OPEN ACCESS

Journal of
Medicinal Plants Research

10 October 2018
ISSN 1996-0875
DOI: 10.5897/JMPR
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

ABOUT JMPR

The Journal of Medicinal Plant Research is published twice monthly (one volume per year) by Academic Journals.

The Journal of Medicinal Plants Research (JMPR) is an open access journal that provides rapid publication (twice monthly) of articles in all areas of Medicinal Plants research, Ethnopharmacology, Fitoterapia, Phytomedicine etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in JMPR are peer reviewed. Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

Contact Us

Editorial Office: jmpr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/JMPR

Submit manuscript online http://ms.academicjournals.me/

Editors

Editorial Board

Journal of Medicinal Plants Research

Table of Contents: Volume 12 Number 25 10 October, 2018

ARTICLES

Antiviral effect of the seaweed *Osmundaria obtusiloba* against the Zika virus 387
Claudio Cesar Cirne-Santos, Caroline de Souza Barros , Caio Cesar Richter Nogueira, Leonardo dos Santos Corrêa Amorim, Renata de Mendonça Campos, Norman Arthur Ratcliffe, Valeria Laneuville Teixeira, Davis Fernandes Ferreira and Izabel Christina Nunes de Palmer Paixão

Malaria parasite clearance rate of crude methanol extract of Cryptolepis sanguinolenta in mice infected with chloroquine sensitive strain of Plasmodium berghei 396
Eze, Chinelo C., Attama, Anthony A., Ibezim, Emmanuel C., Berebon, Dinebari P. and Agbo, Martina C.

Chemical composition, oviposition deterrent and larvicidal activities of the wood extracts of *Tabebuia avellanedae* from the Cerrado of Brazil 404
Jaqueline Cibene Moreira Borges, Eder Alves Pereira e Silva, Tarcísio Castro Alves de Barros, Ilsamar Mendes Soares, Sérgio Donizeti Ascêncio, Rodrigo Ribeiro Fidelis and Raimundo Wagner de Souza Aguiar

Vol. 12(25), pp. 387-395, 10 October, 2018
DOI: 10.5897/JMPR2018.6624
Article Number: B76A50558615
ISSN: 1996-0875

http://www.academicjournals.org/JMPR

ACADEMIC JOURNALS
expand your knowledge

Journal of Medicinal Plants Research

Full Length Research Paper

Antiviral effect of the seaweed *Osmundaria obtusiloba* against the Zika virus

Claudio Cesar Cirne-Santos[1], Caroline de Souza Barros[1,2] , Caio Cesar Richter Nogueira[1,2], Leonardo dos Santos Corrêa Amorim[1], Renata de Mendonça Campos[3], Norman Arthur Ratcliffe[4], Valeria Laneuville Teixeira[2], Davis Fernandes Ferreira[3] and Izabel Christina Nunes de Palmer Paixão[1*]

[1]Laboratório de Virologia Molecular e Biotecnologia Marinha, Programa de Pós-graduação em Ciências e Biotecnologia, Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense 24020-141, RJ, Brasil.
[2]Laboratório Algamar, Departamento de Biologia Marinha, Instituto de Biologia, Universidade Federal Fluminense 24020-141, RJ, Brasil.
[3]Instituto de Microbiologia, Departamento de Virologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-902, Brazil.
[4]Department of Biociences, College of Science Swansea University, SA2 8PP. UK.

Received 13 June 2018; Accepted 30 July 2018

Zika virus (ZIKV), a mosquito-borne member of the family Flaviviridae, is a human pathogen of global significance. Recently, ZIKV, has become a public health problem with increases in numbers of cases and a strong association between ZIKV outbreaks and the spread of cases of Guillain-Barré Syndrome and microcephaly. In this study, the extracts of the seaweed *Osmundaria obtusiloba* (*O. obtusiloba*) (native to the Brazilian coast) against ZIKV using Vero cells was evaluated. The seaweed extract tested inhibited ZIKV replication in a dose-dependent manner at low concentrations with EC_{50} values of 1.82 µg/mL and a selective index (SI) of 288. Other results showed that this extract had significant virucidal effects. In addition, when the extract and Ribavirin were used concomitantly there was a significant synergistic effect. Our promising results suggest that extracts of *O. obtusiloba* are excellent candidates for further studies, and that marine algae are potentially important sources for the development of novel anti-ZIKV agents.

Key words: ZIKA, seaweeds, antiviral activity, marine algae, *Osmundaria obtusiloba.*

INTRODUCTION

The antiviral potential of marine macroalgae is widely recorded. Several studies have demonstrated activity of a number of species of algae against HIV-1 (Cirne-Santos et al., 2008; Barros et al., 2016), HSV-1 (Macedo et al.,

*Corresponding author. E-mail: izabeluff@gmail.com

2012; Mendes et al., 2012; Soares et al., 2012; Barros et al., 2015), HSV-2 (Mendes et al., 2012; Soares et al., 2012) and dengue type 2 strains (Talarico et al., 2007). Thus, marine algae provide a potentially rich source for the discovery of antiviral drugs. In this study, the native algal extract of *Osmundaria obtusiloba* (C.Agardh) R.E.Norris, from the Brazilian coast, was tested for inhibiting the replication of the Zika virus (ZIKV) (Hayashi et al., 2007).

Arthropod-borne viruses, commonly called arboviruses, normally circulate in nature through biological transmission between susceptible vertebrate hosts and blood-feeding arthropods, such as mosquitoes. Studies show that the most important mosquitoes in this transmission are *A. aegypti*, although there is also strong evidence for the role of *A. albopictus* in this process too (Kraemer et al., 2015; Calvez et al., 2016).

The main arboviruses causing disease in humans include the alphaviruses (Togaviridae: Alphavirus), flaviviruses (Flaviviridae: Flaviviruses), Bunyaviruses (Bunyaviridae) and some members of other virus families (Rhabdoviridae and Reoviridae). Currently, of the 534 viruses listed in the International Catalogue of Arboviruses, 214 are known to be, or are probably associated with arthropods, 287 viruses are reported to be possible arboviruses, and 33 are probably or definitely not arboviruses (Gubler, 2001; Iranpour et al., 2016). In total, 134 of the 534 arboviruses have been reported to cause disease in humans and have a global distribution with the majority circulating in tropical areas, where climatic conditions favor transmission throughout the year (Gyawali et al., 2016; Tabachnick, 2016).

Zika virus (ZIKV) is a mosquito-borne and from the genus Flavivirus, family Flaviviridae and clusters with the *Spondweni serocomplex* (Vorou, 2016). Flaviviruses have a positive sense single-strand RNA genome of approximately 11,000 nucleotides in length. The genome contains a long open-reading frame (ORF) that encodes three structural proteins (capsid, precursor membrane and envelope) that form the viral particles and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The non-structural proteins participate in viral replication, virion assembly, and evasion of the host immune response (Lindenbach et al., 2013). The infection in humans produces a self-limiting acute febrile illness with fever, headache, myalgia and rash, very similar to other arboviruses like Dengue Virus and Chikungunya. Therefore, in regions where more than one arbovirus is detected, ZIKV could be circulating but not be notified causing misleading and low incidences recorded (Tappe et al., 2015; Vorou, 2016).

After the ZIKV was first detected in 1947, during a yellow fever surveillance program in Uganda, few reports of the disease were recorded until 2007 when there was a large outbreak in Yap Island Federal Sate of Micronesia (Faye et al., 2014; Hamel et al., 2016). In October of 2013, a large outbreak occurred in French Polynesia where 396 laboratory confirmed cases were reported. Up to now, two main distinct ZIKV geographic lineages have been described (African and Asian) (Musso et al., 2014; Kucharski et al., 2016).

A substantial proportion of ZIKV infections are subclinical, but when clinical symptoms occur, the disease produced was considered moderate and self-limiting. Recent studies, however, have described a strong association between ZIKV outbreak and an increased number of cases of Guillain-Barré Syndrome (GBS) in French Polynesia, indicating a first complication resulting from a ZIKV infection (Cao-Lormeau et al., 2016; Teixeira et al., 2016). There is also strong evidence for the incidence of cases of microcephaly following ZIKV infection of women during pregnancy. These observations have been supported by evidence accrued during different outbreaks and resulted in great fear in pregnant women (Mlakar et al., 2016; Rodrigues, 2016). Other factors associated with ZIKV infection, such as hyperglycemia, among other malignancies are clear demonstrations of the a potent morbidity of this virus (Nielsen and Bygbjerg, 2016).

In May 15, 2015, the Ministry of Health of Brazil confirmed ZIKV circulation in the country after ZIKV identification in 16 samples (eight from Bahia and eight from Rio Grande do Norte) by the National Reference Laboratory. The symptoms that were described as are the most common include, arthralgia, edema of the extremities, slight fever, headache, retro-orbital pain, conjunctival hyperemia and maculopapular rashes, often spreading down the face to the limbs and often itchy, dizziness, myalgia and digestive disorders.(Junior et al., 2015; Heukelbach et al., 2016).

There is no vaccine or specific antiviral therapy for the prevention or treatment of infections by ZIKV (Barrows et al., 2016). A study identified the viral polymerase inhibitor, 7DMA, as an inhibitor of *in vitro* ZIKV replication, and, in virus-infected mice significantly reduced viremia and delayed virus-induced morbidity and mortality (Zmurko et al., 2016). Deng et al. (2016) also showed that an adenosine analog has *in vitro* and *in vivo* activity against ZIKV.

Previous studies have shown different biological activities for *O. obtusiloba* extract such as: *In vivo* tests using BALB/c mice infected with *L. amazonenses* in the control of the dissemination of this parasite (Lira et al., 2016); acute toxicity tests have demonstrated that *O. obtusiloba* extract does not produce significant toxic effects in BALB/c mice (de Souza Barros et al., 2018); and studies have shown that compounds derived from *O. obtusiloba* showed potent antiviral activity against HSV-1

and HSV-2 and had low toxicity to cell cultures (de Souza et al., 2012). In this way, these studies reinforced our perspectives for the accomplishment of this work.

Characterization of the antiviral activity of the crude extract of the algae *O. obtusiloba* against ZIKV was done in this work. It is shown that the extract inhibited ZIKV replication and thus our findings broaden the antiviral scope.

MATERIALS AND METHODS

Seaweed material and extraction

The seaweed *O. obtusiloba,* is a native species of Brazil and was collected by snorkeling at a depth 1-3 m in various coastal sites in Rasa Beach, Armação de Búzios, Rio de Janeiro State (lat. 22° 45'40", long. 41° 54' 32"). The seaweed was separated from sediments, epiphytes, and other associated organisms, washed with sea water and air-dried (approximate ca. temperature 28-30°C for 7-10 days) until the total evaporation of any water. Air-dried seaweed (approximately 100 g) was powdered and exhaustively extracted three times using ethanol for 72 h in the approximate temperature of 28 to 30ºC. The extract was evaporated under reduced pressure, yielding crude extract (15 to 20 mg), of which 2 to 5 mg was used in tests against the ZIKV. The ethanolic extract was chosen due to the efficiency of ethanol in extracting the phenolic compounds from *O. obtusiloba* (Carvalho et al., 2006) and also the low toxicity *in vivo* of this extract (de Souza Barros et al., 2018).

