UNIVERSIDADE ESTADUAL DE SANTA CRUZ - UESC
PROGRAMA DE PÓS-GRADUAÇÃO EM PRODUÇÃO VEGETAL - PPGPV

ARTUR GUSTAVO RIBEIRO DE SOUSA

PHYTOTOXICITY AND CYTOTOXICITY OF LEAVES EXTRACTS OF *Acanthosyris paulo-alvinii* (Santalaceae) TO DIFFERENT PLANT SPECIES

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Linha de Pesquisa: Cultivos em Ambiente Tropical Úmido.
Orientador: Alex-Alan Furtado de Almeida

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Ilhéus, 28 de fevereiro de 2019.

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DEDICATÓRIA

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SUMMARY

RESUMO

FITOTOXICIDADE E CITOTOXICIDADE DE EXTRATOS FOLIARES DE *Acanthosyris paulo-alvinii* (Santalaceae) EM DIFERENTES ESPÉCIES DE PLANTAS

A espécie arbórea *Acanthosyris paulo-alvinii* (mata-cacau), endêmica do sul da Bahia, Brasil, conhecida como hemiparasitaria utilizando o cacaueiro como hospedeiro apresenta propriedades aleloquímicos de natureza desconhecida, que inibe o desenvolvimento de plantas jovens da espécie *Theobroma cacao*, com efeitos provavelmente alelopáticos chegando mesmo a causara sua morte. O presente trabalho objetivou investigar o potencial de fitotoxicidade e citotoxicidade do extrato aquoso de *A. paulo-alvinii* nas plantas modelos *L. sativa* e *A. cepa*, e na capacidade inibitória do crescimento de raízes primárias de *T. cacao* do genótipo CCN51. O extrato seco foi elaborado a partir de folhas de árvores adultas *A. paulo-alvinii* coletadas no arboreto da CEPLAC. O material vegetal foi colocado em incubadora a 60 ° C por 72 horas e depois triturado para posterior elaboração do extrato aquoso, que foi utilizado em variados fracionamentos moleculares e aplicados nos ensaios com *T. cacao* e *Allium cepa*. Para *Lactuca sativa* o extrato foi elaborado a partir de folhas liofilizadas de *A. Paulo-alvinii* e filtrados em tubos centricons com membranas de 5 kDa, 10 kDa e 30 kDa. O efeito inibitório dos extratos aquosos fracionados sobre a germinação de sementes de *L. sativa* foi evidente, especialmente em baixos fracionamentos, sugerindo que moléculas específicas têm efeitos inibitórios na germinação de sementes de *L. sativa.* Adicionalmente, a redução do acúmulo de matéria seca em *L. sativa* também foi observada, mas sem variação estatística na influencia do desenvolvimento de radículas *T. cacao* (CCN51). Em *A. cepa,* os extratos em baixa concentração (0,3%) revelaram baixo potencial de inibição do crescimento meristemático, indicando alterações no potencial mitótico. A espécie *A.paulo-alvinii* possui efeito fito- e citotóxico, atribuído pelo potencial inibitório do extrato aquoso no desenvolvimento de *T. cacau*, genótipo CCN51 prejudicado o seu desenvolvimento. Desta forma, substâncias presentes no extrato aquoso de folhas da espécie *Acanthosyris paulo-alvinii* (mata-cacau) têm exercido atividade alelopática, citotóxica e fitotóxica.

Palavras-chave: citogenética, fracionamento molecular, mata-cacau, mitose.

PHYTOTOXICITY AND CYTOTOXICITY OF LEAVES EXTRACTS OF *Acanthosyris paulo-alvinii* (Santalaceae) TO DIFFERENT PLANT SPECIES

ABSTRACT

The species *Acanthosyris paulo-alvinii* (cacau-killer), endemic to southern Bahia, Brazil, known as hemiparasit using cacao as a host, has allelochemical properties of unknown nature, which inhibits the development of young plants of *Theobroma cacao*, with effects probably allelopathic even going so far as to cause her death. The present work aimed to investigate the phytotoxicity and cytotoxicity potential of the *A. paulo-alvinii* aqueous extract in the L. sativa and *A. cepa* plants, and in the inhibitory capacity of the growth of *T. cacao* primary roots of the CCN51 genotype. The dry extract was elaborated from leaves of adult trees *A. paulo-alvinii* collected in the CEPLAC. The plant material was placed in an incubator at 60 ° C for 72 hours and then ground for further elaboration of the aqueous extract, which was used in various molecular fractions and applied in the tests with *T. cacao* and *A. cepa*. For *Lactuca sativa* the extract was prepared from lyophilized leaves of *A. paulo-alvinii* and filtered in centric tubes with membranes of 5 kDa, 10 kDa and 30 kDa. The inhibitory effect of fractionated aqueous extracts on *L. sativa* seed germination was evident, especially in low fractions, suggesting that specific molecules have inhibitory effects on *L. sativa* seed germination. In addition, the reduction of dry matter accumulation in *L. sativa* was also observed, but without statistical variation in the influence of *T. cacao* root growth (CCN51). In *A. cepa*, extracts at low concentration (0.3%) revealed low potential for inhibition of meristematic growth, indicating changes in mitotic potential. The *A. paulo-alvinii* species has a phyto- and cytotoxic effect, attributed by the inhibitory potential of the aqueous extract in the development of *T. cacau*, genotype CCN51 impaired its development. In this way, substances present in the aqueous extract of leaves of the species *Acanthosyris paulo-alvinii* (cacau-killer) have exerted allelopathic, cytotoxic and phytotoxic activity.

Key-Word: cytogenetics, molecular fractionation, cacau-killer, mitosis.

1. INTRODUCTION

In Brazil there are records of two species belonging *Acanthosyris* genus: *A. paulo-alvinii* Barroso is native to Bahia State and *A. spinescens* can be found in the Southern of Brazil Bolivia, Argentina and Uruguay.The distribution and natural population dynamics of these species have been changing considerably by anthropic actions, which are currently considered endangered species (MAZZINI, 2011). *A. paulo-alvinii* is found in Atlantic Forest ecosystem in the South of the State of Bahia and is classified as a botanical species critically endangered (IBAMA,2007).

Theobroma cacao originated in Amazon region, comprised in the east of Ecuador and the frontier between Brazil, Peru and Colombia, region of greatest genetic diversity (MOTAMAYORET et al. 2008; THOMAS et al. 2012). The genotype CCN 51 (Colección Castro Naranjal) has been widely used in the cacao crop due to higher productivity, large number of almonds and large grease concentration, as well as disease resistance and tolerance to climatic variations (BOZA et al. 2014; HERRMANN et. al. 2014).

The *A.paulo-alvinii* plays an important ecological role in Cabruca agroforestry systems due to its hemiparasitic interaction with *Theobroma cacao* that causes injuries and premature death of host plants. This species locally known as "cocoa-killer" produces haustories that penetrate the roots of *T. cacao,* inducing seedlings death until the ninth month of age. The hemiparasitism of *A. paulo-alvinii* is specific to cocoa tree, with no host oscillation in the root-hemiparasitic interaction Barroso, (1968). Thus, it is suggested that the potential phytotoxicity of *A. paulo-alvinii* promotes *T. cacao* growth inhibition and death Barroso, (1968). Previous phytotoxicity analysis of *A. paulo-alvinii* leaves extracts suggested the existence of toxic molecules, such as alkaloids (CHAVEZ et al. 1997). Although *A. paulo-alvinii* fruits and seeds are edible, and used to feed the native fauna, no toxic effect is known to animals an human health (ULLOA AND JORGENSEN, 1998).

Tests with the *Allium cepa* system have been widely used to evaluate of the cytotoxicity potential of plant extracts chemicals . This evaluation has been recommended and has been applied to test the cytotoxicity potential of several plant extracts (LUZ et al. 2012; NEVES et al. 2014; RIBEIRO et al. 2016; NISHA, 2017). The high sensitivity of the *A. cepa* plant makes it an excellent model for xenobiotic screening in wastewater, heavy metals and

phytotoxic, cytotoxic and genotoxic substances The high sensitivity of *A. cepa* makes the species an excelle nt model to evaluation the xenobiotic, such as heavy metals and phytotoxicity, cytotoxicity and genotoxicity substances. (BONCIUA et al. 2018).Bioassay with *A. cepa* allows the prospection of toxic molecules that affect root growth as response to cell cycle damages promoting physiologic and developmental changes (LEME; MARIN-MORALES, 2009; SINHA; KUMAR, 2014, BIANCHI et al 2016).

