *Ae. aegypti*, it was possible to observe that the extract had effective larvicidal activity in 24 and 48 hours. With a mortality of 84% at 45 µg/mL and 8% at 5 µ/mL in 24 hours; 96% at 45 µ/mL and 26% at 5 µg/mL in 48 hours. Thus demonstrating the extract presents a set of compounds with potential use for the control of this vector of public health importance.

We further evaluated the values of lethal concentrations (LC) 50% and 90% for 24 and 48 hours (Table 4). For 24 h, the LC_{50} was 26.86 µg/mL, and the CL_{90} was 47.55 µg/mL; for 48 h, the LC_{50} was 18.75 µg/mL, and the LC_{90} was 38.93 µg/mL. These values show a remarkable efficiency of the extract that is time- and dose-dependent.

As stated by Geris et al., 2008, plants' secondary metabolites have a range of biological activities due to their structural diversity. These molecules are a continuous source of inspiration for drug discovery. Nevertheless, fungi also produce secondary metabolites with the most varied activities reported, such as antimalarial (Isaka et al., 2007), antibacterial (Hu et al., 2008), antiviral (Linnakoski et al., 2018), antifungal (Hoffman et al., 2008), and leishmanicidal (Cota et al., 2018).

De acordo com Balumahendhiran et al. (2019) also tested the larvicidal activity of the crude extracts from fungi of the *Aspergillus* genus (*A. flavus* and *A. fumigatus*). The extract was tested against three disease-transmitting mosquito species: *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti*. For the extract from *A. flavus*, the authors reported the following (against *Ae. aegypti*): for 24 hours, the LC_{50} was 18.01 µg/mL, and the LC_{90} was 31.05 µg/mL; for 48 hours, the LC_{50} was 50.39 µg/mL, and the LC_{90} was 82.26 µg/mL. For the extract from *A. fumigatus*, in turn, the authors reported: for 24 hours, the LC_{50} was 14.79 µg/mL, and the LC_{90} was 35.40 µg/mL; for 48 hours, the LC_{50} was 35.37 µg/mL, and the LC_{90} was

73.30 µg/mL. Overall, the results of these authors corroborate our's and emphasize the potential of using fungi metabolites for the control of public health importance vectors.

An ethyl acetate extract from the mycelium of the entomopathogenic fungus *Beauveria bassiana* was tested against the four larval stages (LI, L2, L3, and L4) of *Ae. aegypti* by Ragavendran et al. (Ragavendran et al., 2017). The authors observed the larvae mortality within 24 hours of exposure to different concentrations (50, 100, 150, 200, 250, and 300 µg/mL) and reported LC_{50} values of 62.50 (L1), 52.89 (L2), 58.60 (L3), and 47.12 (L4) µg/mL; and LC_{90} values of 314.82 (L1), 236.18 (L2), 247.53 (L3), and 278.52 (L4) µg/mL. While the authors reported for L3 an LC_{50} of 58.60 and an LC_{90} of 247.53 µg/mL, our results of LC_{50} and LC_{90} were 26.86 and 47.55 µg/mL, respectively, suggesting that the extract from *Aspergillus sp.* isolated from *B. excelsa* had a higher potential against *Ae. aegypti* larvae.

According to Montenegro et al. (Montenegro et al., 2006), extracts show promising activity when the mortality is equal or higher than 75% at 250 µg/mL. For Cheng et al. (Cheng et al., 2003), potential substances need LC_{50} values lower than 100 µg/mL to be considered a good larvicidal agent. Here, 84% of mortality was observed within 24 hours at 45 µg/mL. The discovery of new compounds to fight *Ae. aegypti* is attractive since, in the last 15 years, populations of this mosquito developed resistance to the main organophosphate and pyrethroid insecticides in several countries (Rocha et al., 2015). Another disadvantage of the current insecticides is the toxicity caused to the environment.

The insecticidal activity of fungal products is not always attractive for commercial use. For instance, Pradeep et al. (Pradeep et al., 2015) evaluated the larvicidal activity of the isoquinoline-type pigment produced by the fungus *Fusarium moniliforme* against *Ae. aegypti* and *An. stephensi*. They reported LC_{50} of 237.0 µg/mL and 335.6 µg/mL in 24 hours, respectively; these are considered high values for vector control.

In addition to using metabolites produced by endophytic fungi, another approach is using a suspension of conidia, or an association of conidia and neem oil activity against *Ae. aegypti* larvae (Gomes et al., 2015). This demonstrates that using fungi conidia and metabolites, either isolated or associated, can be a valuable tool for vector control and other applications.

In our study, it was also possible to observe external structure changes in the larvae caused by the crude extract of *Aspergillus sp.* (Fig. 3). It is noted a loss of color and loss of segmentation in the treated larva (Fig. 3b), unlike in the control group (Fig. 2a), where is observed segmented abdomen structures (AB) and normal coloration under the optical microscope. We suggest that the extract of *Aspergillus sp*. could interact with the larval chitin cuticle, causing changes that resulted in their death. It is essential to highlight that these changes were not observed in the control group, treated with water and DMSO.

According to (Ureña et al., 2019), several mechanisms may be involved in the mortality of *Ae. aegypti* larvae by insecticides. Some substances act directly on the central nervous system; others can lead to cell damage and, consequently, the death of the larvae because the digestive system tries to eliminate the

compound, leading to extrusion of the peritrophic matrix (Valotto et al., 2011). Red seaweed extract was also reported to affect the larvae's external structure. When compared to the control, the larvae had progressive deformities in their lateral hair, anal papillae, distorted body, loss of color, and changes in the respiratory siphon (Deepak et al., 2019).

These external larval structure changes have also been reported as one of the mechanisms of larvae death in other studies, along with a darker or pale body, cuticle changes, and other larvae aberrations (Araújo et al., 2020; Kuo et al., 2015). The histopathological effect of spores from the fungus *Aspergillus clavatus* against *Culex quinquefasciatus* was also assessed (Bawin et al., 2016). The authors reported that the spores adhered to the external cuticle and the larval digestive tract; this accumulation caused gradual destruction of the digestive epithelium, muscular and connective tissues, and the epidermis right below the cuticle, which together may be accountable to larval death.