Cells and virus

Vero cells (African green monkey kidney) were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, cat. no 11960) supplemented with 5% Fetal Bovine Serum (FBS; Invitrogen), 2 mmol/L L-glutamine (Invitrogen, cat. no. 25030). Antibiotics were added at a final concentrations of 50 units/ml penicillin and streptomycin (Invitrogen, cat. no. 15070). ZIKV (ATCC® VR-1839™) was amplified in C6/36 mosquito cells line from *A. albopictus*, adapted to grow at 28°C, was cultured in L-15 Medium (Leibovitz) supplemented with 0.5% tryptose phosphate broth, 0.03% glutamine, 1% MEM non-essential amino acids solution and 5% FBS.

Cellular cytotoxicity assays

To evaluate the cytotoxic effect of the seaweed extract, VERO cells were cultured in 96-well plates to 90% confluence. The cells were then treated with increasing concentrations (25, 50, 100, 200, 400 and 800 µg/ml) of the crude extracts of algae and incubated for 2 to 3 days in DMEM culture medium with 5% FBS at 37°C in a 5% CO_2 atmosphere. For assessment of cell viability, the MTT method was used as previously described by Mosmann (1983). In the 96-well plate previously treated with the extracts, the MTT reagent [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] Sigma-Aldrich) was added at a concentration of 5 mg/ml and incubated for 3 h at 37°C. The MTT medium was then removed and 100 µM dimethylsulfoxide (DMSO) was added to the plate and incubated for 15 min to dissolve the formazan crystals (Mosmann, 1983).. The plate was read in an ELISA reader at 550 nm absorbance. The percentage of metabolically active cells was compared to the control of extract untreated cells, to determine the cytotoxicity of the compounds. These assays were performed three times independently, each in triplicate.

Plaque reduction assay

VERO cells were cultured in DMEM medium and after confluence were incubated with the ZIKV (MOI of 0.1) for 2 h. Subsequently, the cells were washed with PBS to remove the residual virus and a 2% (w/v) mixture of CMC (Sigma Aldrich) was added with DMEM supplemented with 5% FBS, 5 mmol/L L-glutamine and 0.20% of sodium bicarbonate. Different concentrations of the seaweed extracts were added and incubated at 37°C in a 5% CO_2 atmosphere and analyzed daily for plaque formation. Subsequently, the cells were fixed with 10% formaldehyde, then stained with 1% crystal violet and the plates were examined and plaque formation quantified. The assay was used for evaluation of antiviral activity and for viral titration. The infectious virus titer (PFU/mL) was determined using the following formula: plate count × dilution factor × (1 / inoculation volume).

Antiviral assay

Antiviral activity was evaluated using a virus plaque reduction assay. Vero cells were grown in 24-well plates under conditions described above and subsequently infected with ZIKV (MOI of 0.1) in the absence or presence of different concentrations of the crude seaweed extracts ranging from 1.25, 2.5, 5, 10, 15, 20, 25 or 50 µg/mL, respectively. After 1 h of adsorption at 37°C, residual inoculum was replaced by medium containing 2% methyl-cellulose and the corresponding dose of each extract. Plates were evaluated daily and counted between 5 to 10 days of incubation at 37°C in 5% CO_2. The 50% inhibitory concentration (EC_{50}) was calculated as the extract concentration required reducing the virus plaques by 50%. All experiments were performed twice and each in triplicate.

Viral kinetics and time-of-drug addition studies

Vero cells were cultured in 24 well plates, as above and after 90% confluence, was treated differentially. In some wells, the cells were pretreated with the crude extract at 5 µg/mL, from *O. obtusiloba* for 1, 2 or 3 h prior to infection. Subsequently, these cells were incubated with ZIKV (MOI of 0.1) while in other wells cells were incubated at time 0 (immediately after infection) or at 1, 2 and 3 h post-infection with the extract concentration of 5 µg/mL. Cells were then maintained under the conditions for the plaque assay production at 37°C in 5% in CO_2. Inhibition of viral replication was evaluated in relation to the control cells, incubated without extract in 3 independent experiments in triplicate.

Virucidal effect

A suspension of ZIKV, containing the relative concentration which in culture corresponds to an MOI of 0.1, was incubated with the same volume of algae extract from *O. obtusiloba* at concentrations of 2.5, 5 or 10 µg/mL and incubated in microtiter plates for 2 h at 37°C. The pre-incubated samples were then added to Vero cells in 24-well plates for 2 h, washed and incubated under plate-assay conditions. The virucidal effect was defined by the ability of the compound to inactivate the particles not allowing infection and without generating cytopathic effect as observed in the virus-only control.

Synergistic effect test *O. obtusiloba* plus Ribavirin

For this analysis, Vero cells were cultured in 24-well plates

Table 1. Cytotoxicity (CC_{50}), anti-ZIKV profile (EC_{50}) and selectivity index (SI) of the *O. obtusiloba* extract compared with the Ribavirin control.

Crude extracts	CC_{50}[a] (µg/mL)	EC_{50}[b] (µg/mL)	SI[c]
O. obtusiloba	525 ± 3.11	1.82 ± 0.49	288
Ribavirin	297 ± 4.25	3.95 ± 0.95	75.2

The mean values ± standard deviations are representative of three independent experiments.
[a]Concentration that reduced 50% cytotoxic concentration when compared to untreated controls.
[b]Concentration that reduced 50% of ZIKV replication when compared to infected controls.
[c]Selectivity index was defined as the ratio between CC_{50} and EC_{50} and represents the safety for *in vitro* assays.

subsequently infected with ZIKV MOI 0.1. Subsequently, the infected cells were treated with a high dose of 10 µg / ml extract and 10 µM Ribavirin capable of inhibiting 90% of viral replication. The inhibition with a minimum dose of both 0.5 µg / mL extract and 0.5 µM Ribavirin which are concentrations that inhibit replication below 20% was also evaluated. For the synergism evaluation, we combined the concentrations of the compounds and added the infected cells. After 72 h, inhibition of cytopathic effect was observed by inhibition of viral plaque formation.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey test using the GraphPad Instat version 3 program. A p value of <0.05 was considered statistically significant. The values of $p<0.05$, $p<0.01$ and $p<0.001$ are shown in the figures.

RESULTS

Cytotoxicity and effect of the extract on the ZIKV replication in Vero cells

The cytotoxicity (CC_{50}) of the extract from the algae was assessed by MTT (Sigma-Aldrich), as previously described (Mosmann, 1983) with some modifications. The results in Table 1 show that the *O. obtusiloba* extract produced the best CC_{50} with a value of 525 µg/mL. Subsequently, the antiviral activities of the extract were evaluated. For these analyzes, different concentrations of the extract was tested starting with a concentration having an inhibitory potential of 20 µg/mL and reducing the concentration progressively. The extract inhibited the replication of ZIKV in a dose-dependent manner (Figure 1). The results demonstrate that *O. obtusiloba* extract inhibited above 90% of ZIKV replication in the highest concentrations (20 µg/mL) with low EC_{50} values of 1.82 µg/mL. Based on these data, the Selectivity Index (SI), representing the degree of reliability of the extracts for possible future use, was derived from the relationship between the CC_{50} and EC_{50} levels. The values of EC_{50} and SI presented by *O. obtusiloba* extract, 1.82 µg/mL and 288 respectively, were better and gave significances and times significantly better than those obtained by ribavirin (EC_{50}= 3.95 µg/mL; SI=75.2) used as a control (Table 1).

Virucidal effect

The virucidal activity of *O. obtusiloba* extract was evaluated against ZIKV. The viral suspension was maintained with different concentrations of the extracts (2.5, 5 and 10 µg/mL) for 2 h and then added to Vero cell cultures. The results showed that *O. obtusiloba* had a good inactivation capacity of the virus (virucidal effect). Figure 2 shows that the *O. obtusiloba* extract significantly inhibited ZIKV infectivity at higher levels than Ribavirin at all concentrations tested and at 10 µg/mL of this extract inhibited about 80% of ZIKV replication.

Time of drug addition

To identify the step at which viral replication might be inhibited, time of addition experiments were performed with the compounds administered 5.0 µg/mL at 3, 2, 1 h before infection. Subsequently, the virus was added at time 0, 1, 2, and 3 h after infection and Ribavirin was used as a control (5 µM). At time 0, *O. obtusiloba* extract inhibited over 80% of viral replication. *O. obtusiloba* and Ribavirin maintained an inhibitory effect at the other times but with a decline recorded but even at 3 h after virus infection at least 60% inhibition occurred in viral replication (Figure 3).

Synergism between the extract of *Osmundaria obtusiloba* and Ribavirin

The results obtained clearly show the inhibitory efficiency of the *O. obtusiloba* extract on ZIKA was greatly increased with the addition of Ribavirin and can be attributed to a synergistic effect. As shown in Figure 4, the addition of

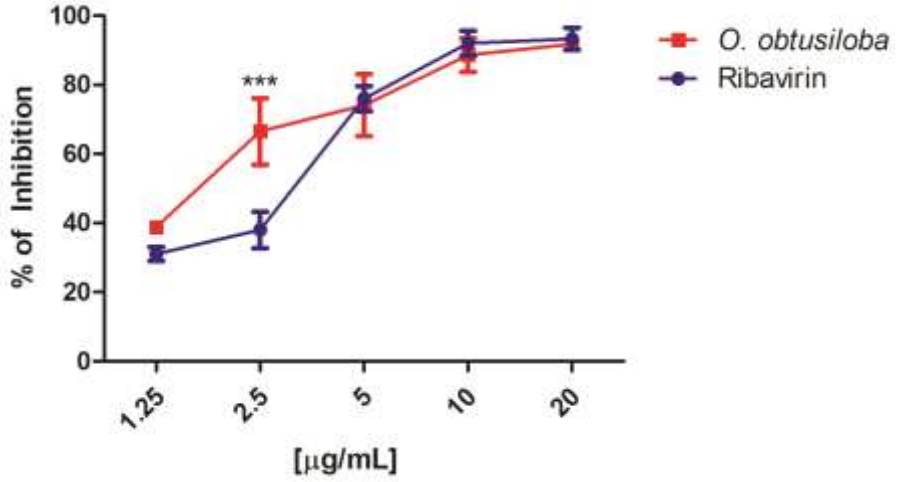


Figure 1. Inhibition of ZIKV replication by *O. obtusiloba* extract. Vero cells were infected with ZIKV (MOI 0.1) and treated at concentrations of 1.25, 2.5, 5, 10 or 20 µg / mL and varying concentrations of Ribavirin as a control. The results were evaluated from three independent experiments in triplicate. Data are presented as percentage of virus titer, when compared to control cells and are expressed as the mean of three experiments ± standard error. Statistical analysis was performed using Tukey test in comparison of *O. obtusiloba* with Ribavirin in each concentration: ***p<0.001.