The analysis of cytotoxicity using *A. cepa* test is accepted by the worldwide scientific community for having shown similar and satisfactory results in others practiced in animal tests and cell cultures (MOURA et al. 2016; SANTANA et al. 2016). Allelopathic, phytochemical and genotoxicity study of aqueous extracts of *Aspidosperma pyrifolium* Mart. and *Combretum leprosum* Mart. in *A. cepa*, confirmed the cytotoxicity (PEREIRA, 2015). Through this test the toxicity that affects the root development, cytotoxicity affecting mitotic index and the presence of alterations in the mitotic cell cycle, such as the existence of micronuclei and chromosomal aberrations are verified (BARBÉRIO et al. 2011; GALUCIO, 2014; FIGUEREDO, 2014). Besides the use of *A. cepa*, *Lactuca sativa* has also been applied in bioassays in the investigation of phytotoxicity and cytotoxicity (SOUZA FILHO et al. 2010; GOMES et al. 2012; RIBEIRO et al. 2012). Tests of inhibition of seed germination and root development of *L. sativa* identified fluorine as the chemical agent responsible for the wastewater toxicity in the galvanizing industry, proving that this test is a practical bioassay to assess the toxicity of complex effluent materials (PARK et al, 2016).

The evaluation of the phytotoxicity potential of plant extracts and also organic and inorganic xenobiotics (ARAGÃO et al. 2017; BAGUR-GONZÁLEZ et al. 2010) have been carried out using *Lactuca sativa* model. In this species the physiological, developmental and growth responses are considerably modified and influenced by the soil or germination medium, mainly in the germinative phases and initial root development. Reduction in both the germination index and the number of divisions of root tips cells were observed in *L. sativa* with the use of *Lepidaploa rufogrisea* extract, a plant of the same botanical family of *L. sativa* (Asteraceae) (ARAGÃO et al. 2017). In addition, *L. sativa* is also considered a model plant for the cytotoxicity by the analysis of chromosomal changes and mitotic cycle cell aberrations caused by the induction of substances present in complex plant extracts (ARAGÃO et al. 2017; ANDRADE-VIEIRA et al. 2014). The present study aimed to investigate the phytotoxicity and cytotoxicity potential of the aqueous extract of *A. paulo-alvinii* in the model plants *L. sativa* and *A. cepa*. In addition, the inhibitory capacity of the *A. paulo-alvinii*

aqueous extract in germination and primary root growth of the CCN51 variety of *T. cacao* was also verified.

2. LITERATURE REVIEW

2. 1 *Acanthosyris paulo-alvinii*

Acanthosyris paulo-alvinii is a tree species belonging to the family Santalaceae and popularly known as cacao-killer. The Santalaceae family is widely distributed in the tropical and temperate zones (KUIJT, 1969). It contains about 480 species distributed in seven genera (NICKRENT, 2011), and in Brazil 69 species are found in eight genera 24 being endemic (DETTKE et al., 2015).

In the south of Bahia, in the municipality of Una and Canavieiras, it was common for farmers to mention the existence of a tree known as a cacao-killer tree which, according to them, caused the death of *Theobroma cacao* when cultivated around its grow (ALVIM; SEESCHAAF, 1971).

Reis (1938) carrying out field studies, it would have as objective to elucidate the supposed negative causes, that the curious tree could exert on the plants of cocoa arriving to cause its death. Thus, he assumed that the roots of the tree excreted toxins which, when absorbed by the roots of the cocoa tree, caused their death. At the time, the same author was unable to determine the species and family of the tree and not exactly what caused the rickets and consequent death of the cocoa trees when planted under its grow. The species was first described by Barroso (1968) as belonging to the genus *Acanthosyris* an endemic genus to South America and with few known species such as *Acanthosyris paulo-alvinii*.

Alvim & Seeschaaf (1968, 1971) observed that cocoa seedlings when grown on the side of cocoa-killer, presented symptoms of yellowing and falling of their leaves from the eighth month and they died in the eleventh month. The authors analyzed the roots of the affected plants and observed that when the lateral roots of the cocoa tree came into contact with the pivoting root of the cocoa tree, small haustories formed that penetrated the cortical tissue of the root.

Chavez et al. (1997) carried out a phytochemical analysis of leaves of the cacao-killer tree and found phytotoxic substances characteristic of allelopathic plants, as well as a new alkaloid that has not yet been known. It is a plant that in the literature was very little quoted due to the lack of data of the species, lacking more research on the species.

When using cocoa-killer leaf extract in *T. cacao* seedlings, Passinho (1995) observed a reduction in plant growth including morphological changes in the root system. It also verified reductions in seed germination rate and *T. cacao* seedlings growth, especially when using 10% (w/v) leaf extract, thus leading to the understanding that the *A. paulo-alvinii* species can release allelochemicals in their host or even in the environment in which they are inserted, thus preventing the survival of *T. cacao* plants. It was also observed the shortening of the pivoting root, stem growth and *T. cacao* leaves.

2.2 *Theobroma cacao*

Native to the American rainforest, the cacao tree belongs to the genus *Theobroma* along with 21 other species (SODRÉ, 2007). *T. cacao* L. is a perennial woody species belonging to the family Malvaceae and its center of diversity extends throughout the region of Central America (MOTAMAYOR et al., 2008; MULLER; VALLE, 2012; PURDY; SCHMIDT, 1996) *T. cacao* within the genus is cultivated because of the economic value of the seeds (ARGOUT et al., 2001, CEPLAC 2001, ALEXANDRE et al., 2015, KONGOR et al., 2016). *T. cacao* is the most to be commercially exploited to produce seeds that after being used will be used in the manufacture of chocolate and derivatives, although other species within the genus have the potential to be used as table fruit (SODRÉ, 2007).

Southern Bahia is responsible for about 70% of Brazil's production with 143,000 tons in an estimated area of 530,000 hectares (IBGE, 2018). When grown in agroforestry systems "cabruca," *T. cacao* plays an important role in environmental preservation, because it is cultivated in the shade of predominantly Atlantic Forest trees, and can also be cultivated in consortium with other species of economic value, thus being considered a sustainable crop (2001). The CCN-51 clone (Castro Naranjal Collection) is the result of crossing the hybrid IMC-67 with the ICS-95 known in Ecuador as "Canellos" being genetically used as parent in breeding programs and cocoa selection for its high productivity, tolerance to climatic variations and diseases, and higher fat concentration in their almonds (BOZA et al., 2014; HERRMANN et al., 2014). By these characteristics it has been widely used in several areas of research.

In the 1970s until 1980 the most used form for the production of cocoa seedlings was the sexuada reproduction, when seeds are used for propagation. A practical way found in the

management of the plants by the producers for the implantation and recovery of areas of planting of the cocoa crop (DIAS, 2001)

One of the most important factors to be observed in the success of seed germination is the maintenance of moisture, which will increase its longevity (DOUSSEAU et al., 2011) Qualified as recalcitrant, *T. cacao* seeds are too sensitive to moisture loss , making it unfeasible for conservation provided by germ viability (CHANGRUN et al., 1999).

2.3 Allelopathy and phytochemicals

The term allelopathy was created by the German Hans Molisch in 1937 which defines the ability of higher or lower plants to cause interference in the environment of other plants through chemical substances, negatively or positively influencing their development (MANO, 2006). Several other authors have used this concept to a greater or lesser extent (RICE, 1984, LOVETT et al., 1992; RIZVI and RIZVI., 1992; SOARES 2000; SANGEETHA et al., 2015 SHAH et al., 2016). However, in 1996 the IAS (International Allelopathy Society) more broadly defined the term allelopathy as a process involving the production of secondary metabolites by plants, as well as the influence exerted by microorganisms, viruses and fungi on the growth and establishment of forest, agronomic and biological systems.

The use of plant extracts with allelopathic potential in bioassay, seed germination and initial development of model plants in the laboratory has been very efficient in the verification of parameters that may suffer interference in their natural environment (COELHO et al., 2011).

Other studies with plants of potential allelopathic effect were found in *Casearia sylvestris* (DICKEL et al., 2007), *Mikania glomerata* (SOUZA et al., 2005), *Achillea millefolium* (DALSENTER et al., 2004; HAIDA et al., 2010). Toxicity was observed in plant and animal cells in many classes of secondary metabolites responsible for allelopathic activities (SOUZA et al., 2003).