In the oviposition deterrence test, it was observed that pregnant females of *Ae. aegypti* preferred laying eggs (46.6%) in the control oviposition site with water and DMSO (0.5%). There was no significant difference among the different concentrations of the extract (ANOVA, $p > 0.05$). However, there was a statistical divergence between the extract doses 20, 35, 45 µ/mL compared to the control.

According to Soonwera and Phasomkusolsil (Soonwera and Phasomkusolsil, 2017), the application of oviposition repellent is an effective strategy for controlling mosquito populations. These authors tested the effect of the essential oil from *Zanthoxynlum limonella* Alston (Rutaceae) against the oviposition of *Ae. aegypti* and *Cx. quinqufasciatus*. They reported that a solution made of 10% of this oil dissolved in ethanol 70% had 100% repellency against *Ae. aegypti* and 99.53% against *Cx. quinquefasciatus*. In the environments receiving insecticides, these agents act as modulators of insect growth (Tilak et al., 2005), by preventing oviposition and consequent population boost, which is especially relevant against insects of medical importance, like *Ae. aegypti*.

In a recent study by Michaelakis et al., 2020, the authors tested an aqueous suspension of spinosad – a natural bioinsecticide toxic to pest and vectors – against *Cx. Pipiens molestus* larvae. However, there was no significant difference between spinosad compared to the control, different from our study, where the crude extract, besides being effective against the larval stage, also could repel the oviposition of pregnant females in 48 hours.

Other natural products also have shown promising results in the oviposition deterrence test against *Ae. aegypti*, such as the extract of *Melanochyla fasciculiflora* (Zuharah et al., 2015), the essential oil, and aqueous extract of *Alpinia purpurata* (Santos et al., 2012). Besides being repellent against the oviposition, these products also were effective against the larval stage. Such compounds can potentially be used in the water for vector control by preventing pregnant females from depositing eggs, or by killing the larvae.

3.4 Molecular docking simulations and biological activity

Retrieving the shapes of GNT inhibitors, I40 and HJIII, it was possible to validate the molecular docking protocols, calculating the RMSD values of 0.36, 0.78 and 0.94 Å, respectively. According to (Gowthaman

et al., 2008; Ramos et al., 2019), the binding mode predicted using docking indicates that when the RMSD is less than 2.0 Å in relation to the crystallographic pose of a respective ligand, the validation is considered satisfactory. The best results of the validation can be seen in Fig. 3.

The residues of active sites for GNT (PDB 4EY6) are around the *α*-helix between the amino acid residues 336–338 and in the *β*-leaf between the amino acid residues 85–87, 121–124 and 202–203. For the ligand it was possible to notice common hydrogen bonds with the residues Tyr124, Glu202 and Ser203. A hydrophobic interaction with residues Trp86, Gly121 and Tyr337 is also highlighted (Cheung et al., 2012).

Generated docking pose made it possible for the ligand to also interact with the amino acid residues of the I40 active site (PDB ID 1QON) around the *α*-helix between the Thr369-Asp375 amino acid residues and included in the *β*-leaf between the amino acid residues Ile82-Trp83. In the ligand, it is possible to observe hydrophobic interactions with the vast majority of residues in Tyr71, Trp83, Tyr370, Phe371, Tyr374 and His380, results that are in agreement with the studies by (Harel et al., 2000).

The interaction sites with the JHIII (PDB 5V13) are around the *α*-helix between the amino acid residues 30–38, 45–51, 60–71, 123–130, 132–136, 138–143 and 280–286 for *β*-leaf between amino acid residues 52–55, 72–73, 144–145 and 276–279. For the ligand, it is possible to observe hydrophobic interactions with all amino acid residues (Kim et al., 2017).

In order to assess whether the interactions showed greater binding affinity than the specific control ligands (I40, GNT and JHIII) for acetylcholinesterase from different organisms (*Drosophila melanogaster* and *Homo sapiens*) and juvenile mosquito hormone (*Ae. aegypti*) it was observed that of molecules submitted to docking, only Aspergillol B, Aspernolide A and Aspulvinone D presented values greater than or equal to the negative controls used. The binding affinity values of the new inhibitors at the acetylcholinesterase receptor can be seen in Fig. 4.

Inhibitors complexed with Aspergillol B (-8.2 Kcal /mol) and Aspulvinone C (-8.5 Kcal /mol) at the acetylcholinesterase receptor showed values close to the pyriproxyfen control, whereas Aspulvinone D had a higher value due to the binding affinity of -9.3 Kcal/mol. Therefore, it is suggested that the molecule is promising for insecticidal activity, since it presents interactions similar to the controls used in the docking study. Thus, comparing the affinity values of Aspulvinone D molecule to GNT control, a difference of ± 0.7 Kcal/mol is observed, and to the others a variation of ± 1.8 to ± 1.5 (Kcal/mol).

When the study was related to the organism of *Drosophila melanogaster*, the Aspernolide A molecule showed a binding affinity of -9.9 Kcal/mol, followed by Aspergillol B with − 9.5 Kcal/mol compared to controls I40 and pyriproxyfen (PDB ID 1QON), according to Fig. 5B.

I40 exhibited a binding affinity of -13.3 Kcal/mol higher than pyriproxyfen of -9.0 Kcal/mol. The Aspergillol B and Aspernolide A molecules showed a higher binding affinity value than the pyriproxyfen (control) used in molecular docking. Thus, when comparing the Aspernolide A compound with the I40

control, a difference of ± 3.4 Kcal/mol was observed, while a variation of ± 3.8 Kcal/mol was observed in Aspergillol B, as shown in Fig. 5B.