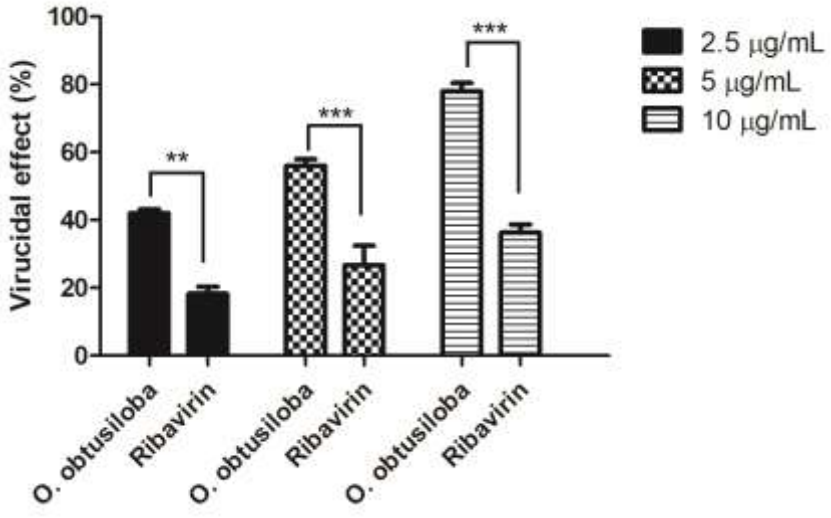


Figure 2. Virucidal effect. Viral suspension (ZIKV) was incubated with *O. obtusiloba* extract at the concentrations of 2.5, 5 or 10 µg/ mL for 2 hours and then added to Vero cells. Data are presented as percentage of virus titer, when compared to Ribavarin control cells and are expressed as the mean of three experiments ± standard error. Statistical analysis was performed using Tukey test in comparison of *O. obtusiloba* with Ribavirin in each concentration: **p<0.01; ***p<0.001.

subdoses of *O. obtusiloba* extract (0.5 µg / mL) resulted in only *ca.* 20% virus inhibition, and, similarly, 0.5 µM doses of Ribavirin recorded *ca.* 15% inhibition. However, when the seaweed extract and Ribavirin were given together

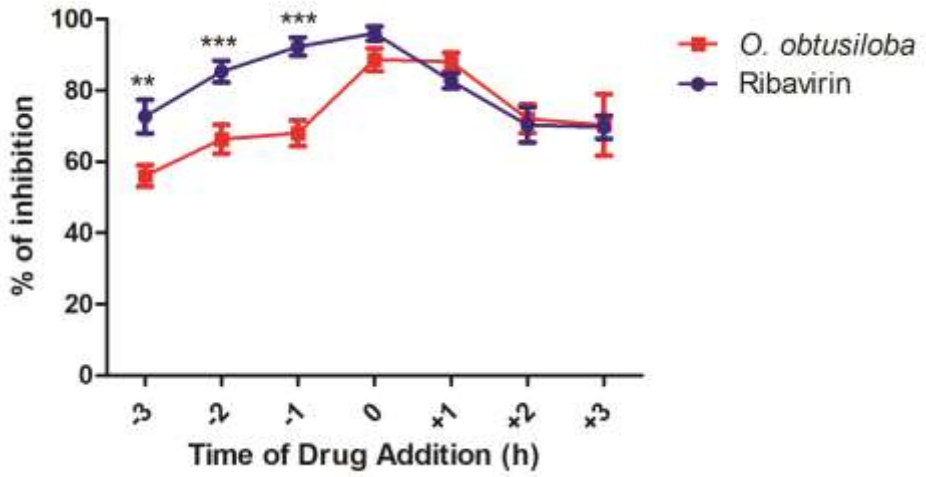


Figure 3. Effect of the addition of *O. obtusiloba* extract on replication over time. Monolayers of Vero cells were infected with ZIKV at an MOI of 0.1 at time zero. At the times indicated, extract or ribavirin was added to a final concentration of 10 µg /mL for extract or 10µM / mL, respectively. Data are presented as percentage of virus titer, when compared to control cells and are expressed as the mean of three experiments ± standard error. Statistical analysis was performed using Tukey test in comparison of *O. obtusiloba* with Ribavirin in each time: **p<0.01; ***p<0.001.

and associated with the lowest concentrations, the inhibitory effect was potentiated and was inhibiting almost 3 times more than the effect of both added at 0.5 µM alone, generating an inhibition of the replication of the ZIKV of approximately 90%, and thus showing characterizing a strong synergistic effect.

DISCUSSION

In this study, the results demonstrate that *O. obtusiloba* extract inhibited viral replication significantly when cells were treated with various concentrations of the extract and that inhibition was dose-dependent generating an EC_{50} of 1.82 µg/mL. In addition, there was a low cytotoxicity of the extract on Vero cells resulting in a CC_{50} of 525 µg/mL. Interestingly, the extract showed a selectivity index (SI) of 288 which, as demonstrated in the literature, is described as good for SI compounds with values greater than 100 (Silva et al., 2011; Zandi et al., 2011).

The literature records a considerable number of studies of antivirals for Dengue that has very significant results (Zandi et al., 2012). However, few studies have been undertaken for other arboviruses that have results as significant as those on Dengue. ZIKA was focused on, which has been associated with severe syndromes (Alvarado-Socarras et al., 2018; Barbi et al., 2018). Previously seaweed extracts have been reported with little cytotoxicity (Alencar et al., 2014) but with considerable antiviral activity, for example, in studies with HIV (Nogueira et al., 2016) and against Herpes (de Souza Barros et al., 2017).

In the present work, initially the seaweed extract was evaluated for virucidals activity and shown to inactivate the ZIKA particles. The studies demonstrated that the extract of *O. obtusiloba* inactivated the viral particles up to 80% in concentrations of up to 10 µg/mL. Thus, additional studies of this virucidal compound are necessary to develop new strategies for the preparation of preventive measures.

Looking for specific characteristics of the mechanism of action of the extract, studies such as the time of addition of the extracts (Time of Addition Experiment) showed that the *O. obsutiloba* used at different times both pre- and post-infection has great potential to inhibit the replication of ZIKV, around 60% after treating up to 3 h prior to infection. At time 0, however, the addition of the extract was concomitant with the infection of the cells, and resulted in 90% inhibition of the virus replication. Even if the extract was added up to 3 h after infection, there was still inhibition of around 80% of the virus. As far as infections are concerned, the signs and symptoms can initially be confusing and the follow-up may be delayed but a drug capable of treating late infections would be an

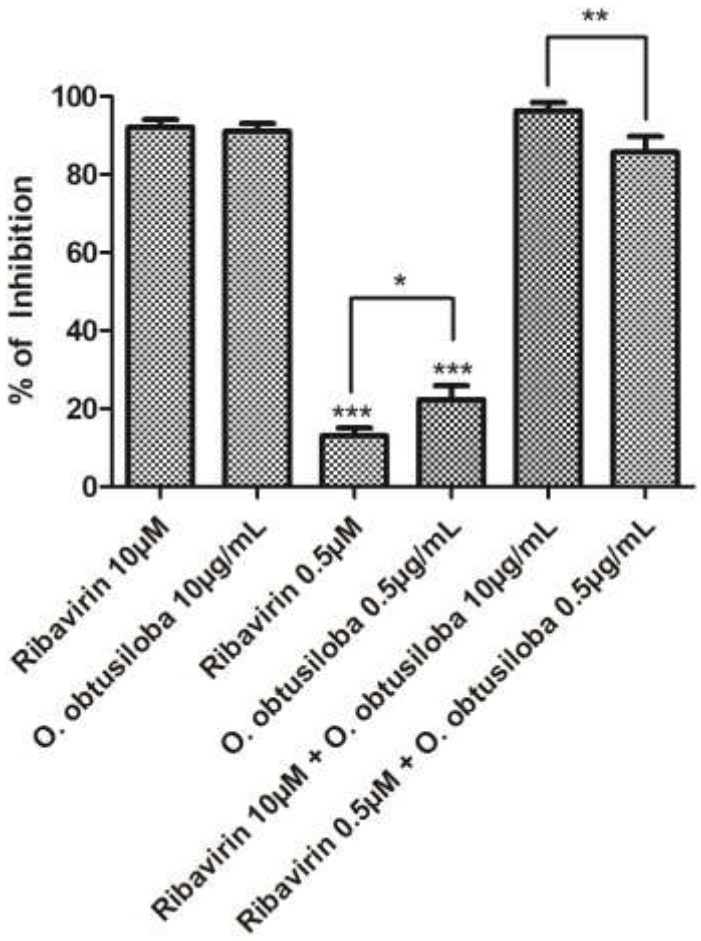


Figure 4. Evaluation of the synergistic effect in combination of the extract of *O. obtusiloba* and Ribavirin. Monolayers of Vero cells were infected with ZIKV at an MOI of 0.1 and subsequently treated with the extract and Ribavirin subdoses (0.5µg/mL and 0.5µM, respectively) and concentrations of 10µg/mL and 10µM. In addition, combinations of the extract and Ribavirin were also tested at both concentrations for assessment of synergism. Evaluation of the synergistic antiviral effect was determined by the inhibition of cytopathic effects by plaque assay. Data are presented as percentage of virus titer, when compared to control cells and are expressed as the mean of three experiments ± standard error. Statistical analysis was performed using Tukey test: * $p<0.05$; **$p<0.01$; **$p<0.001$.

important additional strategy. These data suggest the potential of *O. obtusiloba* as an algal extract candidate for future development. Similarly, Zmurko et al. (2016) performed time of drug addition studies against ZIKV with the 7 DMA viral polymerase inhibitor, but without pretreatment and with posttreatment at different times up to 24 h. They showed that the addition of the compound to the infected cells could be delayed up to ~10 h after infection without much loss of antiviral potency (Zmurko et al., 2016).

There is much interest in searching for combinations of drugs for the inhibition of virus replication as described for Dengue (Yeo et al., 2015), HSV-1 (Mancini et al., 2009), and also for Chikungunya (Mishra et al., 2016). These analyzes are performed in order to reduce the concentration of substances used and to optimize the treatments making them more effective and less toxic. The results herein demonstrated an important synergistic effect by the combination of Ribavirin and the *O. obtusiloba* extract (Figure 4), since the use of both

combined in subdoses were able to inhibit viral replication three times more than both evaluated separately.

Conclusions

The findings showed that the crude *O. obtusiloba* seaweed extract tested had activity against ZIKV, demonstrating that marine algae are an interesting source for drug discovery and the development of novel anti-ZIKV agents. Extracts of *O. obtusiloba* all gave very promising results, and are candidates for further studies to isolate their active factors and better elucidate their mechanisms of action. In summary, *O. obtusiloba* extract has anti-ZIKV with a particularly significant virucidal effects and synergistic effect in combination with Ribavirin. Currently, there are no vaccines or specific drugs for prevention and treatment of ZIKV infection. The results demonstrate the importance of the marine environment in the search for antivirals drugs with activity against ZIKV.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial support and for Productivity Fellowships to ICNPP and VLT (443930/2014-7 and 304070/2014-9). ICNPP and VLT (E-26/201.442/2014) also thank Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) for the Cientista do Nosso Estado Fellowship. CCCS thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the Postdoc fellowship and CSB thanks FAPERJ for the Postdoc fellowship (E-26/201.344/2016) in the postgraduate program in sciences and biotechnology of UFF (PPBI-UFF).

REFERENCES

Alencar DB, Silva SR, Pires-Cavalcante KM, Lima RL, Pereira Junior FN, Sousa MB, Viana FA, Nagano CS, Nascimento KS, Cavada BS, Sampaio AH ,Saker-Sampaio S (2014). Antioxidant potential and cytotoxic activity of two red seaweed species, amansia multifida and meristiella echinocarpa, from the coast of northeastern brazil. Anais da Academia Brasileira de Ciencias 86(1):251-263.

Alvarado-Socarras JL, Idrovo AJ, Contreras-Garcia GA, Rodriguez-Morales AJ, Audcent TA, Mogollon-Mendoza AC, Paniz-Mondolfi A (2018). Congenital microcephaly: A diagnostic challenge during zika epidemics. Travel medicine and infectious disease.

Barbi L, Coelho AVC, Alencar LCA , Crovella S (2018). Prevalence of guillain-barre syndrome among zika virus infected cases: A systematic review and meta-analysis. The Brazilian Journal of Infectious Diseases 22(2):137-141.