Among the secondary metabolites that have allelopathic potential, fatty acids, aromatic acids, phenolic acids, aldehydes, phenols, alkaloids, flavonoids, tannins, etc, have already been identified. (COUTINHO et al., 2010, SINGH, SAHARAN and BHANDARI, 2014).

In the literature phytochemical studies of the species *Myrcia uniflora*, *Myrcia multiflora* and *Myrcia citrifolia*, and the isolation of the flavonoids myricitrin, myrciacitrins I, II, III, IV and V, myrciafenonas A and B and eucalyptine, triterpene β-amirin and (2), 2 ', 4', 6 '-trihydroxyacetophenone (GOTTLIEB et al., 1972; MATSUDA et al., 2002; BATISTA et al., 2011; FERREIRA et al., 2011) terpenes (ADAMS et al., 2011; GMINSKI et al., 2010); alkaloids (MULACAet al., 2011; RAKBA et al., 1999; JIMÉNEZ et al., 2008).

2.4 Bioassays with *Allium cepa*

The bioassay using meristematic cells of *Allium cepa* was developed by Levan (1938) and is considered an option of fundamental importance for the investigation of the cytotoxic and genotoxic potential of chemical products, plant extracts, industrial waste and contaminated water.

The *Allium cepa* test provides results that aim to evaluate chromosomal and mitotic alterations, besides the occurrence of inhibition of cell division due to the cytotoxic elements present in the plants. This test is also used to ascertain physical characteristics such as root size and shape, color change and microscopic characteristics (FRESCURA, 2012).

The bioassay using *A. cepa* radicles allows the prospection of toxic agents in the growth medium, capable of interacting and damaging the cell cycle, promoting considerable changes in root growth and development (LEME; MARIA-MORALES, 2009;, BIANCHI, MANTOVANI, MARIN-MORALES, 2016).

Tests using A. strain as a bioindicator of cytotoxicity of substances used in *Olea europaea* cannabis allowed the verification that high concentrations of the preservative is potentially cytotoxic (SILVA et al, 2013). Allelopathic, phytochemical and genotoxic studies of aqueous extracts of *Aspidosperma Pyrifolium* and *Combretum leprosum* in *A. cepa* confirmed the cytotoxicity (PEREIRA, 2015). This test is used to verify the toxicity affecting root development, cytotoxicity by mitotic index, and the presence of alterations in the mitotic cell cycle, such as the presence of micronuclei and chromosomal aberrations and asynchrony in anaphase (BARBÉRIO et al., 2011; GALUCIO, 2014; FIGUEREDO, 2014).

In this study, we used a bioassay sample with *A. cepa* to infer cytotoxic parameters in the observation of alterations in the cell division index and, consequently, in the reduction of the mitotic index, when related to normal root growth (MARCANO et al., 2004). The mitotic

index suggests a cell division within the appropriate standards (BECAVELLO et al., 2012). Bioassay using as an instrument *A. cepa* was validated as a useful utility in studies of the cytotoxic and genotoxic potential of contaminated water, chemicals, industrial effluents and plant extracts (CUCHIARA et al., 2012).

Other investigations using tests with the roots of *A. cepa* in contact with industrial wastewater, found chromosomal abnormalities in the cells of *A. cepa*, the presence of fragmented chromosomes, anaphase bridges, and delayed chromosomes (SIDDIQUI et al., 2011). Studies on the possible cytotoxic and genoxic effects using the infusion of *Plectranthus babatus* leaves through the *A. cepa* biotest showed that when the highest concentrations of tea were used, there was an increase in the frequency of chromosomal aberrations and consequent cell death (BEZERRA et al., 2016).

Several studies report the use of bioassay using *A. cepa* as an important instrument when using extracts and infusions of plants and their genotoxicity in increasing and reducing the propagation of cells, indicating mutagenic potential (KNOLL et al., 2006). The test of the effect of plant extracts evaluated through the analysis of meristematic cells of *A. cepa* can be applied as an efficient and low-cost alternative (BECAVELLO et al.,2012; LOUVATEL et al.2014).

2.5 Bioassays with *Lactuca sativa*

One of the main variables used to evaluate whether a plant has allelopathic characteristics is the germination test with seeds, among them the *Lactuca sativa*. In order to obtain reproducible answers, it is necessary that the tests be performed in a controlled environment (MANO, 2006).

Tests with *L. sativa* based on the inhibition of twinning and the influence of root length extracts identified fluorine as the chemical agent responsible for the waste water toxicity of the galvanizing industry, proving that said test is a practical bioassay to evaluate the toxicity of complex effluent materials (PARK et al, 2016).

When testing the allelopathic effect of *Sapindus saponaria* fruit on the germination of *L. sativa* seeds, a significant effect of the aqueous extract on the germination process was verified (GRIZI, GUALTIERE, 2011). When the foliar extracts of the species *Peltophorum dubium* and *Psychotria leiocarpa* were used in bioassay, changes in the mean germination time of *L. sativa* were observed (MARASCHIN-SILVA and AQUILA, 2006). The

allelopathic activity using an aqueous extract in the 5% and 7.5% concentrations of *Araucaria angustifolia* in bioassay in the germination of *L. sativa* was confirmed by the inhibition of seed germination around 80%, probably due to the presence of allelochemical compounds (SILVA et al., 2014).

Using *L. sativa* seeds in bioassay, using the aqueous extract of *Ocotea odorifera* at 1% concentration inhibited seed germination by 70%. Still using the aqueous extract at the concentration of 1% of the species *Cryptocarya moschata*, the same effect of inhibition of the *L. sativa* seeds was observed, but with a greater intensity in relation to the control, that is, 77%. It was also observed, atrophy or even the absence of the radicle and hypocolite decrease (LEYSER et al., 2009).

3 MATERIAL AND METHOD

3.1 Collection and preparation of *Acanthosyris paulo-alvinii* extract

Acanthosyris paulo-alvinii leaves were obtained from adult trees in a Cabruca agroforestry systemat the CEPEC-CEPLAC (Centro de Pesquisas do Cacau-Comissão Executiva do Plano da Lavoura Cacaueira) in Ilhéus in the Bahia State and stored in paper bags for transportation to the Plant Physiology Laboratory of UESC (Universidade Estadual de Santa Cruz), Ilhéus, BA. Plant samples were placed in an incubator at 60° C for 72 hours and then weighed and ground in a grinding mill for the preparation of the dried extract. The dried extract was used for the preparation of the aqueous extract in specific concentrations for the of phytotoxicity and cytotoxicity tests.

3.2 Phytotoxicity potential of *Acanthosyris paulo-alvinii* on germination and root growth of *Lactuca sativa*

In fractionation procedure 6 mL centricons with 5 kDa, 10 kDa and 30 kDa membranes were used to obtain the fractionated aqueous extract of *A. paulo-alvinii*. To prepare the extract the standard procedure was applied using 1.5 g of the leaves maceration of *A. paulo-alvinii* in 15 mL of ultrapure water (100 mg / mL). After filtration on filter paper the extract was centrifuged at 15.000 for 15 min at 4° C and the supernatant was collected and boiled for 20 min. The extract was centrifuged again at 15.000g for 15 min at 4° C and the supernatant collected for use. For the 30 kDa fractionation 6 mL of the extract was added to the top of the 30 kDa centricon. The supernatant extract was centrifuged initially for 15 min, then for another 5 min at 4000g until all the liquid extract was filtered and that all the liquid crosses the membrane being retained in the inferior part of the centricon. The fraction retained at upper part was recovered with a 6 mL water jet against the membrane and named of F> 30. The liquid that crossed the membrane, the one retained at bottom of tube, was named F <30. An aliquot of 1.5 mL of the F <30 fraction was reserved. For fractionation at 10 kDa, 4.5 ml remaining of F <30 in centricon 10 kDa were used. The aliquot 4.5 ml of F10-30 and 4.5 ml of F <10 were obtained. The aliquot F10-30 (~ 4.5 mL) and a 1.5 mL of F <10 were reserved.

For fractionation at 5 kDa ~ 3 mL remainder of F <10 at the centricon of the 5 kDa was used. After complete filtration, F5-10 in 3 mL and F5 in 3 mL were recovered.