In relation to the 5V13 complex (*Ae. aegypti*), the Aspergillol B and (+)-*N*-deoxymilitarinone showed a higher binding affinity value compared to JHIII (control) used with a value of -9.4 and – 9.7 Kcal/mol, respectively. The results of the affinity values can seen in Fig. 5.

JHIII showed a binding affinity of -8.9 Kcal/mol lower than the pyriproxyfen of -10.6 Kcal/mol. However, a binding affinity variation of ± 1.2 kcal/mol of Aspergillol B and ± 0.9 Kcal/mol of (+) - *N*-deoxymilitarinone is observed compared to pyriproxyfen (control).

Based on the data found, we propose that the molecules present in the extract of the fungus *Aspergillus* sp. are able to bind to the active sites of the proposed targets. The Aspulvinone D molecule has a greater affinity for the human acetylcholinesterase active site, while Aspernolide A has a greater affinity for the active site of the acetylcholinesterase binding protein in e (+) - *N*-deoxyilitarinone for the youth hormone. Aspergillol B was the common molecule for both cases, considering the mode of binding in all study targets, thus the promising dual effect is observed, both for the inhibition of the enzyme acetylcholinesterase (human and insect) as well as the juvenile hormone.

Molecules with insecticidal potential can irreversibly inhibit the production of acetylcholinesterase, such an enzyme is responsible for the hydrolysis of acetylcholine (Ach) which ends the propagation of the nervous impulse. The inhibition of the enzyme acetylcholinesterase is the initial mechanism for a substance considered to have a potential insecticide in the larval phase, considering this knowledge cited by several authors it is essential to observe interactions at the active site of the acetylcholinesterase complex, in which three important characteristics are present in order to understand the mechanism of elucidation of the biological action of enzyme production.

In the Aspulvinone C and Aspulvinone D molecules used in the docking study, similar interactions were detected in the control molecules in relation to the acetylcholinesterase active site, located around the α-helix between the amino acid residues Ser203, Tyr337 and Phe338 and in the β-leaf between the Trp86, Gly121 and His447 residues, as shown in Fig. 6.

Figure 6- Interactions of GNT (**A**), Pyriproxyfen (**B**) and potential inhibitors Aspulvinone C (**C**) and Aspulvinone D (**D**) with active site of the acetylcholinesterase receptor. *

*Dashed black lines indicate hydrogen bonds, saline bridges and interactions with metal. Solid green lines show hydrophobic interactions and the dashed green lines show π-π and π-cations interactions.

According to (Meriç, 2017) at the AChE active site, the catalytic triad (Ser203, Glu334 and His447) is found in the lower portion of the active site, surrounded by three important parameters for catalytic activity: the acyl sac (residues of Phe295, Phe297 and Phe338), the oxy-anion channel (nitrogen from the main chain of residues Gly121, Gly122 and Ala204) and the choline binding site (Trp86 eTyr337). The most significant contributions of the interactions in the docking study were observed in the Aspulvinone C

molecule, in which the contribution of the catalytic triad represented by His447 and the connection with the Trp86 hill is notable. Other not so common interactions were also observed with the Tyr72 and Tyr341 residues, these interactions end up stabilizing the ligand in the active center of receptor. The increase in binding affinity, in turn, ends up inactivating the enzyme acetylcholinesterase by competition with an active site with GNT.

The interactions observed in controls I40 and pyriproxyfen in the docking study were also similar in the Aspergillol B and Aspernolide A molecules in relation to the acetylcholinesterase site, located around the α-helix between the amino acid residues Tyr370-Tyr374 and the β-leaf in the residue Trp83, as shown in Fig. 7.

Interactions of potential inhibitors with the amino acid residues Trp71, Trp83, Tyr370, Phe371 and His480 of acetylcholinesterase are similar to those reported in the literature (Fournier et al., 1993; Gnagey et al., 1987). The best evaluated inhibitors in binding affinity parameters were Aspergillol B and Aspernolide A, considering that the interactions were similar to those observed in controls I40 and pyriproxyfen for Tyr71, Trp83, Tyr370 and Tyr374 residues, contributing to the increase in binding affinity. The less common interactions between the inhibitors were Glu80, Gly150 and Phe371, and these electrostatic contributions help stabilize the protein's active site.

Aspergillol B molecule with a free energy of -7.74 Kcal/mol has a hydrogen bond with the amino acid residue Tyr370 and an π-π interaction with Trp83, the latter being also similar at I40. Aspernolide A molecule with the greatest potential for insecticidal activity has greater free energy than the pyriproxyfen control (-9.35 Kcal/mol) and with *π-π* interaction with a similar Tyr370, however, the interactions are more intense in the inhibitor favored by the reduction the distance. The conformation of inhibitors at the active site is influenced by the distances of interactions with amino acid residues.

In the juvenile hormone receptor complexed with JHII, the Aspergillol B and (+) - N-deoxymilitarinone molecules had interactions similar to the control molecules present in the amino acid residues located around the α-helix between the amino acid residues Tyr33, Leu37, Val51, Val68 and Tyr129 and β-leaf between Trp53 and Phe144 residues, as shown in Fig. 8

The Aspergillol B molecule with a free energy of -8.57 Kcal/mol has hydrophobic interactions with the Tyr33, Val51, Val65 and Ser69 residues and π-π interactions with Trp33 and Trp53. The molecule with the greatest potential to inhibit the enzyme acetylcholinesterase is (+) -*N*-deoxymilitarinone, as it has significant similar interactions and free energy of -11.79 Kcal/mol compared to the controls used. There are less common interactions for the Tyr129 and Tyr155 residues which contribute to greater stability of the molecule at the receptor.

In the complex of the juvenile hormone protein, the ligand JHIII is present in the binding pouch of the *N*-terminal domain, the conformation of the crystal being identical to the three chains of the protein. In JHIII, the presence of an epoxy group located in the center of the domain is observed and a methyl ester is oriented towards the surface. The epoxy group forms hydrogen bonds with the phenolic hydroxyl of

Tyr129 and the rest of the isoprenoid chain is surrounded by hydrophobic side chains including those of Phe144, Tyr64, Trp53, Val65, Val68, Leu72, Leu74, Val51 and Tyr33 [48].