Barros CdS, Cirne-Santos CC, Garrido V, Barcelos I, Stephens PRS, Giongo V, Teixeira VL ,de Palmer Paixão ICN (2016). Anti-hiv-1 activity of compounds derived from marine alga canistrocarpus cervicornis. Journal of Applied Phycology 28(4):2523-2527.

Barros CS, Teixeira VL, Paixão IC (2015). Seaweeds with anti-herpes simplex virus type 1 activity. Journal of Applied Phycology 27(4):1623-1637.

Barrows NJ, Campos RK, Powell ST, Prasanth KR, Schott-Lerner G, Soto-Acosta R, Galarza-Muñoz G, McGrath EL, Urrabaz-Garza R, Gao J (2016). A screen of fda-approved drugs for inhibitors of zika virus infection. Cell Host and Microbe 20(2):259-270.

Calvez E, Guillaumot L, Millet L, Marie J, Bossin H, Rama V, Faamoe A, Kilama S, Teurlai M, Mathieu-Daudé F (2016). Genetic diversity and phylogeny of aedes aegypti, the main arbovirus vector in the pacific. PLoS Neglected Tropical Diseases 10(1):e0004374.

Cao-Lormeau V-M, Blake A, Mons S, Lastère S, Roche C, Vanhomwegen J, Dub T, Baudouin L, Teissier A, Larre P (2016). Guillain-barré syndrome outbreak associated with zika virus infection in french polynesia: A case-control study. The Lancet 387(10027):1531-1539.

Carvalho LRD, Guimarães SM, Roque NF (2006). Sulfated bromophenols from osmundaria obtusiloba (c. Agardh) re norris (rhodophyta, ceramiales). Brazilian Journal of Botany 29(3):453-459.

Cirne-Santos CC, Souza TML, Teixeira VL, Fontes CFL, Rebello MA, Castello-Branco LRR, Abreu CM, Tanuri A, Frugulhetti IC, Bou-Habib DC (2008). The dolabellane diterpene dolabelladienetriol is a typical noncompetitive inhibitor of hiv-1 reverse transcriptase enzyme. Antiviral Research 77(1):64-71.

de Souza Barros C, Garrido V, Melchiades V, Gomes R, Gomes MWL, Teixeira VL, de Palmer Paixão ICN (2017). Therapeutic efficacy in BALB/C mice of extract from marine alga canistrocarpus cervicornis (phaeophyceae) against herpes simplex virus type 1. Journal of Applied Phycology 29(2):769-773.

de Souza Barros C, Gomes MWL, Gomes RdSP, Melchiades V, Nogueira CCR, Cirne-Santos CC, esar, Garrido V, Pinto CEC, Teixeira VL, de Palmer Paixao ICN (2018). Acute toxicity evaluation of ethanol extract of red algae, osmundaria obtusiloba, in balb/c mice. Journal of Medicinal Plants Research 12(17):217-221.

de Souza LM, Sassaki GL, Romanos MTV, Barreto-Bergter E (2012). Structural characterization and anti-hsv-1 and hsv-2 activity of glycolipids from the marine algae osmundaria obtusiloba isolated from southeastern brazilian coast. Marine Drugs 10(4):918-931.

Deng YQ, Zhang NN, Li CF, Tian M, Hao JN, Xie XP, Shi PY, Qin CF (2016). Adenosine analog nitd008 is a potent inhibitor of zika virus. In Open forum infectious diseases. Oxford University Press 3(4).

Faye O, Freire CC, Iamarino A, Faye O, de Oliveira JVC, Diallo M, Zanotto PM (2014). Molecular evolution of zika virus during its emergence in the 20 th century. PLoS Neglected Tropical Diseases 8(1):e2636.

Gubler DJ (2001). Human arbovirus infections worldwide. Annals of the New York Academy of Sciences 951: 13-24.

Gyawali N, Bradbury RS, Taylor-Robinson AW (2016). Do neglected australian arboviruses pose a global epidemic threat? Australian and New Zealand Journal of Public Health 40(6):596-596.

Hamel R, Liégeois F, Wichit S, Pompon J, Diop F, Talignani L, Thomas F, Desprès P, Yssel H, Missé D (2016). Zika virus: Epidemiology, clinical features and host-virus interactions. Microbes and Infection 18(7-8):441-449.

Hayashi L, de Paula EJ, Chow F (2007). Growth rate and carrageenan analyses in four strains of kappaphycus alvarezii (rhodophyta, gigartinales) farmed in the subtropical waters of são paulo state, brazil. Journal of Applied Phycology 19(5): 393-399.

Heukelbach J, Alencar CH, Kelvin AA, de Oliveira WK, de Góes Cavalcanti LP (2016). Zika virus outbreak in brazil. The Journal of

Infection in Developing Countries 10(02):116-120.
Iranpour M, Moghadam AR, Yazdi M, Ande SR, Alizadeh J, Wiechec E, Lindsay R, Drebot M, Coombs KM, Ghavami S (2016). Apoptosis, autophagy and unfolded protein response pathways in arbovirus replication and pathogenesis. Expert Reviews in Molecular Medicine 18 p.
Junior VLP, Luz K, Parreira R, Ferrinho P (2015). Zika virus: A review to clinicians. Acta Medica Portuguesa 28(6):760-765.
Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, Moore CG, Carvalho RG, Coelho GE , Van Bortel W (2015). The global distribution of the arbovirus vectors aedes aegypti and Ae. Albopictus. Elife 4:e08347.
Kucharski AJ, Funk S, Eggo RM, Mallet H-P, Edmunds WJ, Nilles EJ (2016). Transmission dynamics of zika virus in island populations: A modelling analysis of the 2013–14 french polynesia outbreak. PLoS Neglected Tropical Diseases 10(5):e0004726.
Lindenbach B, Murray C, Thiel H, Rice C (2013). Flaviviridae. Fields Virology 1:712-746.
Lira M-LF, Lopes R, Gomes AP, Barcellos G, Verícimo M, Osako K, Ortiz-Ramirez FA, Ramos CJB, Cavalcanti DN, Teixeira VL (2016). Anti-leishmanial activity of brazilian green, brown, and red algae. Journal of Applied Phycology 28(1):591-598.
Macedo NRPV, Ribeiro MS, Villaça RC, Ferreira W, Pinto AM, Teixeira VL, Cirne-Santos C, Paixão IC, Giongo V (2012). Caulerpin as a potential antiviral drug against herpes simplex virus type 1. Revista Brasileira de Farmacognosia 22(4):861-867.
Mancini DAP, Torres RP, Pinto JR, Mancini-Filho J (2009). Inhibition of DNA virus: Herpes-1 (hsv-1) in cellular culture replication, through an antioxidant treatment extracted from rosemary spice. Brazilian Journal of Pharmaceutical Sciences 45(1):127-133.
Mendes GdS, Bravin IC, Yoneshigue-Valentin Y, Yokoya NS, Romanos MTV (2012). Anti-hsv activity of hypnea musciformis cultured with different phytohormones. Revista Brasileira de Farmacognosia 22(4):789-794.
Mishra P, Kumar A, Mamidi P, Kumar S, Basantray I, Saswat T, Das I, Nayak TK, Chattopadhyay S, Subudhi BB (2016). Inhibition of chikungunya virus replication by 1-[(2-methylbenzimidazol-1-yl) methyl]-2-oxo-indolin-3-ylidene] amino] thiourea (mbzm-n-ibt). Scientific Reports 6:20122.
Mlakar J, Korva M, Tul N, Popović M, Poljšak-Prijatelj M, Mraz J, Kolenc M, Resman Rus K, Vesnaver Vipotnik T, Fabjan Vodušek V (2016). Zika virus associated with microcephaly. New England Journal of Medicine 374(10):951-958.
Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65(1-2):55-63.
Musso D, Nilles E, Cao-Lormeau V-M (2014). Rapid spread of emerging zika virus in the pacific area. Clinical Microbiology and Infection 20(10):O595-O596.
Nielsen KK, Bygbjerg IC (2016). Zika virus and hyperglycaemia in pregnancy. The Lancet 387(10030):1812.
Nogueira CCR, de Palmer Paixão ICN, Cirne-Santos CC, Stephens PRS, Villaça RC, de Souza Pereira H, Teixeira V, Laneuville E (2016). Anti-hiv-1 activity in human primary cells and anti-hiv-1 rt inhibitory activity of extracts from the red seaweed acanthophora spicifera. Journal of Medicinal Plants Research 10(35):621-625.
Rodrigues LC (2016). Microcephaly and zika virus infection. The Lancet 387(10033):2070-2072.
Silva A, Morais S, Marques M, Lima D, Santos S, Almeida R, Vieira I, Guedes M (2011). Antiviral activities of extracts and phenolic components of two spondias species against dengue virus. Journal of Venomous Animals and Toxins Including Tropical Diseases 17(4):406-413.
Soares AR, Robaina M, Mendes GS, Silva TS, Gestinari L, Pamplona OS, Yoneshigue-Valentin Y, Kaiser CR, Romanos MTV (2012). Antiviral activity of extracts from brazilian seaweeds against herpes simplex virus. Revista Brasileira de Farmacognosia 22(4):714-723.
Tabachnick WJ (2016). Climate change and the arboviruses: Lessons from the evolution of the dengue and yellow fever viruses. Annual Review of Virology 3:125-145.
Talarico LB, Duarte ME, Zibetti RG, Noseda MD, Damonte EB (2007). An algal-derived dl-galactan hybrid is an efficient preventing agent for in vitro dengue virus infection. Planta Medica 73(14):1464-1468.
Tappe D, Nachtigall S, Kapaun A, Schnitzler P, Gunther S ,Schmidt-Chanasit J, (2015). Acute zika virus infection after travel to malaysian borneo, september 2014. Emerging infectious diseases 21(5):911-913.
Teixeira MG, da Conceição N. Costa M, de Oliveira WK, Nunes ML, Rodrigues LC (2016). The epidemic of zika virus–related microcephaly in brazil: Detection, control, etiology, and future scenarios. American Journal of Public Health 106(4):601-605.
Vorou R (2016). Zika virus, vectors, reservoirs, amplifying hosts, and their potential to spread worldwide: What we know and what we should investigate urgently. International Journal of Infectious Diseases 48:85-90.
Yeo KL, Chen Y-L, Xu HY, Dong H, Wang Q-Y, Yokokawa F, Shi P-Y (2015). Synergistic suppression of dengue virus replication using a combination of nucleoside analogs and nucleoside synthesis inhibitors. Antimicrobial Agents and Chemotherapy 59(4):2086-2093.
Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR ,Abubakar S (2012). Novel antiviral activity of baicalein against dengue virus. BMC Complementary and Alternative Medicine 12(1):214.
Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR, Abubakar S (2011). Antiviral activity of four types of bioflavonoid against dengue virus type-2. Virology Journal 8(1):560.
Zmurko J, Marques RE, Schols D, Verbeken E, Kaptein SJ, Neyts J (2016). The viral polymerase inhibitor 7-deaza-2'-c-methyladenosine is a potent inhibitor of in vitro zika virus replication and delays disease progression in a robust mouse infection model. PLoS Neglected Tropical Diseases 10(5):e0004695.