In this procedure eight treatments were obtained with the approximate volumes below: TC = water, F1 = 5 mL crude extract, F2 = 4.5 mL F> 30, F3 = 1.5 mL F <30, F4 = 1.5 mL F10-30, F5 = 1.5 mL F <10, F6 = 3 mL F5-10 and F7 = 3 mL F <5. The lowest volume was used between treatments F4, F5 and F6 and completed to 3 mL, defined as the final volume that was used for all treatments in the bioassay. The extracts were transferred to 8.5 cm diameter Petri dishes lined with filter paper (Aton), in each plate distributed in a completely randomized design, 20 *L. sativa* seeds covered with filter paper of porosity 3 microns 0.5% mesh (Aton) were placed. Every 24 hours, germination and root growth were evaluated for a maximum of 72 hours.

To verify the influence of the aqueous extract on germination in quantitative terms, the germination percentage of *L. sativa* seeds was calculated in response to each treatment. Statistical analysis was performed using the Genes software (CRUZ, 2008), and ANOVA was used to verify the significant difference, followed by the Scott-Knott group at 5% probability, for the evaluation of the data of dry mass (mg) of seeds and primary root.

3.3 Phytotoxicity potential of *Acanthosyris paulo-alvinii* to germination and roots grow in *Theobroma cacao*

Seeds of CCN51 cocoa variety were used for the phytotoxicity experiment in *Theobroma cacao*. This genotype is cultivate extensively studied in research because its resistant to diseases, relatively easily find, with number and form of seeds usually homogeneous and high homozygosity.

The aqueous extracts of *A. paulo-alvinii* used for germination tests were made in the following concentrations: 4%; 6%; 8%; 10%; 12% and 14% using the dry leaves and 100 ml of ultrapure water (w/v) and distributed in 250 ml Erlenmeyer. Afterwards, they were filtered on quantitative filter paper and placed in 1.8L glass measuring 20 cm x 20 cm, lined with filter paper (Aton). The pulp of 50 seeds were removed with the aid of sawdust, and the skin of the seed was removed with the aid of a scalpel. The fruits were obtained from the CEPEC-CEPLAC.

All seeds were evaluated and photographed every 24 hours and the total time evaluated was up to 72 hours, in which was observed roots' emission that were measured with a digital pachometer. The experimental design was completely randomized in which 50 seeds of *T. cacao* were used for each recipient to seven treatments. The root metric data were submitted to ANOVA and the means of the treatments were compared by the Scott-Knott group at 5% probability using the Genes software (CRUZ, 2008).

3.4 Cytotoxicity potential of *Acanthosyris paulo-alvinii* in meristematic root cells of *Allium cepa*

For the cytotoxicity test we used bulbs of *A. cepa* (2*n* = 16) of approximate 4 cm of diameters, which were purchased commercially in Itabuna in the Bahia State. The experimental design were completely randomized using15 bulbs in 50 ml plastic cups per treatment. Fist the treatments consist of four concentrations 0.1%; 0.3%; 1% and 2% in ultrapure water (w/v). Each treatments was boiled for 1 hour and also in room temperature. For the experimental control only ultrapure water was used. Solution and water were renewed every 24 hours. The extracts were renovate every day and the optimal size for the roots was observed to application in the analyzes. Roots were collected after 48 hours of grow in the different aqueous extracts concentrations. At the end of 72 hours all treatments were collected and fixed in Carnoy (ethanol: acetic acid [3: 1]) and remained at room temperature for 24 hours to be stored at 8° C until analysis.

For cytological analyzes fixed roots of each sample were placed in Petri dish with distilled water for five min. Roots were dried and placed in the 5N HCl solution for a 20 minutes and placed in water for another five minutes. The material was placed on microscope slide and sectioned the apical meristematic that was squashed in one 45% acetic acid drop according to the protocol proposed Guerra and Souza (2002). The cytological material was covered with a 20x20 mm coverslip and submerged in liquid nitrogen to remove the coverslip. After drying, slides were 2% Giemsa stained for 20 minutes. After drying Neomount mounting medium (Merck) was used for permanent slide production.

The cytological preparations were observed under Leika optical microscope. For each treatment the total of ten slides were prepared, followed by counting of whole cell population per slide. The evaluation of cytotoxic potential was performed with the determination of normality and cellular abnormalities involving the mitotic cycle and the quantitative variation

in the number of roots growth. The following parameters were analyzed: mitotic index, calculated by the number of cells in mitotic division as a function of the total cells number observed. Chromosomal aberrations in the mitotic cell cycle were investigated in metaphase, anaphase and telophase. The data were submitted to analysis of variance and Scott-Knott grouping test at 5% of probability was used by Genes software for mean clustering (CRUZ, 2008).

4 RESULTS

4.1 Phytotoxicity potential of *Acanthosyris paulo-alvinii* on germination and root growth in *Lactuca sativa*

Seeds of *Lactuca sativa* in the control treatment using water showed 100% germination. However, the lowest fractionation of *A. paulo-alvinii* aqueous extract (F1) was able to inhibit 90% of the *L. sativa* seeds germination (Figure 1). In F2 and F3 fractions, germination was inhibited in only 15% and 20%. In the F4 to F6 fractions the inhibition of seed germination was about 10%. Lower inhibition of germination was observed in F7, the higher molecular fractionation used in our study, with only 5% inhibition of *L. sativa* seed germination.

We verified statistical significant difference in the dry mass evaluation of a pool of five seeds plus the primary roots of *L. sativa* germinated at different aqueous extract fractionation (ANOVA, $p < 0.01$), with the variation coefficient of medium magnitude (Table 1). The mean root dry mass was 4.82 mg at the end of the seven days of germination. However, the control showed higher mean dry mass (6.06 mg) with statistical difference in relation to the other treatments by Scott-Knott group at 5% probability. In turn, the fractionations F3 to F7 did not differ statistically from dry mass values of roots with 4.08 mg and 5.55 mg, respectively. On the other hand, F1 fractionation showed the lowest average dry root mass (3.53 mg), statistically lower than other treatments.