Predicting the biological activity of molecules submitted via the web PASS server (Harel et al., 2000) resulted in the data shown in Table 3. The reference molecules (pyriproxyfen, I40, GNT and JHIII) showed insecticidal or similar activity, corroborating the results of the literature (Braga and Valle, 2007; Harburguer et al., 2009; Kim et al., 2017; Olmstead and LeBlanc, 2003; Paul et al., 2006; Sullivan and Goh, 2008).

In the molecules analyzed in the biological activity prediction study, only Aspergillol B showed a satisfactory prediction compared to the controls used in the molecular docking study, in which Pa was 0.187 and similar to other bioactive molecules known for insecticidal activity, acetylcholinesterase and acetylcholine inhibitor when Pa > Pi.

4 Conclusion

Results of this study suggest that metabolic from crude extract of endophytic fungus *Aspergillus* sp. isolated from *Bertholletia excelsa* was effective against drug-resistant bacteria strains of *S. aureus*, demonstrating that the crude extract of *Aspergillus* sp. can also be used as an antimicrobial agent. Moreover, the metabolites present in the extract are potential natural larvicides to control the vector *Ae. aegypti*, the findings of the molecular docking study demonstrated the mechanism of action of the compound Aspergillol B by inhibiting the enzyme acetylinesterase (-7.74 Kcal / mol). Therefore, this compound can serve as a new class of product with environmentally friendly larvicidal activity.

Declarations

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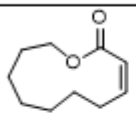
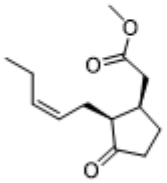
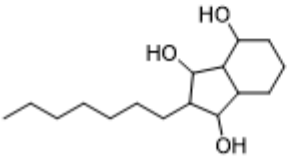
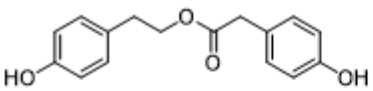
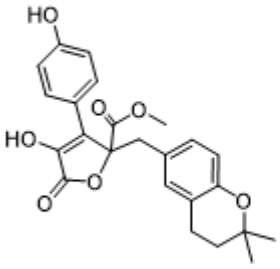
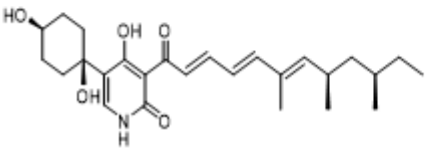
Tables

Tabela 1- Protocols data used for molecular docking validation.

Enzyme	Inhibitor	Grid center	Grid size (points)
AChE (PDB code: 4EY6)	(-)-Galanthamine	X = -10.733 Y = -43.626 Z = 29.535	60 x 32 y 24 z
AChE (PDB code: 1QON)	9-(3-Iodobenzylamino)-1,2,3,4-Tetrahydroacridine	X = 33.218 Y = 67.677 Z = 9.118	60 x 32 y 26 z
Juvenile hormone (PDB code: 5V13)	methyl (2E,6E)-9-[(2R)-3,3-dimethyloxiran-2-yl]-3, 7-dimethylnona-2,6-dienoate	X= 214.732 Y= 2.286 Z= 353.677	60 x 36 y 24 z

Table 2. Compounds identified by LC-MS / MS from the crude extract of *Aspergillus* sp. isolated from *Bertholletia excelsa*.

Number	Compound	Empirical formula	Estrutura química	Expected neutral mass
01	2-deceno-*d*-lactone	$C_{10}H_{16}O_2$		168.115029755
02	Methyl jasponic	$C_{13}H_{20}O_3$		224.1412445049
03	2-heptyloctahydro-1*H*-indene-1,3,4-triol	$C_{16}H_{30}O_3$		270.2194948239
04	Aspergillol B	$C_{16}H_{16}O_4$		272.1048589996
05	Aspernolide A	$C_{24}H_{24}O_7$		424.1522031217
06	(+)-*N*-deoxymilitarinone A	$C_{26}H_{37}NO_5$		443.2671732992

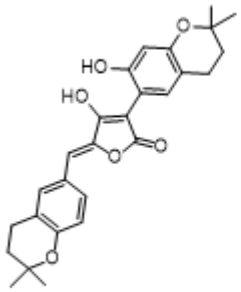
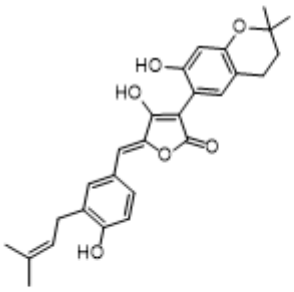
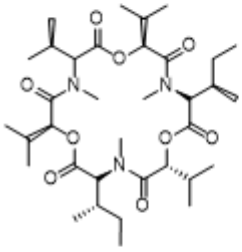
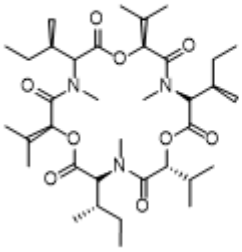
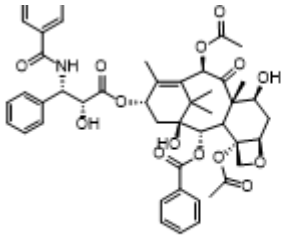
07	Aspulvinone C	$C_{27}H_{28}O_6$		448.188588627
08	Aspulvinone D	$C_{27}H_{28}O_6$		448.188588627
09	Enniatin A1	$C_{35}H_{61}N_3O_9$		667.4407805688
10	Enniatin A	$C_{36}H_{63}N_3O_9$		681.4564306326
11	Paclitaxel	$C_{47}H_{51}NO_{14}$		853.3309553465

Table 3. Larvicidal activity (LC_{50} and LC_{90}) for *Aedes aegypti* in 24 and 48 hours.