Vol. 12(25), pp. 396-403, 10 October, 2018
DOI: 10.5897/JMPR2018.6647
Article Number: DFE517058617
ISSN: 1996-0875

http://www.academicjournals.org/JMPR

ACADEMIC JOURNALS
expand your knowledge

Journal of Medicinal Plants Research

Full Length Research Paper

Malaria parasite clearance rate of crude methanol extract of *Cryptolepis sanguinolenta* in mice infected with chloroquine sensitive strain of *Plasmodium berghei*

Eze, Chinelo C.[1]*, Attama, Anthony A.[2]*, Ibezim, Emmanuel C.[1], Berebon, Dinebari P.[1] and Agbo, Martina C.[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

Received 17 July, 2018; Accepted 16 August, 2018

This study aims to determine the malaria parasite clearance rate of crude methanol extract of *Cryptolepis sanguinolenta* in mice infected with chloroquine sensitive strain of *Plasmodium berghei*. *P. berghei* was injected in mice and left for 3 days for establishment. Blood sample collected and diluted with phosphate buffer saline was used for infection. Five (5) groups of animals (mice) were used in this study each containing 5 animals each. The body weights of the entire animal were recorded before and after treatment. Group 1 (normal control), Group 2 (positive control, untreated malaria-passaged mice), Group 3 (standard control, malaria -passaged mice treated with 25 mg/kg body weight of chloroquine), Group 4 (malaria-passaged mice treated with 200 mg/kg body weight of extract), and Group 5 (malaria-passaged mice treated with 400 mg/kg body weight of extract). Hematological assessments were carried out before the experiment, 5 days after infection and after treatment. The percentage of parasite load in malaria passaged mice was found to be significantly ($p < 0.05$) lower in animals treated with mid and high doses of the extract when compared to control groups. Before treatment, no significant ($p > 0.05$) elevation was observed in the body weight of mice. On day 5 after infection, dose-dependent significant ($p < 0.05$) decrease was observed in the test groups. After treatment period, the body weights of the animals exhibited dose-dependent increase. The study thus revealed that *Cryptolepis sanguinolenta* root extracts possesses antimalarial activity in the *in vivo* mice model and has the ability of re-establishing the blood cells by boosting and stabilizing the blood parameters.

Key words: *Cryptolepis sanguinolenta,* chloroquine, *Plasmodium berghei,* malaria and clearance rate.

INTRODUCTION

Malaria caused by one of five protozoan parasites belonging to the genus *Plasmodium* spp: *Plasmodium*

*Corresponding author. E-mail: chinelo.eze@unn.edu.ng. Tel: +234 803 775 9532.

vivax, Plasmodium malariae, Plasmodium falciparum, Plasmodium ovalis, or Plasmodium knowlesi, though treatable or curable has plagued humanity throughout known history and human prehistory accounting for high mortality statistics in the tropics. In 2015, malaria was responsible for 212 million clinical cases, 429,000 deaths globally with most of the deaths estimated to have occurred in the African Region (92%), followed by the South-East Asia Region (6%) and the Eastern Mediterranean Region (2%) (World Health Organization (WHO), 2016). Malaria is not restricted to low and middle-income countries, but endemic in poor populations globally. In a report by (World Health Organization (WHO), 2011), *P. falciparum* causes the most severe malaria and is predominantly endemic in Africa; *P. vivax* causing life-threatening symptoms, is the prevalent species in South-East Asia, Latin America, Western Pacific and the Eastern Mediterranean, while *P. malariae* and *P. ovale* are less prevalent, and cause less severe disease in human hosts.

However, despite the geometric increasing threat of malaria parasites to lives globally, successful control of the disease is achievable, which includes vector (anopheles' mosquito) control methods and evaluation of traditionally used herbal remedies (Akuodor et al., 2017) as well as effective case management.

Chloroquine has been a highly effective medicine for treatment and prevention of malaria. However, use of the classical drugs of chloroquine and primaquine in the control of the main causative agents of malaria; *P. falciparum* and *P. vivax*, has been exasperated by the resistance of the malarial parasites to these drugs (Olorunniyi and Morenikeji, 2014). This has necessitated the use of "novel" phytomedicines as alternative and effectual antimalarial agents from natural products.

The root of the plant *Cryptolepis* (*Cryptolepis sanguinolenta* (Lindl.) Schlecter, Asclepiadaceae or Periplocaceae is derived from the root of *C. sanguinolenta*; Syn *C.triangularis* N.E Br., and *Pergularia sanguinolenta* Lindl. The plant was named by a man called Kanyanga Cimanga. The anti-plasmodial efficacy of aqueous root extract *C. sanguinolenta* in the treatment of malaria is well-known in West African ethnomedicine. The root has a bitter taste and has been in use in traditional herbal medicine for treatment of malaria in Eastern Nigeria for several decades. In Nigeria, the Vernacular names of *C. sanguinolenta* is Paran pupa (Yoruba), Gangnamau (Hausa) (Osafo et al., 2017) and Akpa-oku (Igbo).

Detailed morphology and pharmacological active alkaloids of *C. sanguinolenta* have been described elsewhere (Osafo et al., 2017; Barku et al., 2012). *C. sanguinolenta* is native to West Africa and is found in countries like Ghana, Nigeria, Cote d'Ivoire, Guinea, Guinea-Bissau, Mali, Senegal, Sierra Leone, Angola, Congo, Uganda, and Cameroon (Ajayi et al., 2012). Cryptolepine, the major bioactive alkaloid in the root bark of *C. sanguinolenta* is basically responsible for anti-plasmodial activity against *P. falciparum* chloroquine sensitive strain D-6 (Barku et al., 2012) and is presently a potential antimalarial lead.

Abay et al. (2015) observed that currently, promising results has been obtained with clinical research conducted on Qing hao (*Artemisia annua*, Democratic Republic of Congo trials), Totaquina (*Cinchona* spp., Multicounty trials) and Phyto-laria (*C. sanguinolenta*, Ghana trial) showing a parasite clearance at days 5 – 7 after treatment of 70 - 100, 92 - 100 and 100%, respectively.

In a prospective descriptive open trial on Ghanaian patients with acute uncomplicated falciparum malaria, Bugyei et al. (2010) appears to report the only antimalarial clinical trial of *C. sanguinolenta* roots when used as a single herb formulation conveniently packaged as tea-bag sachets (PHYTO-LARIA®). More than half of the patients were cleared of *P. falciparum* parasitaemia within 72 h, with mean clearance of 82.3 h (Bugyei et al., 2010). However, the use of root and root bark decoction of *C. sanguinolenta* by traditional medical practitioners in Nigeria and other parts of West Africa in antiplasmodial (antimalarial), anticancer, antihyperglycaemic, antifungal, antihypertensive, antibacterial, anti-inflammatory, tuberculosis, hepatitis and antidiabetic treatment has been reported (Forkuo et al., 2016; Osafo et al., 2017).

Researchers uses *Plasmodium berghei* infection of laboratory mouse strains as a model to simulate human malaria because of its similarity to the *Plasmodium* species (*P. falciparum, P. vivax, P. malaria and P. ovale*) which cause human malaria, distinctive similarity of its life-cycle to the species that infect humans, and its potentials to causes disease in mice which have symptoms similar to those observed in human malaria (Oluyemi and Folayele, 2017).

The present study investigates the malaria parasite clearance rate (antimalarial potential) of crude methanol extract of *C. sanguinolenta* in mice infected with Chloroquine sensitive strain of *P. berghei* by assessing the percent reduction in parasitaemia and changes in haematological parameters.

MATERIALS AND METHODS

Collection and authentication of plant material

C. sanguinolenta roots were collected from Orba in Udenu Local Government Area of Enugu State during the rainy season (June - July, 2017). Identification and authentication were done by Mr Alfred Ozoiko, a taxonomist with the International Center for Ethnomedicine and Drug Development (Inter CEDD) Nsukka with Voucher no. interCEED 042.

Processing of *Cryptolepis sanguinolenta* root

The freshly harvested roots of *C. sanguinolenta* were chopped into smaller pieces, washed thoroughly with clean water and sun-dried

for up to one month to reduce the moisture content. The roots were milled powdered and sieved through a 2.0 mm sieve size to remove larger particles and fiber. The processed root was then stored in sterile and air-tight container for further use.

Animals and parasite used for the study

Male Swiss albino mice (25 ± 2 g body weight and 6 – 8 weeks old) were used for this study. The animals were obtained from the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Science, University of Nigeria animal house. The mice were housed in a wooden cage with wire netting for proper ventilation. Saw dust was used as beddings. The mice were allowed to acclimatize to the laboratory conditions for 2 weeks and given food and water ad libitum.

The use of animals in this study was in accordance with the guidelines approved by the Animal Ethical Committee, University of Nigeria, Nsukka.

Chloroquine sensitive strain of *P. berghei* was obtained from the National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja, Nigeria. The *P. berghei* parasite was maintained for the duration of this study by continuous serial passage of blood from infected to uninfected mice which served as donor mice on a weekly basis.

Preparation of extract

1 kg ground powdered roots were weighed subjected to extraction with 80% methanol by cold maceration. 500-ml volume of methanol was poured into the container containing the powdered root and left for 24 h. This was filtered with gauze and a funnel into a vessel. The filtrate in a closed container was poured into evaporating dishes and left to dry at 35°C. After extraction, 4 g of dried extract were obtained, kept in a clean container and placed in the refrigerator at 4°C.

Preparation of drug

The test drug (Chloroquine Phosphate) used for this experiment were prepared in aqueous solution and administered orally at single dose of 25 mg/kg body weight for three (3) consecutive days.

Inoculation of mice

The mice were injected intraperitoneally with 0.2 ml suspension of 10^6 parasitized erythrocytes (*P. berghei*) and were left for 3 days for establishment. Blood sample was collected and diluted with Phosphate buffer saline (PBS) and used for infection.

Experimental groups

Five (5) groups of animals (mice) were used in this study each containing 5 animals each. The body weights of all the animals were recorded before and after treatment.

Group 1 - Normal control received no extract but 2 ml per 100 kg body weight of sterile distilled water.
Group 2 - Positive control, untreated malaria-passaged mice,
Group 3 - Standard control, malaria-passaged mice treated with 25 mg/kg body weight of Chloroquine.
Group 4 - Malaria-passaged mice treated with 200 mg/kg body weight of extract, and
Group 5 - Malaria-passaged mice treated with 400 mg/kg body weight of extract.

Measurement of parasitaemia

The measurement of parasitaemia was determined as described elsewhere (Adetutu et al., 2016). The malaria clearance rate was conducted by preparing a thin blood film of blood. Briefly, a thin blood film stained with Giemsa stain was prepared on the fifth day for each mouse. The stain was allowed to dry completely. The percentage of red blood cells (RBCs) infected with malaria parasites were determined microscopically using the x100 objective with immersion oil in 10 different fields on each slide. The % Parasitemia and % Suppression were calculated using the formula (Birru et al., 2017):

$$\% \text{ Parasitaemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC count}} \times 100 \quad (1)$$

$$\% \text{ Suppression} = \frac{\text{Mean \% Parasitaemia of Negative Control} - \text{Mean \% Parasitemia of Tre}}{\text{Mean \% Parasitaemia of Negative Control}}$$

Determination of body weight

The animals were sacrificed after 14 days by euthanization using diethyl ether. Body weights before and after administrations were recorded using sensitive electronic balance.

Red blood cell (RBC) count

The red blood cell count was conducted using counting chamber (haemocytometer). Briefly, 4 ml of RBC diluting fluid was added to 20 µl of well mixed anti-coagulated blood and was allowed to stand for 5 min for destruction of WBC. The counting chamber and cover slip were assembled making sure they are completely clean and dry. The blood sample was remixed with capillary and the grid filled with the blood sample. It was left undisturbed for 2 min to allow the red blood cell to settle. These were examined microscopically with a 40x objective in 5 different boxes with chambers.