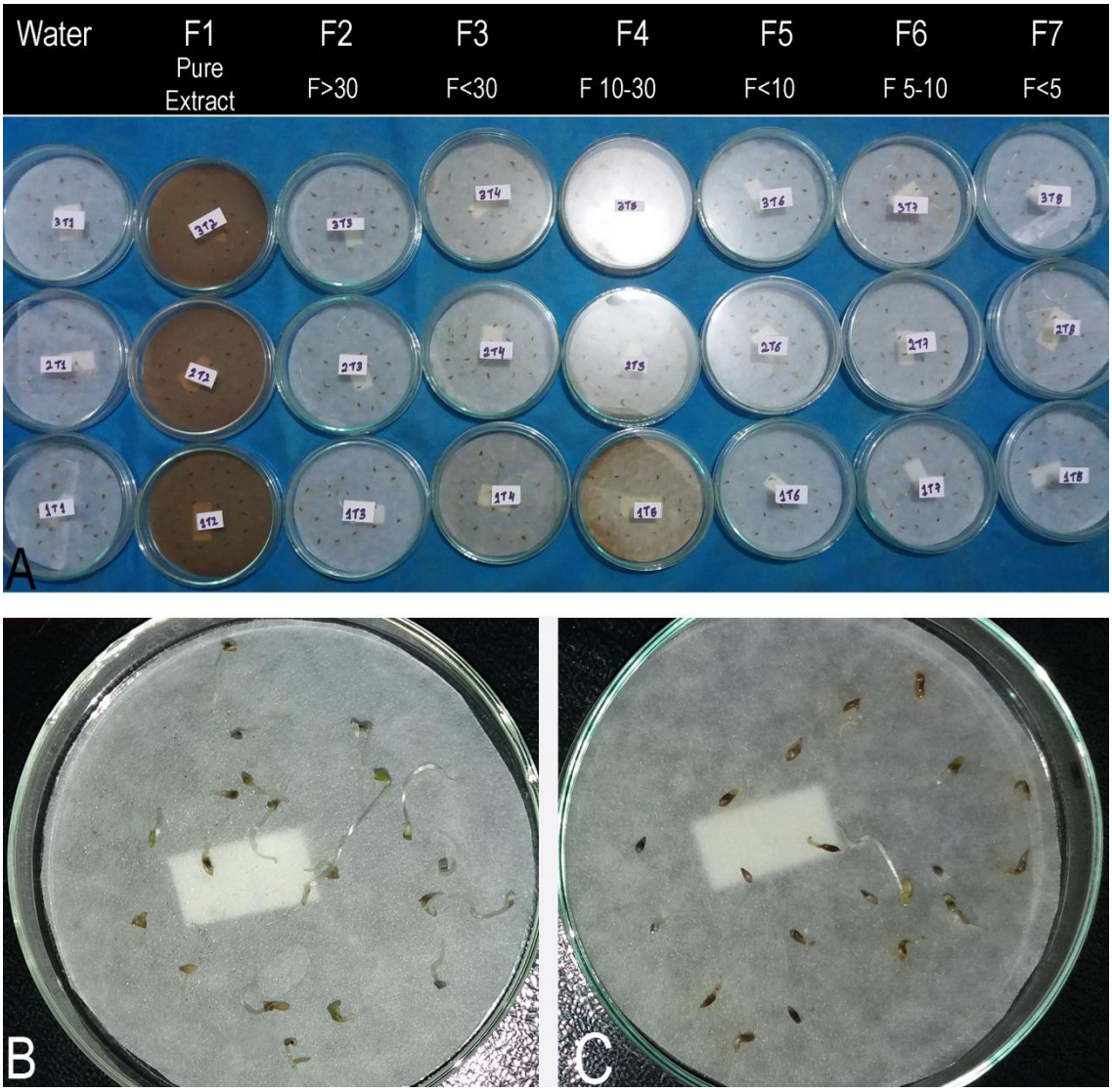

Figure 1. A) Fractionation experiment of *Acanthosyris paulo-alvinii* extract and its influence in *Lactuca sativa* seeds germination. The denominations F represent the centricon membrane fractions, where 30, 10 and 5 are equivalent to the molecular weight in kD.B) Plate control with water. C)Highest molecular fractionation F7

Table 1.Variance analysis for dry mass of *Lactuca sativa* roots grown in medium with different fractionation of *Acanthosyris paulo-alvinii* aqueous extract.

SV	**DF**	**MQ**
Fractions	7	0.000797*
Error	87	0.000292
Total	94	
CV (%)	35.36	
Mean	4.829	

** significant effect to p<0.01
(SV) source of variation; (DF) degree of freedom; (MQ) mean square; (CV%) coefficient of variation.

4.2 Phytotoxicity potential of *Acanthosyris paulo-alvinii* on germination and root growth in *Theobroma cacao*

The evaluation of the cytotoxic potential of *A. paulo-alvinii* aqueous extract in *T. cacao*, at seven different concentrations also showed significant influence in cocoa seeds germination (Figure 2)Significant effect was confirmed by ANOVA ($p < 0.01$) for both evaluation, number of days and variation in concentration of the aqueous extract used in seeds germination of CCN51 cocoa variety (Table 2). The mean germinated root length was 10.61 mm with coefficient of variation of medium magnitude (34.13) varying considerably compared to the control treatment.

Table 2. Analysis of variance for the roots growth of *Theobroma cacao* CCN 51 variety in different concentrations of *Acanthosyris paulo-alvinii* aqueous extract.

SV	**DF**	**MQ**
Day	3	21383.01*
Concentration	6	3419.51*
Error	1390	13.13
Total	1399	
CV (%)	34.13	
Mean	10.61	

*significant effectto $p<0.01$
(SV) source of variation; (DF) degree of freedom; (MQ) mean square; (CV%) coefficient of variation.

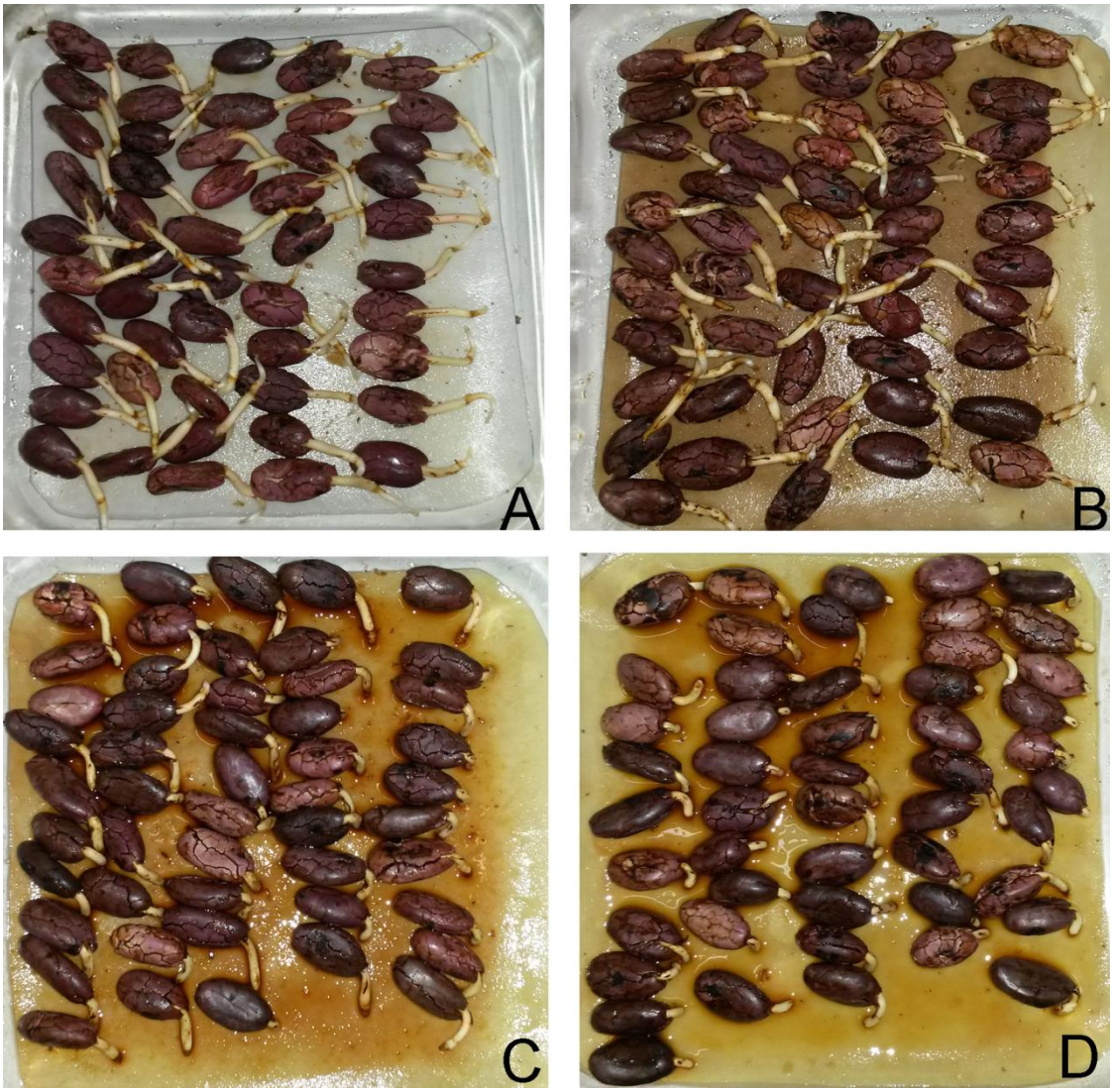

Figure 2. Sample of the phytotoxicity assay promoted by *Acanthosyris paulo-alvinii* aqueous extract in *Theobroma cacao* seeds, CCN51 cocoa variety. A) Control with water; *A. paulo-alvinii* aqueous extract at B) 4% concentration, C) 8% concentration, D) 14% concentration.

The Scott-Knott clustering at 5% probability (Table 3) revealed that the control treatment, without the aqueous extract of *A. paulo-alvinii*, allowed the germination and roots growth to largest length values (16.82 mm) with statistical difference between other aqueous extract concentrations. The concentrations of the aqueous extract at T6% to T12% did not indicate variation of the average length of roots between them, being in the same statistical group according to Scott-Knott analysis. However, the treatment with the highest concentration, T14% generated considerable reduction in roots mean length (6.45 mm) with lower values compared to lower concentration treatments.

Table 3. Mean values of roots length (mm) of CCN51 cocoa variety seeds treated with different concentrations of *Acanthosyris paulo-alvinii* aquous extracts, in six different treatments with groups of 50 seeds each, with replicates for each treatment.

Treatment	Mean
Control	16.82a
T4%	15.72b
T6%	11.18d
T8%	8.66d
T10%	7.73d
T12%	7.73d
T14%	6.45e

Means with the same letters are statistically at the same group by Scott-Knott clustering at 5% probability.