Aspergillus sp.	*Aedes aegypti*	
	24 h	48 h
LC_{50} (µg/mL)	26.86	18.75
C.I (µg/mL)	19.98 ± 35.28	11.12 ± 25.88
LC_{90} (µg/mL)	47.55	38.55
C.I (µg/mL)	38.20 ± 70.27	30.55 ± 59.64

*. C.I. = confidence interval

Table 4- Biological activity prediction of the molecules selected by virtual screening approaches.

Inhibitors	Pa[1]	Pi[2]	Biological activity
Pyriproxyfen	0.586	0.003	Insecticide
GNT	0.376	0.154	Acetylcholine neuromuscular blocking agente
I40	0.025	0.005	Acetylcholine transporter Inhibitor
JHIII	0.336	0.011	Insecticide
Aspulvinone D	–	–	–
Aspulvinone C	–	–	–
Aspergillol B	0,187	0,042	Insecticide
Aspernolide A	–	–	–
(+)-*N*-deoxymilitarinone	–	–	–

1-probability to be active; **2**-probability to be inactive.

Figures

Figure 1

Fungus Aspergillus sp. isolated from Bertholletia excelsa Humn. & Bonpl.

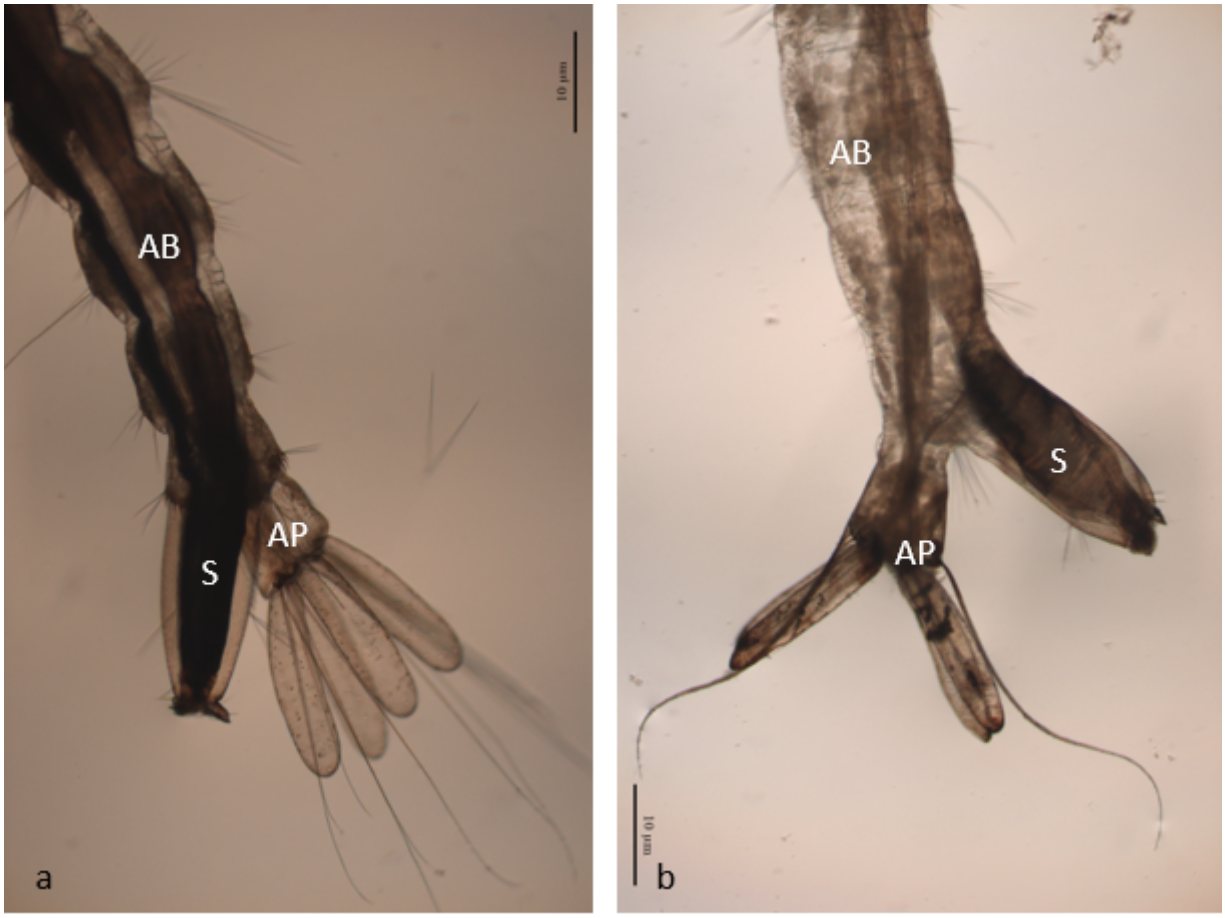


Figure 2

Control Aedes aegypti larva (a) and treated with crude extract of Aspergillus sp (b) presenting the following abdomen (AB), anal papilla (AP) and respiratory siphon (S) structures.