White blood cell (WBC) count

The white blood cell count was performed using counting chamber (haemocytometer). Aliquot (380 µL) of WBC diluting fluid (Turk's solution) is added to 20 µLof well mixed anti coagulated blood to give a 1 in 20 dilutions. It was allowed to stand for 5 min for the Turk's solution to destroy the RBC and stain the nuclei of the WBCs, making them easier to see and count. The Neubauer counting chamber and the cover slip were assembled making sure they are completely clean and dry. The blood sample was remixed with capillary and the grid filled with the blood sample. It was left undisturbed for 2 min to allow the white blood cell to settle; thereafter, it was covered with the slid, placed on microscope stage and viewed with 40x objective. The cells in the four large corner squares of the chamber were counted.

Determination of hemoglobin (Hb)

Aliquot (20 µl) of well mixed blood in anti-coagulant bottle was carefully measured and dispensed into 4 ml drapkin neutral diluting fluid. It was left at room temperature, protected from sunlight for 4-5 min. The colorimeter was zeroed with drapkin fluid as blank and the absorbance of the test blood sample was read at 540 nm.

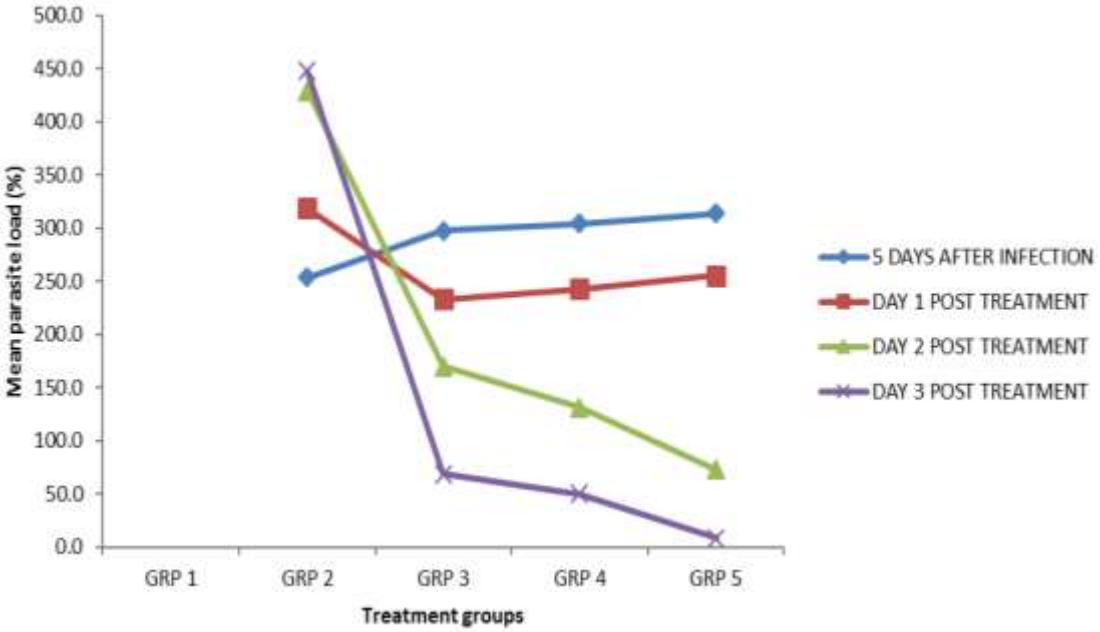


Figure 1. Effect of methanol extract of *Cryptolepis sanguinolenta* roots on percentage of parasite load in malaria-passaged mice. Group 1 = Normal control; Group 2 = Positive control (Untreated malaria-passaged mice); Group 3 = Standard control (Malaria-passaged mice treated with chloroquine); Group 4 = Malaria-passaged mice treated with 200 mg/kg b.w. of extract; Group 5 = Malaria-passaged mice treated with 400 mg/kg b.w. of extract.

Determination of pack cell volume (PCV)

A plane capillary tube was filled up to two-third with well mixed EDTA anti coagulated blood and the unfilled end was sealed with a sealant material. The packed cell volume (PCV) was determined by centrifuging heparinized blood in a micro hematocrit tube at 10,000 rpm for 5 min to separate the blood into a layer of volume of packed red blood cells which when divided by the total volume of the blood sample gives the PCV. The % PCV was read on the hematocrit reader before infection, after infection and after treatment. The PCV of each mouse was determined using the formula:

$$\text{PCV} = \frac{\text{Volume of packed RBC per volume of blood}}{\text{Total volume of blood}} \times 100 \quad (3)$$

White cell count

The Leishman staining technique was used. A drop of the fluid was placed on one end of the glass slide using an applicator. Another glass slide was used to make a smear of the fluid on the glass slide using the push wedge technique. The stain was made to cover the film and then left to stand for 2 min. Thereafter, distilled water, twice the quantity of stain was used to flood the thin film; the setup was rocked gently for 2 min and then allowed to stand for another 15 min before rinsing the stain. The slide was left to air-dry and then examined on the microscope using the oil immersion objective lens of x100 magnification. The cells were counted and differentiated on morphology basis using a tally counter.

Statistical analysis

The data were expressed as the mean ± standard deviation of the mean (SD). Statistical analysis was carried out employing t – test and one-way ANOVA following Dunnett's multiple comparison test.

RESULTS

Effect of methanol extract of *Cryptolepis sanguinolenta* roots on percentage of parasite load in malaria-passaged mice

On day 5 after infection, significantly ($p < 0.05$) higher percentage of parasite was observed in all groups except Group 1 (normal control group). On day 1, 2 and 3 post treatment, significant ($p < 0.05$) reduction were observed in the parasite of Groups 4 and 5 passaged mice treated with 200 mg/kg b.w and 400 mg/kg b.w. of the extract, respectively when compared to the parasite of Groups 2 (malaria-passaged untreated) and 3 (malaria-passaged treated with Chloroquine) mice. There were no significant ($p > 0.05$) differences between Groups 4 and 5 malaria-infected mice administered low (200 mg/kg b.w.) and high (400 mg/kg b.w.) doses of the extract, respectively (Figure 1).

Effect of methanol extract of *Cryptolepis sanguinolenta* roots on the body weight of malaria-passaged mice

Before treatment, the weights of all the animals in all groups were observed and recorded. The results on day

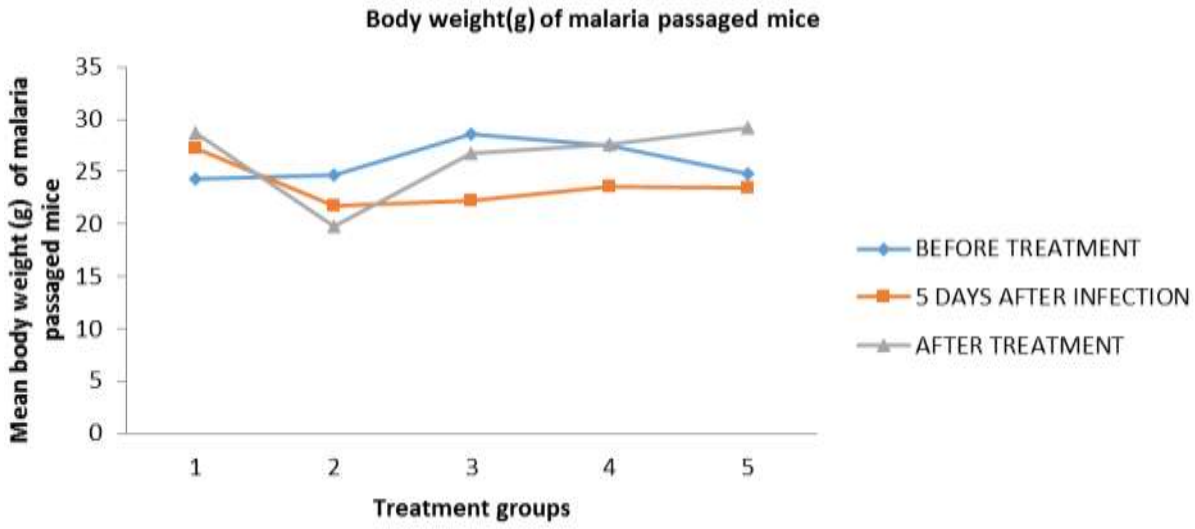


Figure 2. Effect of methanol extract of *Cryptolepis sanguinolenta* roots on the body weight of malaria-passaged mice. Group 1 = Normal control; Group 2 = Positive control (Untreated malaria-passaged mice); Group 3 = Standard control (Malaria-passaged mice treated with chloroquine); Group 4 = Malaria-passaged mice treated with 200 mg/kg b.w. of extract; Group 5 = Malaria-passaged mice treated with 400 mg/kg b.w. of extract.

5 after infection revealed relative decrease in their weights except in Group 1 (normal control). After the treatment period, the body weights of rats from Groups 2 to 5 exhibited dose-dependent increase. Significantly ($p < 0.05$) higher body weights of mice in all test groups were observed compared to the body weights of mice in the control groups. However, non-significant difference ($p > 0.05$) was observed across all groups except Group 2 mice that were passaged but untreated (Figure 2).

Effect of methanol extract of *Cryptolepis sanguinolenta* roots on blood parameters of malaria-passaged mice

Figure 3A, shows the result of the effect of methanol extract of *C. sanguinolenta* roots on packed cell volume of malaria-passaged mice. Non-significant difference ($p > 0.05$) was observed in the packed cell volume (PCV) of all the groups before the treatment period. Dose-dependent significant ($p < 0.05$) reduction was observed in all groups except in Group 1 (normal control), on day 5 after infection. Results obtained after the treatment period showed significantly ($p < 0.05$) higher PCV of Group 5 mice compared to the PCV of mice in all the groups except Group 2. On the other hand, Group 5 mice had PCV that was found to be significantly ($p < 0.05$) lower than the PCV of Group 1 mice. The PCV of Group 4 mice was found to be significantly ($p < 0.05$) lower and higher than the PCV of Groups 1 and 2 mice, respectively.

Figure 3B is the result of the effect of methanol extract *C. sanguinolenta* roots on red blood cell volume of malaria-passaged mice and dose-dependent decrease and increase in red blood cell (RBC) count were observed for day 5 after infection and after treatment periods in Groups 4 and 5 administered 200 and 400 mg/kg b.w. of the extract, respectively as shown in Figure 3. Before treatment, non-significant ($p > 0.05$) reduction was witnessed in RBC count in all the groups compared to the normal control (Group 1) mice. After the treatment period, it was observed that the RBC counts in Groups 4 and 5 exhibited significant ($p < 0.05$) elevation compared to the RBC counts of Group 2 mice that were malaria-passaged but untreated. On the other hand, Group 5 mice had significantly ($p < 0.05$) higher RBC count than the RBC counts of Groups 2 and 3 mice representing untreated and that treated with choloroquine, respectively.

Figure 3C shows the results of the effect of methanol extract *C. sanguinolenta* roots on white blood cell count of malaria-passaged mice and the total white blood cell (WBC) on day 5 after infection recorded the highest count across the groups except Group 2 which represented positive control group, while the lowest WBC count was recorded after treatment in all the groups. Observation before treatment period recorded non-significant ($p > 0.05$) decreases in WBC count of the mice in the test groups (Groups 4 and 5) malaria-passaged mice administered 200 and 400 mg/kg b.w. of the extract compared to all the control groups.

After treatment, significant ($p < 0.05$) reduction was found in the total WBC counts of Groups 4 and 5 mice compared to the total WBC counts of Groups 1, 2 and 3 mice that represented normal control mice, malaria-passaged untreated and Chloroquine-treated groups, respectively.