4.3 Cytotoxicity potential of *Acanthosyris paulo-alvinii* in meristematic root cells of *Allium cepa*

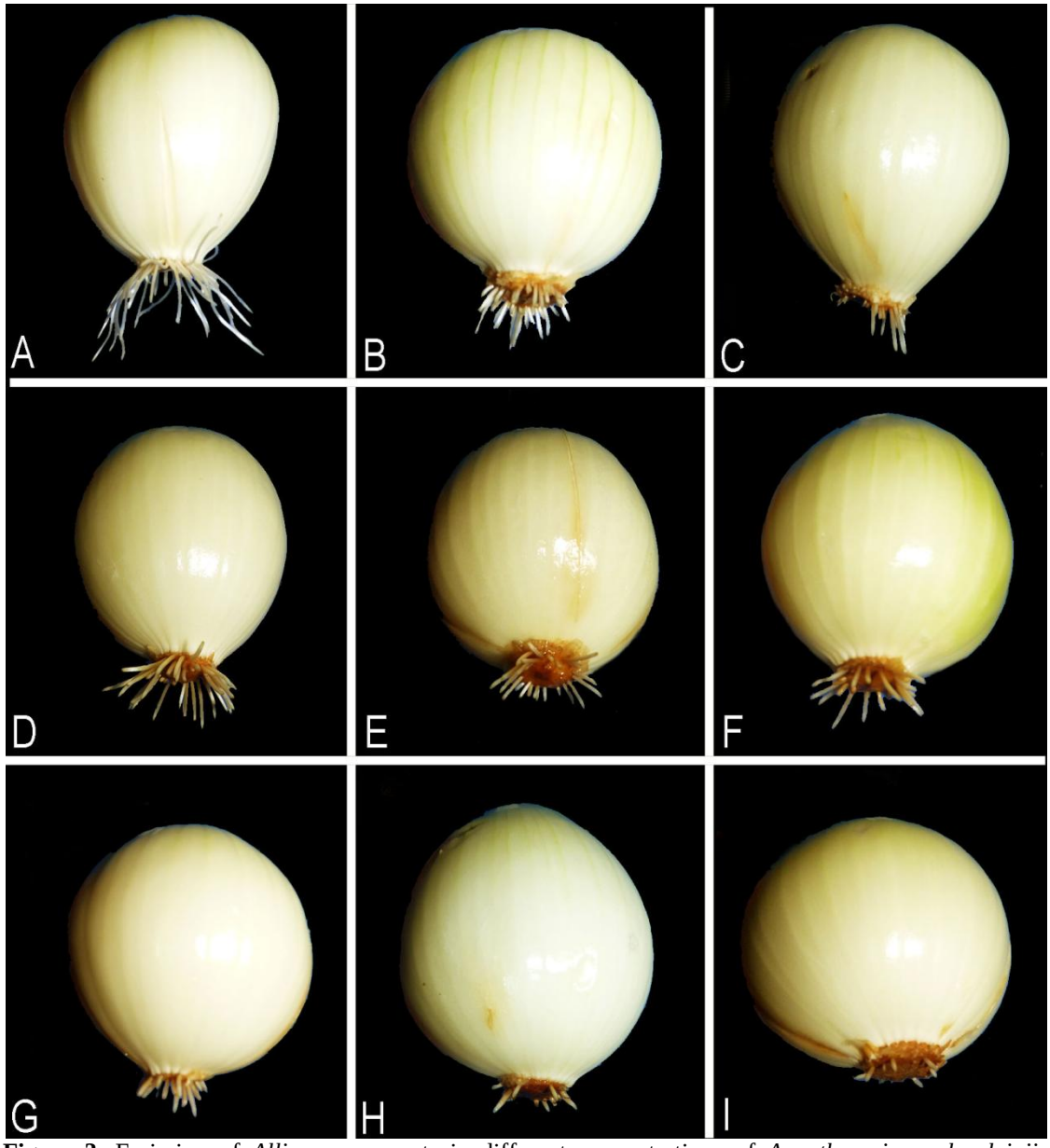

Figure 3. Emission of *Allium cepa* roots in different concentrations of *Acanthosyris paulo-alvinii* aqueous extract. A) control treatment; B) treatment 0.3% unboiled (NB); (C) treatment 0.3%, boiled; (D) treatment 0.6%, unboiled; (E) treatment 0.6%, boiled ; (F) treatment 1%, unboiled; (G) treatment1%, boiled ; (H) treatment 2%, boiled ; (I) treatment 2%, unboiled.

The number and length of *Allium cepa* roots showed considerably variation among the different dilutions of *A. paulo-alvinii* aqueous extract (Figure 3). The variation in roots length showed a significant difference ($p < 0.01$) with coefficient of variation of mean magnitude (Table 4). The average length of roots was 9.97 mm, ranging from 22.92 mm to 3.07 for control and with aqueous extract treatment at concentration of 2% obtained without boiling (Table 5).

Table 4. Analysis of variance for roots length (mm) of *Allium cepa* grown in different treatments of *Acanthosyris paulo-alvinii* aqueous extract.

SV	DF	MQ
Concentration	8	560.681366 *
Error	126	15.028083
Total	134	
CV (%)	38,86	
Mean	9.97	

*significant effectto p<0.01
(SV) source of variation; (DF) degree of freedom; (MQ) mean square; (CV%) coefficient of variation.

The treatments with or without boiling did not present significant statistical variations in root grow and length (Table 5). The tendency observed was in relation to the increase of the concentration of dry extract in both preparations and the proportional effect in relation to increase the aqueous extract concentration in length reduction of *A. cepa* roots. In this way, three statistically different groups were formed by the mean roots length as reveled by the Scott-Knott test at 5% probability (Table 5).

Table 5. Mean root length (mm) of *Allium cep*a bulbs as a function of the different concentrations of *Acanthosyris paulo-alvinii* aqueous extract. (NB) - unboiled and (B) - boiled. Eight different treatments with groups of 15 bulbs with repetitions for each treatment.

Tratament	Root length average
Controle	22.92a
NB0.3%	11.87b
NB0.6%	13.87b
NB1%	6.67c
NB2%	3.07d
B0.3%	12.13b
B0.6%	8.08c
B1%	7.43c
B2%	3.69d

Means with the same letters are statistically at the same group by Scott-Knott clustering at 5% probability.

The cytotoxicity analysis in *Allium cepa* root tips meristematic cells submitted to different treatments of *A. paulo-alvinii* aqueous extract showed variation in number of mitotic cycle cell phases per treatment, with a significant reduction in number of cells in mitotic division (prophase, metaphase, anaphase and telophase) (Table 6). It was also observed higher mean number of cells in prophase per slide/meristematic root's cell, followed by metaphase, anaphase and lastly, few cells in telophase (Table 6).

The mitotic index in meristematic root tips cells varied considerably among the treatments, reducing gradually with increasing the concentration of the aqueous extract of *A.*

paulo-alvinii (Table 7, Figure 4). The highest mitotic index (0.21) was observed in control treatment. However, about 75% of the mitotic index reduction was noticed with the concentration of 0.3% to 0.6%, both unboiled or boiled. Lower mitotic index values were observed at higher concentrations, regardless of the preparation mode of the aqueous extract with the mitotic index at 0.04 and 0.02 in 1% and 2% aqueous extract concentration, respectively.

The Scott-Knott test at 5% probability, revealed four statistically distinct groups for the number of prophases in relation to treatments, with the control containing the mean of 810.40 cells in this mitotic phase (Table 7). Based on the control treatment, the number of prophase cells was reduced by 73.32% with the 0.6% boiled treatment. In contrast, the number of prophase cells in the 2% boiled treatment revealed the greatest decrease in the number of cells at this stage with a reduction of 93.7% compared to the control treatment. A standard reduction in the number of phases the cell division was observed, due to the increase concentration the aqueous extract for the other phases of mitosis. The absence of a statistical relation between the effect of the number of cell cycle phases and the unboiled and boiled preparation of the aqueous extract was observed in comparison of cells in telophase (Table 7).