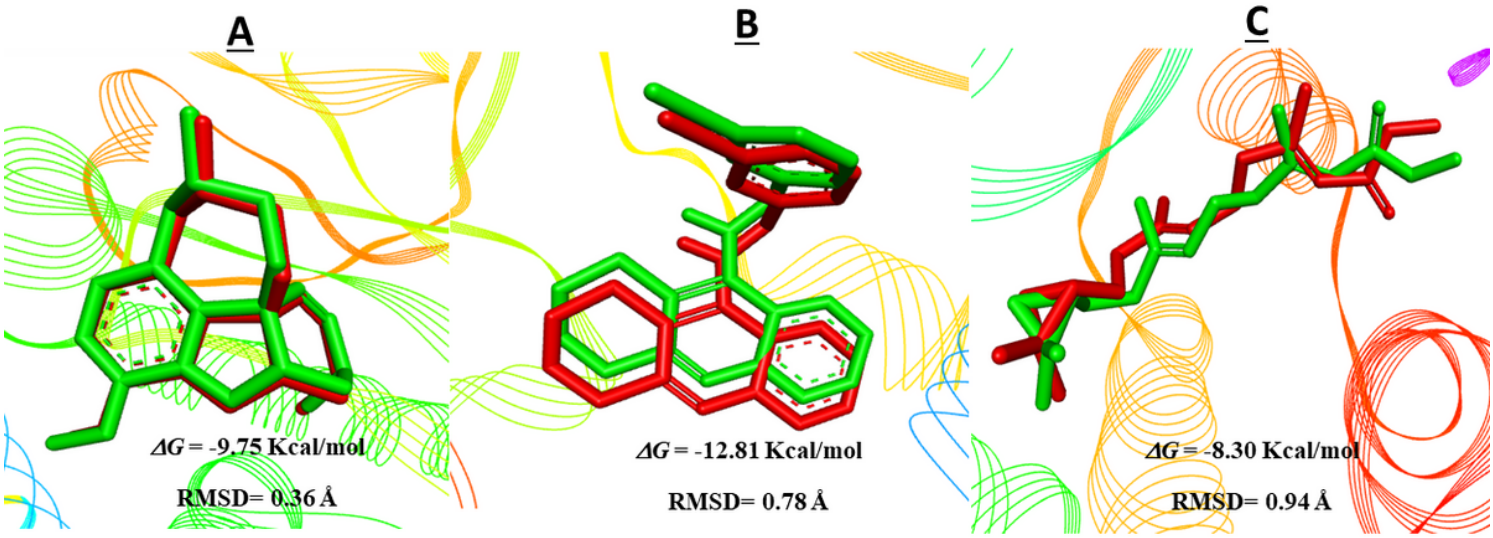


Figure 3

Shapes overlay of crystallographic ligands (in green) with the calculated shape (in red): (A) GNT and (B) I40 and (C) JHIII.

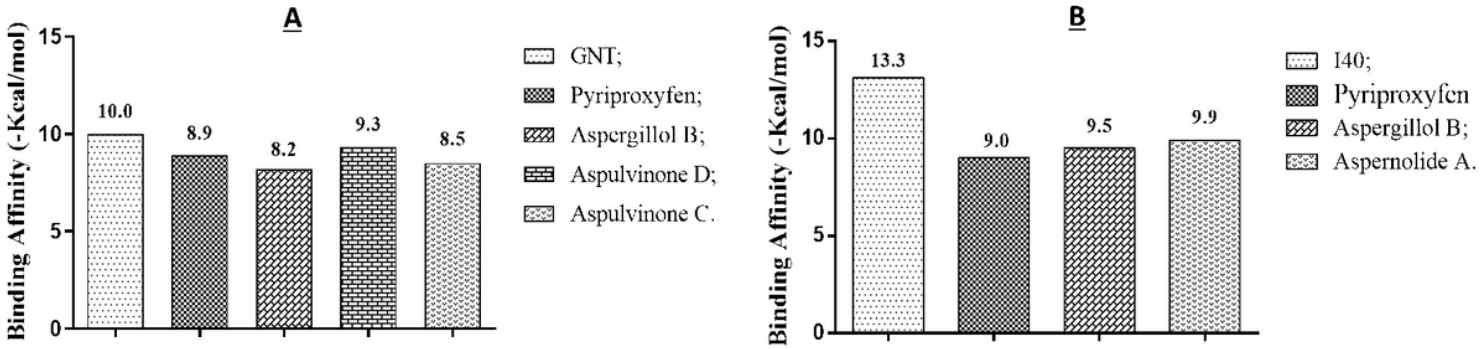


Figure 4

Results of binding affinity of the compounds with (A) human acetylcholinesterase; (B) insect acetylcholinesterase.