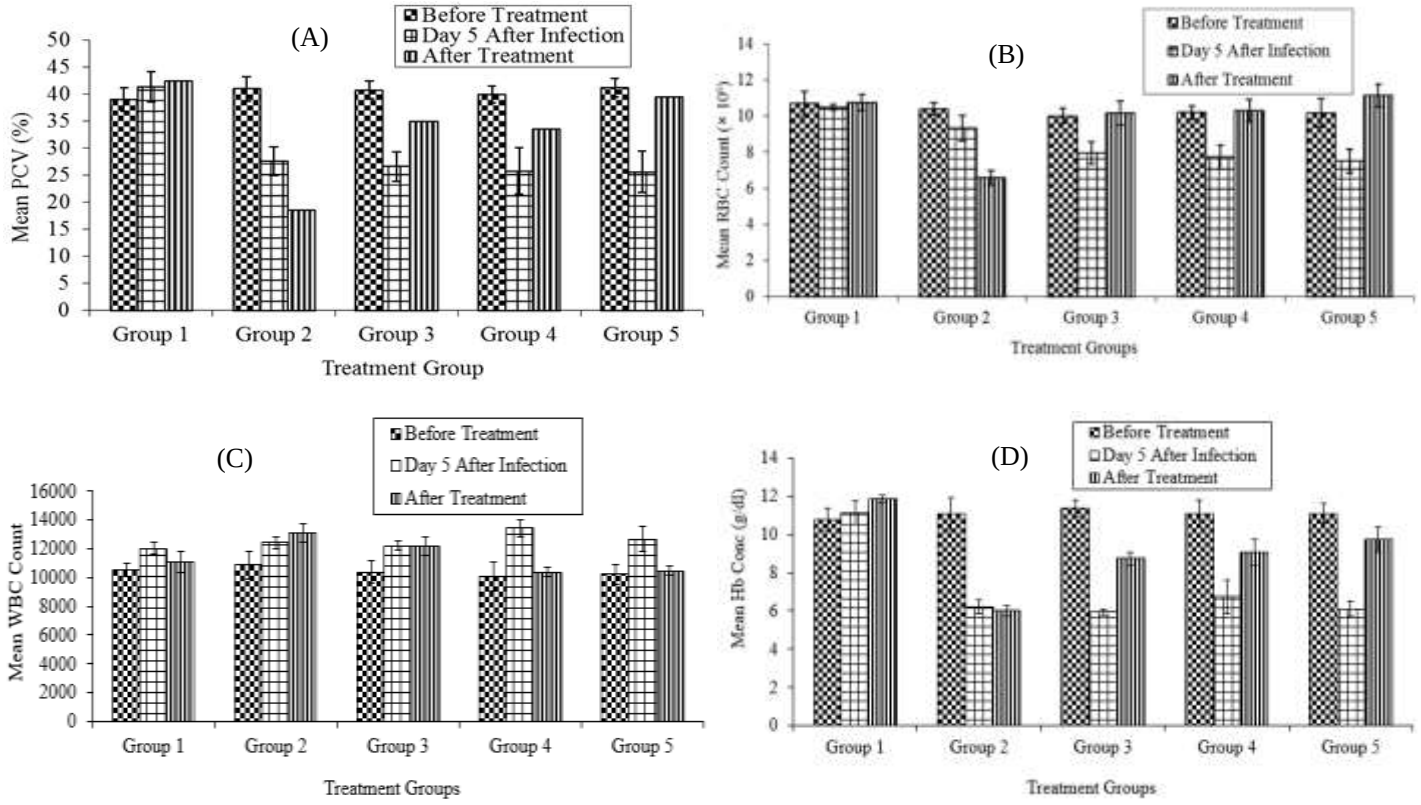


Figure 3. Effects of methanol extract and fraction of *Cryptolepis sanguinolenta* roots on (A) pack cell volume (B) Red blood cell count (C) White blood cell count, and (D) Hemoglobin concentration of malaria passaged mice. Group 1 = Normal control; Group 2 = Positive control (Untreated malaria-passaged mice); Group 3 = Standard control (Malaria-passaged mice treated with chloroquine); Group 4 = Malaria-passaged mice treated with 200 mg/kg b.w. of extract; Group 5 = Malaria-passaged mice treated with 400 mg/kg b.w. of extract.

Figure 3D shows the result of the effect of methanol extract *C. sanguinolenta* roots on haemoglobin concentration of malaria-passaged mice and the highest and lowest haemoglobin (Hb) concentrations were observed in mice of all the groups except normal control mice (Group 1) before the treatment period and 5 days after infection, respectively. Non-significant differences ($p > 0.05$) were witnessed in the haemoglobin concentration of mice Groups 4 and 5 treated with low and high doses of the extract, respectively compared to the haemoglobin concentration of mice in normal, positive and standard control groups.

Results recorded after treatment period showed significantly ($p < 0.05$) lower level of Hb concentration in Groups 4 and 5 mice compared to the Hb concentration of mice in the normal control group. Conversely, significantly ($p < 0.05$) higher concentration of Hb was witnessed in the test animals compared to that of Group 3 mice (positive control).

DISCUSSION

The aqueous methanol extract of *C. sanguinolenta* showed a high percentage of malaria parasite clearance rates in Chloroquine sensitive strain of *P. berghei* comparable to the standard Chloroquine drug. Initially, percentage of parasite clearance was very low during treatment with the methanolic extract of *C. sanguinolenta* roots but higher during the final stage of treatment. Importantly, our results indicated that the inhibition by the extract of *C. sanguinolenta* roots was effective than the positive control (untreated malaria-passaged mice) and standard control (malaria-passaged mice treated with chloroquine) used for treatment of malaria. It was remarkable to observe that the parasite clearance by the extract was better than the positive control, an established drug (chloroquine) commonly used for treatment of malaria. Similar observation was reported by Bugyei et al. (2010) in which *C. sanguinolenta* was better at fever and malaria parasite clearance than chloroquine treated patients in a clinical study. The results showed that the percentage of parasite load in malaria passaged mice was found to reduced significantly in animals treated with mid (200 mg/kg body weight of extract) and high doses (400 mg/kg body weight of extract) of the extract when compared to control groups. This rapid parasite clearance by the extract may be due to its early

Tmax, quick clearance from the plasma, possibly accumulating in vital organs and extending plasma residence time (Donkor, 2016). This suppressive antiplasmodial effect of the methanol extract of *C. sanguinolenta* root on the multiplication of *P. berghei* portends that these extracts are a potential source for new antimalarial drugs.

Evaluation of the methanol extract of *C. sanguinolenta* roots on the body weight of malaria-passaged mice showed that 5 days after infection, the body weights of the malaria-passaged mice administered with chloroquine, 200 and 400 mg/kg b.w. of the extract witnessed relative decrease. The reduction in body weight gain is a simple and sensitive index of toxicity after exposure to toxic substances (Pillai et al., 2011).

A dose-dependent decrease was observed in treated mice compared to that of normal control mice. However, the result showed that after the treatment period, the body weights of mice from all the groups except the normal control exhibited dose-dependent increase. Plants with antimalarial activity are expected to prevent body weight loss in infected mice resulting from rise in parasitaemia (Nardos and Makonnen, 2017).

Thus, higher body weights of mice in all test groups were observed compared to the body weights of mice in the control groups. However, non-significant difference was observed across all groups except Group 2 mice that were passaged but untreated. This result is consistent with (Birru et al., 2017), in which neither the extract doses nor the standard drug-treated group demonstrated statistically significant difference in body weight compared to the control group. The discrepancy was attributed to imbalance of the extract to potentiate protective effect and the cumulative pathophysiologic changes associated with the infection (Birru et al., 2017).

The effect of methanol extract of *C. sanguinolenta* roots on various blood parameters such as pack cell volume (PCV), red blood cell count (RBC), white blood cell count (WBC) and haemoglobin concentration (Hb) of malaria-passaged mice were evaluated. A reduction in concentration of PCV, RBC and Hb are useful indicators of clinical malarial anemia and frequently monitored as drug efficacy against Plasmodium infection (Yeo et al., 2017).

In the present study, PCV was measured to assess the efficacy of the extract and chloroquine in preventing haemolysis due to increasing parasitemia level. There was no observable difference in the packed cell volume (PCV) of all the groups before the treatment period.

However, dose-dependent reduction was observed, on day 5 after infection among Groups 4 and 5 malaria-infected mice treated with 200 and 400 mg/kg b.w. of the extract compared to the PCV of normal control mice (Group 1). This suggests that the malaria parasite actually attacked the erythrocyte (RBC) and reduced their PCV which upon treatment with extract, regenerated the blood cells leading to improved PCV. We observed a relative reduction in the PCV of the test mice compared to that of Groups 2 and 3 mice which were found to be non-significant. This finding is consistent with Ajayi et al. (2012) and Adetutu et al. (2016) who observed that neither the extracts nor the chloroquine significantly prevented the reduction of PCV as compared to the untreated group. The inability of the extract to prevent the reduction of PCV may be attributed to the anti-haemolytic potential of saponin found in the *C. sanguinolenta* roots.

Non-significant differences were observed in the haemoglobin concentration of mice treated with low and high doses of the extract respectively compared to the haemoglobin concentration of mice in normal (Group 1), positive (Group 2) and standard (Group 3) control groups.

Several researchers (Ajayi et al., 2012; Donkor, 2016; Osonwa et al., 2017; Madukaku et al., 2015) have reported similar findings that haemoglobin concentrations were not significantly different from the parasite control treated groups. Conversely, higher concentration of haemoglobin witnessed in the test animals compared to that of Group 3 mice (positive control) agreed with other reports (Ekaidem and Akpan, 2016; Parker et al., 2016).

Plasmodium parasite strains have high affinity for RBCs and feeds on it. Initially, a dose-dependent decrease in red blood cell (RBC) count were observed for day 5 after infection but gradual increase was recorded after treatment periods in Groups 4 and 5 administered 200 and 400 mg/kg b.w. of the extract respectively. In an earlier report, Ajayi et al. (2012) observed that *C. sanguinolenta* did not alter red cells (RBCs) and its related indices in rats treated with 250 mg/kg b.w. Changes in RBC are the most typical feature of malarial infections (Yeo et al., 2017).

The lowest WBC count was recorded after treatment in all the groups. After 5 days of infection, the total WBC count of Group 4 malaria-passaged mice that were treated with 200 mg/kg b.w. of the extract was found to be elevated significantly compared to the total WBC counts of the mice in the Group 5 mice treated with 400 mg/kg b.w. of the extract and control groups. After treatment, significant reduction was found in the total WBC counts of Groups 4 and 5 mice compared to the total WBC counts of Groups 2 and 3 mice that represented malaria-passaged untreated and chloroquine-treated groups respectively.

These findings support earlier reports by (Ajayi et al., 2012) that aqueous root extracts of *C. sanguinolenta* and its major alkaloid cryptolepine had little or no effect on RBC, WBC, Hb concentration, and platelets when administered to rats for 3 or 7 days compared to the control.

In a similar study but with different extracts, Adetutu et al. (2016) and Parker et al. (2016) observed an insignificant difference in the WBC counts of all groups. The results of the present study imply that the *C. sanguinolenta* root extracts enhanced the normal status of the WBC.