Changes in mitotic cell cycle were observed in all treatments, except in the interface with intact interphase nucleus and without micronuclei detected in any treatment (Figure 5A). However, metaphases with chromosomes out of the metaphasic plate, without spindle orientation, and with aberrant condensation pattern were also observed (Figure 5C and D), mainly in the 0.3% boiled extract treatment. Anaphasic bridge was the most frequent evidence chromosome alteration in all treatments, but was absent in control, which showed no changes in the cell cycle quality (Figure 5F - H). Lower rate of aberrations in the mitotic cycle at high concentrations 1% and 2% of *A. paulo-alvinii* aqueous extract was observed.

Table 6. Variance analysis for number of cells in different mitosis phases of *Allium cepa* in function the concentration of *Acanthosyris paulo-alvinii* dry extract.

		MQ			
SV	**DF**	**Prophase**	**Metaphase**	**Anaphase**	**Telophase**
Concentration	8	562987.52*	21864.97*	3865.20*	327.55*
Error	81	44495.77	2828.30	444.45	60.66
Total	89				
CV (%)		61.50	58.75	57.00	51.02
Mean		219.24	71.53	24.23	7.9

*significanteffectto p<0.01
(SV) source of variation; (DF) degree of freedom; (MQ) mean square; (CV%) coefficient of variation

Table 7. The mean values of the cell cycle stages of *Allium cepa* roots treated with different concentrations of infusions for three days, containing *Acanthosyris paulo-alvinii* extracts in eight different treatments.

Treatment	Mitotic index	Prophase	Metaphase	Anaphase	Telophase
Control	0.21	821.40a	159.50a	61.05a	18.80a
NB0.3%	0.07	167.30b	107.40b	39.33b	11.50b
NB0.6%	0.06	162.70b	91.00b	31.12b	11.00b
NB1%	0.04	142.80c	37.20d	10.03d	4.60c
NB2%	0.02	51.80d	24.32d	7.00d	1.80d
B0.3%	0.06	209.10b	69.34c	24.10c	8.20b
B0.6%	0.07	219.60b	99.80b	37.00b	9.02b
B1%	0.04	138.30c	33.90d	6.40d	5.00c
B2%	0.02	64.10d	17.20e	3.30d	1.20d

Means with the same letters are statistically at the same group by Scott-Knott clustering at 5% probability.

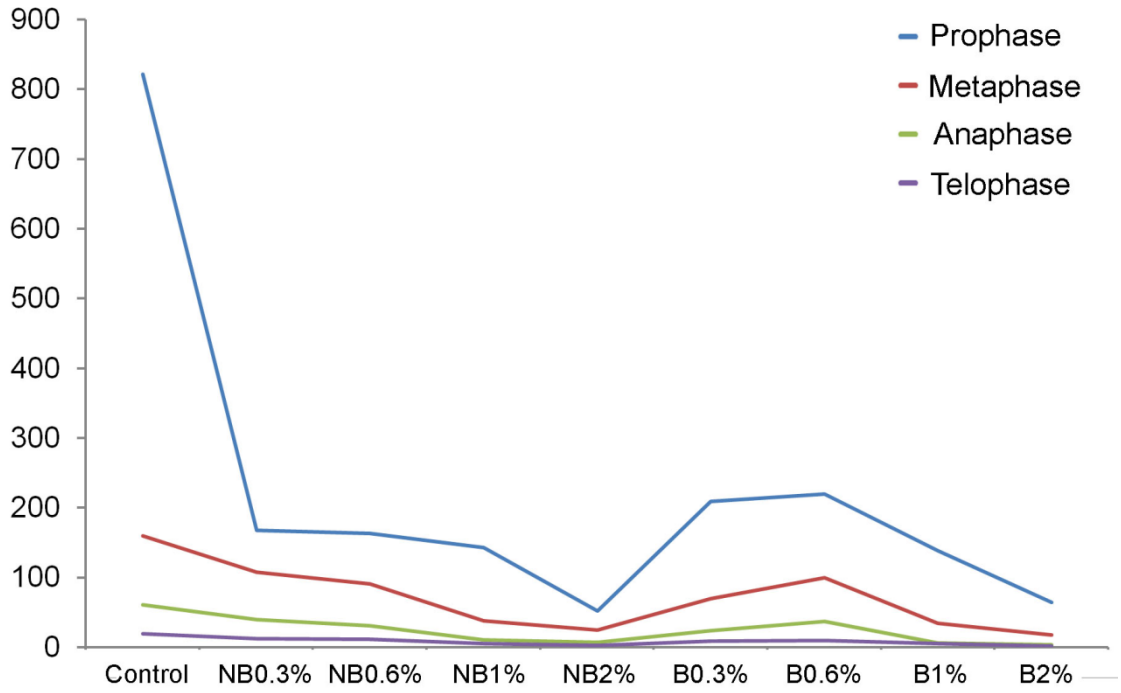

Figure 4. Number of root meristematic cells of *Allium cepa* in specific phase the mitotic cell cycle submitted to growth in different concentrations of *Acanthosirys paulo-alvinii* aqueous extract.

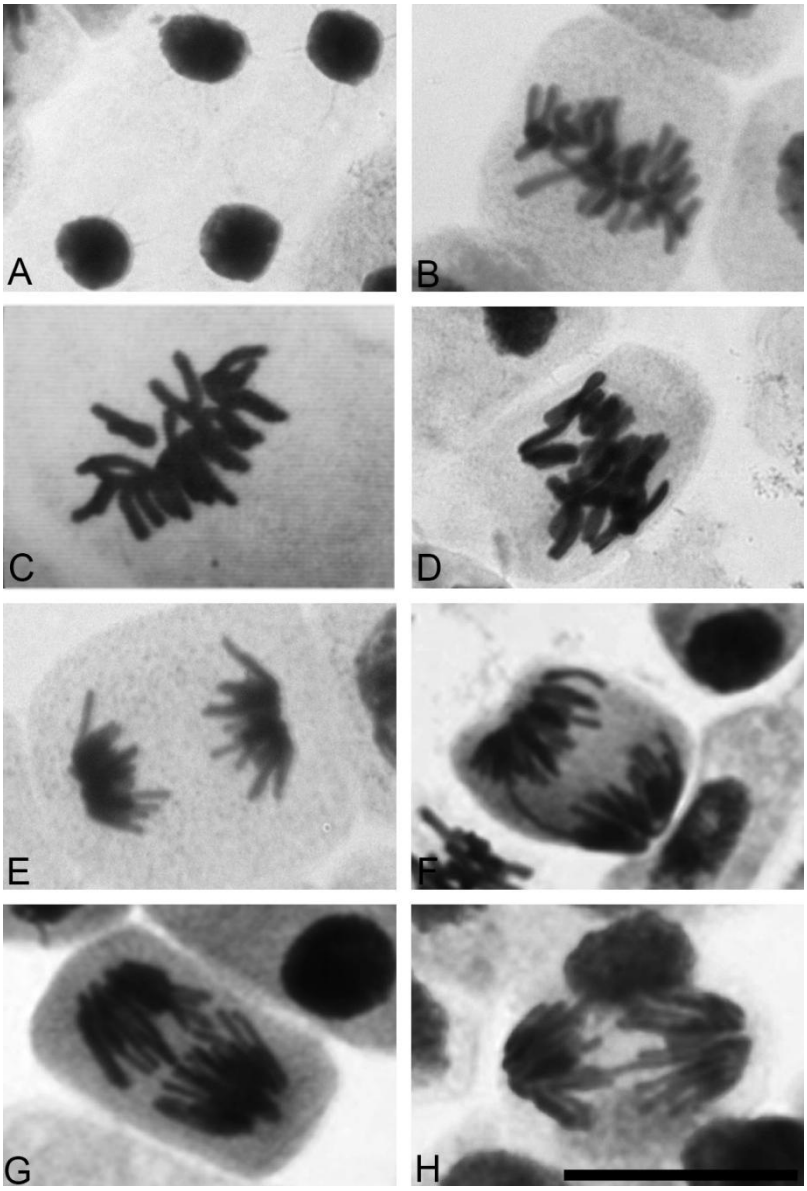

Figure 5.(A) Interphase nucleus of 0.6% unboiled treatment; (B) metaphase in control; (C - D) metaphase of material submitted to 1% boiled treatment and 2% unboiled treatment, respectively. (E) anaphase in control treatment; (F) anaphase submitted to 0.3% boiled treatment; (G) anaphase of material submitted to the 1% boiled treatment; (H)anaphase of material submitted to 2% unboiled treatment. Bar = 10 μm.