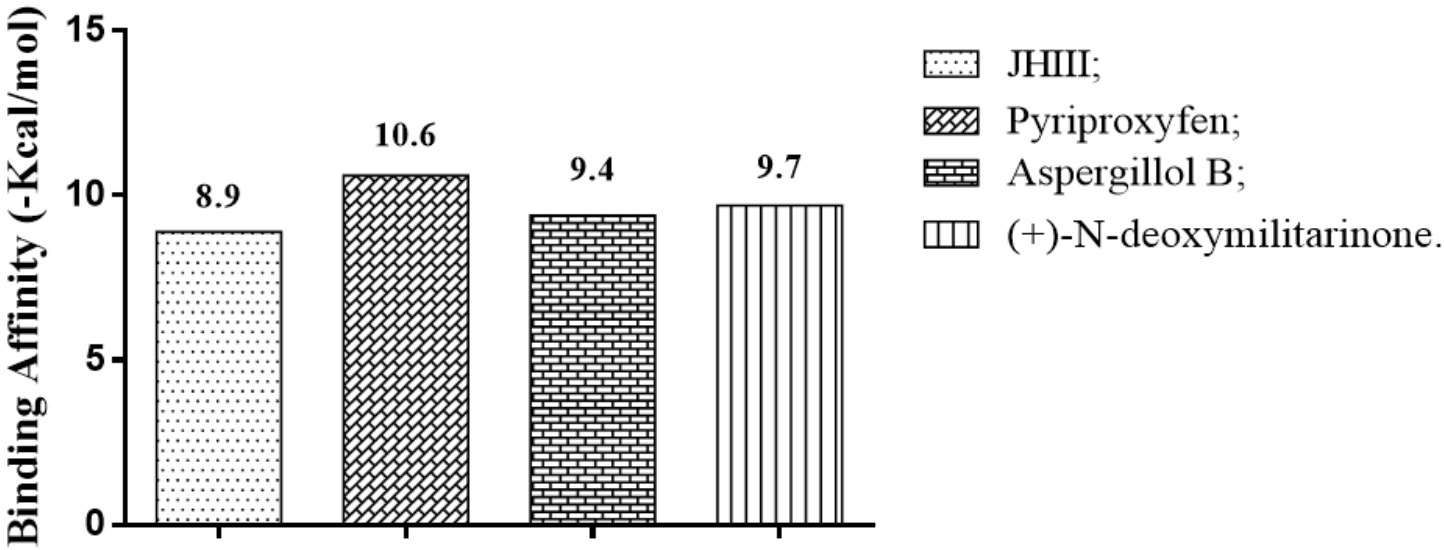
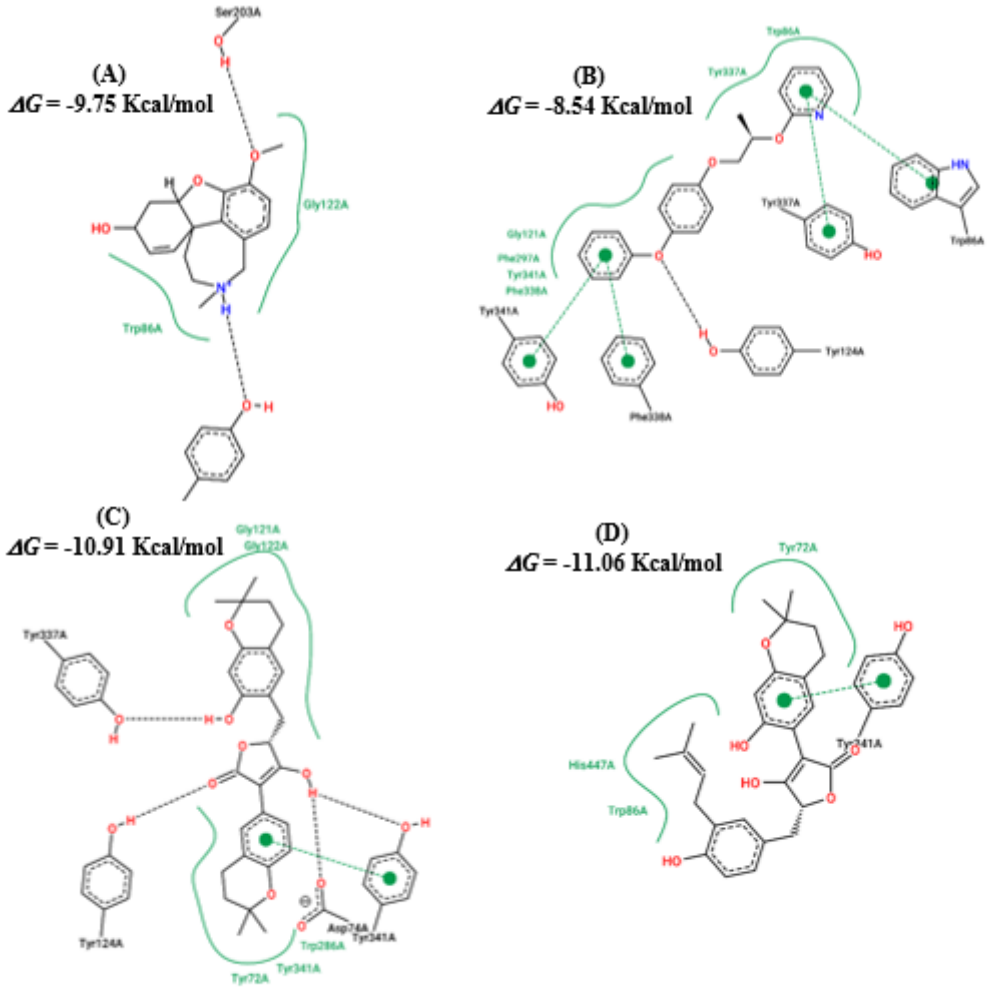


Figure 5

Results of binding affinity of the compounds with the juvenile hormone receptor.



*Dashed black lines indicate hydrogen bonds, saline bridges and interactions with metal. Solid green lines show hydrophobic interactions and the dashed green lines show π-π and π-cations interactions.

Figure 6

Interactions of GNT (A), Pyriproxyfen (B) and potential inhibitors Aspulvinone C (C) and Aspulvinone D (D) with active site of the acetylcholinesterase receptor. *

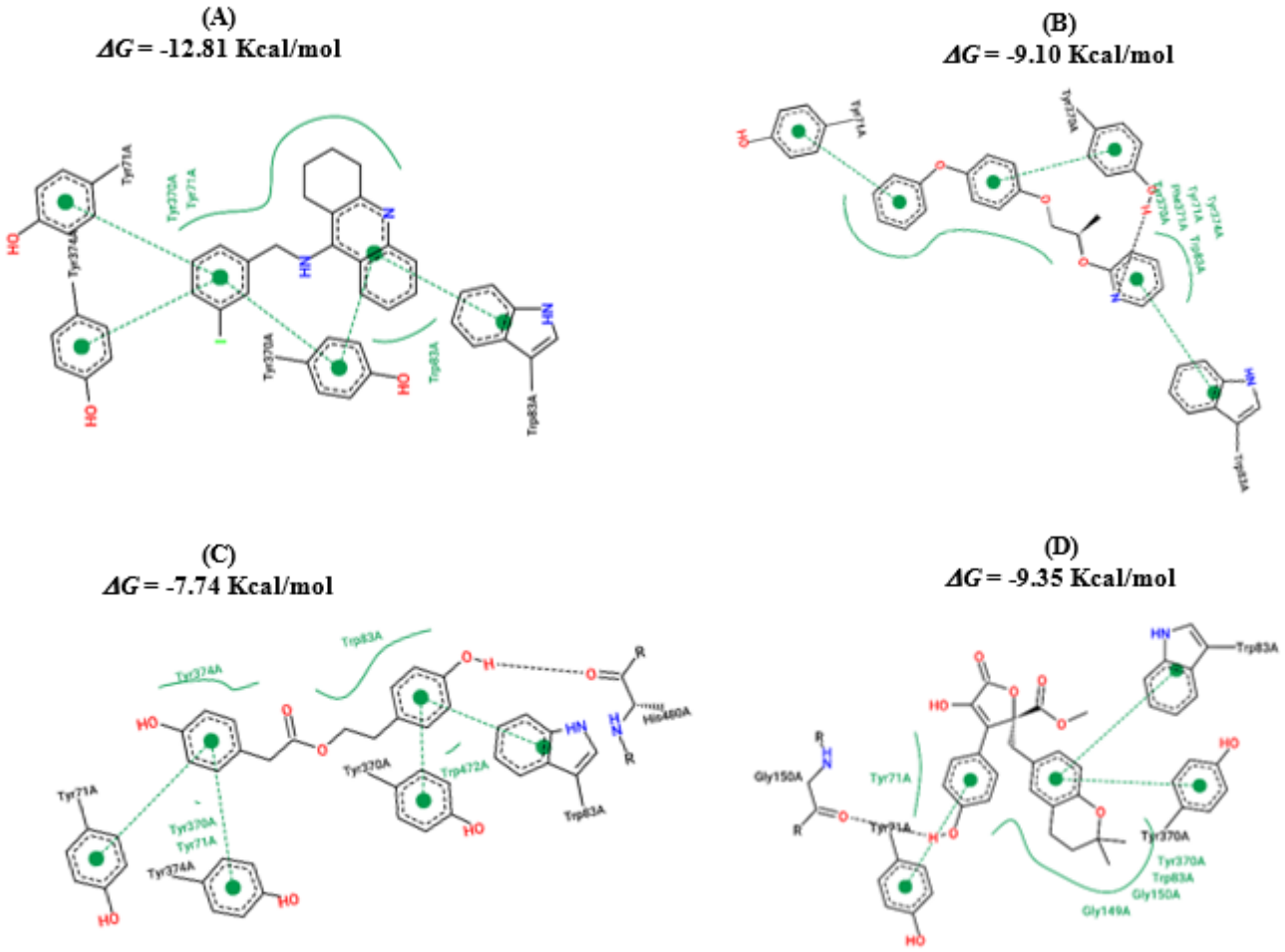


Figure 7

Interactions of controls I40 (A), Pyriproxyfen (B) and potential inhibitors Aspergillus B (C) and Aspernolide A (D) with insect acetylcholinesterase active site.

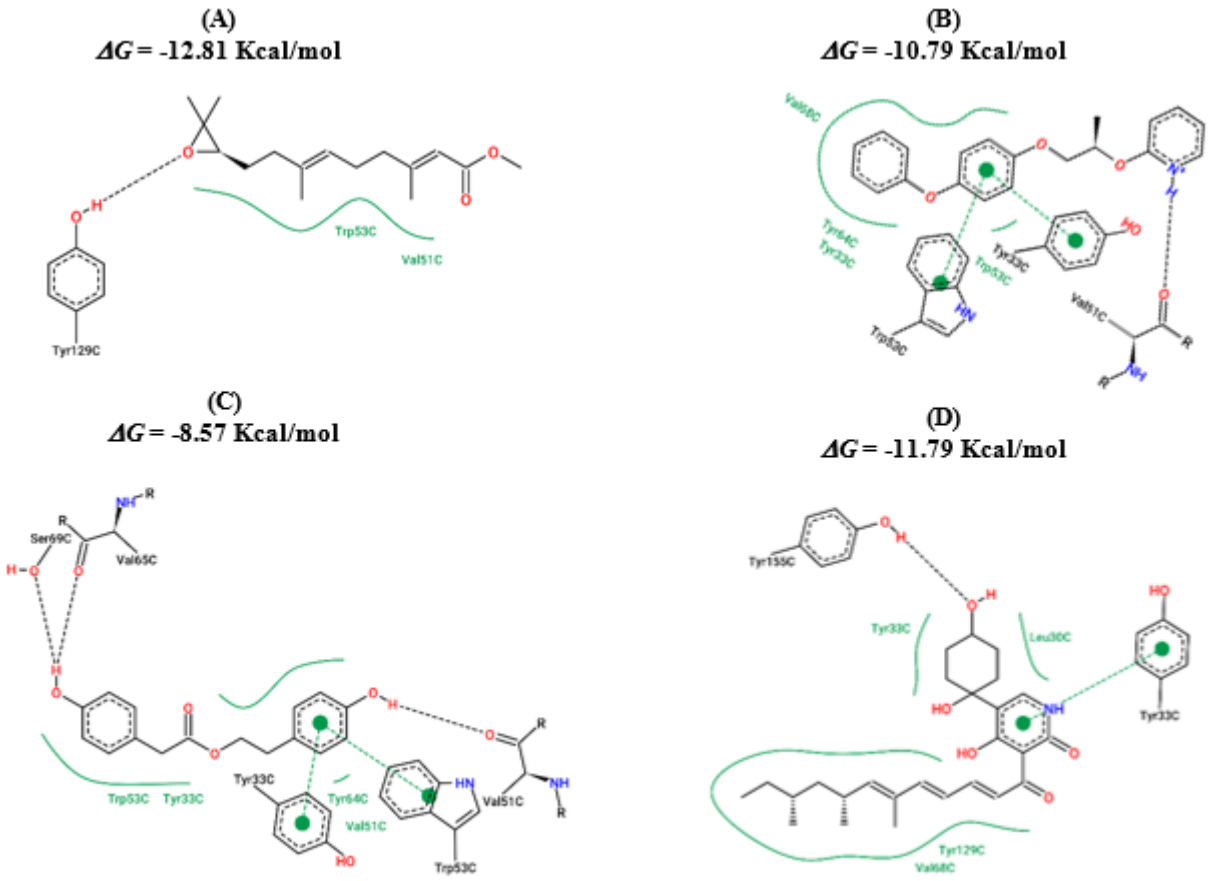


Figure 8

Interactions of the controls JHIII (A), Pyriproxyfen (B) and potential inhibitors Aspergillol B (C) and (+) -N-deoxymilitarinone (D) with active site of juvenile hormone receptor.

Supplementary Files

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- GA.png