5 DISCUSSION

In the present study the phytotoxicity and cytotoxicity potentials of aqueous extracts of *Acanthosirys paulo-alvinii* were efficiently evaluated in three species from different botanical families, including *Theobroma cacao*, a species thatshows an important ecological interaction with *A. paulo-alvinii* under natural conditions. *Acanthosirys paulo-alvinii* has several qualitatively varied organic compounds such as alkaloids, isoperinoids, phenols, proteins and other secondary compounds. The role and action of specific molecules in the direct relationship of hemiparasitic interaction with cacao is still unknown (CHAVEZ, 1997). However, the presence of haustories and roots with specific morphology favors hemiparasitism with *T. cacao* (ALVIM AND SEESCHAAF 1971), indicating the necessary host-hemiparasite contact for the interaction. Cocoa was introduced in southern Bahia for commercial purposes around 1830 (MENDES COSTA, 2012, CEPLAC, 2013), an exotic species to the region. However, cacao is so far known as the only species in the natural environment to be affected by interaction with *A. paulo-alvinii*, a species endemic to the southern region of Bahia (BARROSO, 1968).

In the evaluation of phytotoxic potential in *L. sativa* the extract was fractioned in different kDa, while the evaluation in *T. cacao* was performed with the application the non-fractionated brute aqueous extract. The different gradients obtained with fractionations revealed reduction in germination with the lower degree of fractionation , indicating that higher degree of fractionation reduce the inhibitory effect of the germination by reducing qualitatively and quantitatively the molecules in the medium, especially with greater molecular weight. This is consistent with results found in other plant extracts phytotoxicity evaluation, as for example when *L. sativa* seeds were exposed to aqueous extracts of fresh and dry leaves of *Duranta repens* L. and showed delayed germination (TUR et al. 2010). Other analyzes of phytotoxicity, cytotoxicity and genotoxicity on L. sativa have shown the reduction of the germination index when applied to Jatropha curca (Euphorbiaceae) oil used as biofuel. In this case, the presence of phorbol esters are the main clue to the toxic effect of the oil of this species (ANDADE-VIEIRA et al. 2014). Studies with leaf extracts of *Rhus typhina* showed the effect of allelochemicals dispensed in the ecosystem visibly causing an allelopathic effect on *L. sativa* (WANG et al. 2017).

In the *A. paulo-alvinii* leaf surface a large variety of organic and inorganic metabolite compounds are washed by rain or dew action that may have an allelopathic effect in the soil *(*TUKEY, 1969). Nevertheless, the presence of haustories playing a role in the contact with cacao expands the molecular possibilities in this ecological interaction.. In the cacao tree, germination inhibition has been previously observed in concentrations equal to or greater than 4% (PASSINHO, 1995). This was only observed in concentrations higher than 8% in our study. The phytotoxicity effect of the extracts of the leaves of the in seeds germination, may be related to presence of mainly two chemical classes: isoprenoids and phenols. However, it has been speculated to be the result of the interaction the compounds present in aqueous extract, indicating a complex means of action that leaf extracts hason *T. cacao* seeds (PASSINHO, 1995). In the present work, total inhibition of germination was not observed even at high concentrations of leaf extracts.

The growth of primary roots is also a parameter explored in the study of phytotoxicity and the allelopathic effect of plant extracts (FERREIRA AND AQUILA 2000). In the present work, the presence the ultra filtered and fractionated extract of *A. paulo alvinii* in the medium of *L. sativa* root growth showed that they had a significant influence on growth. It was verified that all the evaluated extract concentrations, showed a reduction in the size of roots with the reduction of the fractionation, i.e. higher concentrations of the total extract proportionally reduce the root growth. This was more evident in the *T. cacao* assay using higher concentrations of the aqueous *A. paulo-alvinii* extract .Reduction of root growth in different has been observed in allelopathic studies. using *Mimosa bimucronata* aqueous extract, showing that complex substances can have similar performances in more than one botanical group, in this case, there being no species-specific response (FERREIRA AND AQUILA 2000).

Currently, there is a search for new alternatives for the control of invasive plants, preferably by natural forms of control. In this sense, plant with allelopathic properties can be used as natural herbicides, being less harmful to ecological systems (MATSUMOTO et al. 2010). Aqueous extracts of *A. paulo alvinii* leaves presented a phytotoxic effect on *L. sativa*, since there was a reduction in the parameters of germination and initial growth when compared to the control. Among the treatments, the lower fractionation of the leaf extract of *A. paulo alvinii* presented higher phytotoxicity levels, since, there was greater inhibition of all the. Contrasting with the dry matter of *L. sativa* seeds and roots, the length of primary rootlets in *T. cacao* was reduced in concentrations higher than 4% in comparison to the control. In this

sense, *L. sativa* seeds were more sensitive to *A. paulo-alvinii* extracts due to the inhibition of germination and dry matter reduction even when submitted to ultra fractionated extracts.

The *Allium cepa* species is one of the most widely used a model plant in tests for cytotoxicity and genotoxicity evaluation for organic and inorganic compounds (BONCIU et al. 2018). This species firstly evaluated in 1938 after methodological standardizations carried out by Levan *A. cepa* have gained space in these surveys due to its efficiency, practicality and speed in , indicating cytogenetic and cytotoxicity potential in the comparison of control and treatments (FISKESJÖ, 1985; RANK, 2003). A series of studies corroborates the use extracts *Allium cepa* as an important test in the evaluation of genotoxicity for extracts and infusions the medicinal plants (BAGATINI; SILVA; TEDESCO, 2007; FACHINETTO et al. 2007).

The meristematic cellular of *A. cepa* responses had similar characteristics independent of the preparation of the *A.paulo alvinii* aqueous extract, suggesting that proteins, thermo-unstable molecules and volatile substances may not be acting in the induction the root length reduction of *A. cepa.* The most notorious effect of the reduction is obtained at higher concentrations, as evidenced by the statistically significant variation between the treatments as a function of the concentration the *A. paulo-alvinii* aqueous extract. It is common to observe changes in development of roots when extracts of plants known by their allelopathic interaction are included in the growth medium (BONCIU et al. 2018). However, the reduction of seed germination potential is not always the rule, for example, extracts of three weeds (*Cynodon dactylon*, *Cyperus rotundus* and *Sorghum halepense*) with allelopathic function promoted changes in the development of *Solanum lycopersicum* (tomato) seedlings and in *Oryza sativa* (rice), but only in the first species the seed germination potential was affected (FERREIRA AND AQUILA, 2000).

The evaluation the mitotic index was carried out with the purpose of verifying the capacity the aqueous extract of *A. paulo-alvinii* to promote meristematic inhibitions in mitotic cell cycle of *A. cepa*. In the control treatment 21% of cells were in mitotic phase as expected in normal cell division in the species. On the other hand, the reduction of the mitotic index, regardless of the preparation of the extract, reached 75% of the mitotic potential, for example, with the aqueous extract of *A. paulo-alvinii* in the concentration of 0.3%.

In the evaluation of chromosomal changes in the mitotic phases anaphase bridges were observed with great frequency in all treatments. In addition, chromosomal segregation changes were observed in metaphase and telophase in lower proportion. These alterations

suggest changes in the chromosomal behavior caused by changes in microtubular dynamics and achromatic spindle movement. The wide occurrence of anaphase bridges suggests chromosomal break, but the presence of micronucleus was not observed (RANK, 2003).

In *A. cepa* submitted to treatments with the infusion of leaves of *Plectranthus barbatus* family Lamiaceae (Brazilian boldo) revealed that, the two major concentrations induced the formation of chromosomal aberrations, confirming that recurrent use can lead to cellular damages. However, the responses are dose dependent, and the frequency with which the tissues are exposed to plant extracts (BEZERRA et al. 2016). In *A. cepa*, the damage evaluation or cell division disturbances including the risk evaluation of aneuploidy, besides being sensitive and presenting good correlation with other test systems, has good reproducibility (FISKESJÖ, 1985; BONCIU et al. 2018).

6 CONCLUISONS

The phytotoxicity effect of *Acanthosirys paulo-alvinii*, was demonstrated. The aqueous extracts in varied dosages promoted the inhibition of germination in *L. sativa* and reduction of the accumulation of dry mass, indicating the abnormal pattern of cell growth,. On the other hand, when submitted to higher concentrations of the same extract, *T. cacao* seeds of the CCN 51 genotype showed the reduction in germination and root development, definitively proving the phytotoxic and cytotoxic capacity of the aqueous extract of *A. paulo-alvinii*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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