Conclusion

The study confirmed that *C. sanguinolenta* root extracts possesses anti-malarial activity in the *in vivo* mice model and has the ability of re-establishing the blood cells by boosting and stabilizing the blood parameters. The results of this investigation, thus, likely support the ethnomedicinal use of this plant in Eastern Nigeria for the treatment of malaria.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

REFERENCES

Abay SM, Leonardo L, Nisha D (2015). Plasmodium transmission blocking activities of *Vernonia amygdalina* extracts and isolated compounds. Malaria Journal 14(1):288.

Adetutu A, Olorunnisola OS, Owoade AO, Adegbola P (2016). Inhibition of in vivo growth of *Plasmodium berghei* by *Launaea taraxacifolia* and *Amaranthus viridis* in Mice. Malaria Research and Treatment.

Ajayi AF, Akhigbe RE, Adewumi OM, Olaleye SB (2012). Haematological evaluation of *Cryptolepis sanguinolenta* stems ethanolic extract in rats. International Journal of Medicine and Biomedical Research 1(1):56-61.

Akuodor GC, Ezeonu CT, Essien AD, Asika EC, Chilaka KC, Nwachukwu DC, Nwobodo NN (2017). *In vivo* antiplasmodial activity of Bombax buonopozense root barks aqueous extract in mice infected by *Plasmodium berghei*. Journal of Traditional Chinese Medicine 37(4):431-435.

Barku VYA, Opoku-Boahen Y, Dzotsi EY (2012). Isolation and pharmacological activities of alkaloids from *Cryptolepis sanguinolenta* (Lindl) schlt. International Research Journal of Biochemistry and Bioinformatics 2(3): 58-61.

Birru EM, Geta M, Gurmu AE (2017). Antiplasmodial activity of *Indigofera spicata* root extract against *Plasmodium berghei* infection in mice. Malaria Journal 16(1):198.

Bugyei KA, Boye GL, Addy ME (2010). Clinical efficacy of a tea-bag formulation of *Cryptolepis sanguinolenta* root in the treatment of acute uncomplicated falciparum malaria. Ghana Medical Journal 44(1).

Donkor AF (2016). Efficacy, Pharmacokinetics and safety evaluation of *Cryptolepine-Artemisinin* based combinations in the management of uncomplicated malaria (Doctoral dissertation).

Ekaidem IS, Akpan HD (2016). Adverse Haematological Changes in Plasmodium Infected Mice Following Treatment with *Azadirachta indica* (Neem) Leaf Extract. Journal of Advances in Medical and Pharmaceutical Sciences 9(2):1-8.

Forkuo AD, Ansah C, Boadu KM, Boampong JN, Ameyaw EO, Gyan BA, Ofori MF (2016). Synergistic anti-malarial action of cryptolepine and artemisinins. Malaria Journal 15(1):89.

Madukaku CU, Chimezie OM, Chima NG, Hope O, Simplicius DN (2015). Assessment of the haematological profile of children with malaria parasitaemia treated with three different artemisinin-based combination therapies. Asian Pacific Journal of Tropical Disease 5(6):448-453.

Nardos A, Makonnen E (2017). *In vivo* antiplasmodial activity and toxicological assessment of hydroethanolic crude extract of Ajuga remota. Malaria Journal 16(1):25.

Olorunniyi OF, Morenikeji OA (2014). *In vivo* antimalarial activity of crude aqueous leaf extract of *Pyrenacantha staudtii* against *Plasmodium berghei* (NK65) in infected mice. African Journal of Pharmacy and Pharmacology 8(12):342-345.

Oluyemi OF, Folayele OT (2017). Effects of some micronutrients on mice infected with *Plasmodium berghei*. Journal of Public Health and Epidemiology 10(1):21-27.

Osafo N, Mensah KB, and Yeboah OK (2017). Phytochemical and Pharmacological Review of *Cryptolepis sanguinolenta* (Lindl.) Schlechter. Advances in Pharmacological Sciences.

Osonwa UE, Majekodunmi SO, Osegbo E, Udobre A (2017). Enhancement of Antimalaria Effect of Mefloquine by Chlorpheniramine. American Journal of Medicine and Medical Sciences 7(6):248-264.

Parker EJ, Mary CU, Nwodo Okwesili FC, Ikechukwu UR (2016). Hematological Responses and Percentage Parastaemia in Malaria-Infected Mice Treated with Ethanol Extract of *Zapoteca portoricensis* Roots. Biochem Anal Biochem 5(252):2161-1009.

Pillai P, Suresha P, Mishrab G, Annapurnaa M (2011). Evaluations of the acute and sub-acute toxicity of the methanolic leaf extract of *Plectranthus amboinicus* (Lour) Sprengin Balb C mice. European Journal of Experimental Biology 1:236-245

World Health Organization (WHO) (2011). World Malaria Report, Geneva, Switzerland pp1-79.

World Health Organization (WHO) 2016. World Malaria Report, Geneva, Switzerland pp1-186.

Yeo SJ, Liu DX, Kim HS and Park H (2017). Anti-malarial effect of novel chloroquine derivatives as agents for the treatment of malaria. Malaria Journal 16(1):80.

Vol. 12(25), pp. 404-414, 10 October, 2018
DOI: 10.5897/JMPR2018.6650
Article Number: 45CEE2758619
ISSN: 1996-0875

http://www.academicjournals.org/JMPR

ACADEMIC JOURNALS
expand your knowledge

Journal of Medicinal Plants Research

Full Length Research Paper

Chemical composition, oviposition deterrent and larvicidal activities of the wood extracts of *Tabebuia avellanedae* from the Cerrado of Brazil

Jaqueline Cibene Moreira Borges[1], Eder Alves Pereira e Silva[2], Tarcísio Castro Alves de Barros[3], Ilsamar Mendes Soares[1], Sérgio Donizeti Ascêncio[1], Rodrigo Ribeiro Fidelis[3] and Raimundo Wagner de Souza Aguiar[1*]

[1]Department of Biotechnology, Biodiversity and Graduate School of Biotechnology of Amazonia (Bionorte) Gurupi, Federal Universiy of Tocantins, Brazil.
[2]Department of Biotechnology, Federal University of Tocantins, Gurupi, TO, Brazil.
[3]Department of Vegetable Production, Federal University of Tocantins (UFT)-Campus de Gurupi, Brazil.

Received 20 July, 2018; Accepted 20 August 2018

Tabebuia avellanedae **is an important timber source belonging to the family of Bignoniaceae. The latter is known for its richness in terms of variety of bioactive chemical constituents, and it has been used in folk medicine for treatment of various diseases. The aim of this work was to investigate the chemical composition, oviposition deterrent and larvicidal activities of the wood extracts of** ***T. avellanedae*** **from the Cerrado of Brazil. Extracts of acetone, ethyl acetate and ethanol from** ***T. avellanedae*** **were obtained using various extraction methods. Quantitative analysis of phytochemical screening confirmed the presence of phenols and tannins in the wood extracts, however, anthraquinones, coumarins and alkaloids were absent. The toxicity of** ***T. avellanedae*** **extracts against 3rd instar larvae of** ***Aedes aegypti*** **using maceration and Soxhlet extraction methods was analyzed. The acetone and ethyl acetate extracts obtained by Soxhlet extraction were more toxic against 3rd instar** ***A. aegypti*** **larvae, with $CL_{50}$of 100.1 and 151.0 µg/mL, respectively. The mortality values (LT_{50} and LT_{95}) were 38.66 and 66.74 min for ethyl acetate extract, respectively, and 53.47 and 119.96 min for acetone extract, respectively. In all cases, the assay showed that all extracts presented mortality of 100% to 3rd instar larvae after 12 h. The oviposition assay showed that gravid** ***A. aegypti*** **females laid their eggs preferentially in the control ovitraps. The ethanol extract at 333.3 µg/mL strongly deterred oviposition by 89.89% while the ethyl acetate and acetone extracts presented 89.04 and 68.10% deterrence, respectively. The bioactive compounds in** ***T. avellanedae*** **make it a potential source for the control of** ***A. aegypti*** **vectors, without promoting deforestation of trees.**

Key words: Mosquitoes, larvae, toxicity, Ipê-roxo, *Aedes aegypti*, phytochemical, phenolic compounds.

INTRODUCTION

The mosquito *Aedes* (Stegomyia*) aegypti* (Linneus, 1792) (Diptera: Culicidae) transmits dengue and is

*Corresponding author. E-mail: rwsa@mail.uft.edu.com.

responsible for the transmission of other virus diseases, including yellow fever, chikungunya and Zika virus (Olagnier et al., 2016). Dengue, Zika and chikungunya viruses deserve special attention particularly because these viruses are currently causing negative impacts on public health and economic damage around the world (Mayer et al., 2017). To date, vaccines and medications for treatment of dengue are not available. Disease prevention solely depends on the elimination of mosquito by killing larvae with larvicides (Kumar et al., 2010).

Many insecticides may be used to control mosquitoes, however, many of them are not selective and can harm beneficial insects, increase the resistance of these insects and produce environmental contamination (Moreira et al., 2016). For the past several decades, the problem of vector resistance to insecticides has developed in many vector-borne disease endemic areas throughout the world. Therefore, new compounds to overcome this problem are being developed. Botanical insecticides are less likely to bio-accumulate, as they are biodegradable (Bosire et al., 2014).

Insecticidal plants and their compounds have been intensively screened in terms of their insecticidal properties (Rajeswary and Govindarajan, 2014; Tennyson et al., 2015; Thongwat et al., 2017). The use of phytochemicals is one such strategy that may be suitable for mosquito control (De Omena et al., 2007; Ribeiro et al., 2009; Garcez et al., 2009). The family Bignoniaceae, order Lamiales (Dahlgren, 1989), consists of about 120 genera and 800 species. Among these species, the chemistry of *Tabebuia* spp. has been extensively studied, following its use in popular medicine, and several biologically active constituents have been isolated, including furanonaphtoquinones, quinones, benzoic acid, cyclopentene dialdehyde, flavonoids, iridoids, phenolic glycosides and naphthoquinones (Alonso, 2004). Other chemical compounds of *Tabebuia* spp. have investigated for their biological properties. Cavalcanti et al. (2015) reported the antimicrobial activity of β-lapachone encapsulated into liposomes against methicillin-resistant *Staphylococcus aureus* and clinical strains of *Cryptococcus neoformans*.

Tabebuia avellanedae has several popular names including *pau d'arco*, *ipê*, *ipêroxo*, *lapacho*, *tahuari*, *taheebo*, trumpet tree, *tabebuia ipê* and *tajy*. It has been used for its anti-inflammatory and antioxidant activities. An anticancer effect of a series of furanonaphthoquinones based on the naphtho [2,3-b] furan-4,9-dione skeleton were detected in water extracts of the inner bark (Zhang et al., 2015).

The constituents (particularly quinones) of several *Tabebuia* spp. have been shown to have potent larvicidal activity against *Toxocara canis in vitro*, indicating the relevance of studies in this area (Santos-Mota et al., 2015). Lapachol, an important representative of the quinone group, is isolated from plants of the Bignoniaceae family.Recent research by Kim et al. (2013) demonstrated high larvicidal activity of methanolic extractsand fractions of the bark of *T. avellanedae* against the mosquito species *A. aegypti*, *Culex pipiens pallens* (Coquillett, 1898) (Diptera: Culicidae) and *Ochlerotatus togoi* (Theobald, 1907) (Diptera: Culicidae).

Studies by Jiménez-González et al. (2013) provided evidence supporting the use of *Tabebuia* species for treating infectious diseases. Nevertheless, little work has been done on activity of *T. avellanedae* wood residues against *A. aegypti* mosquito larvae. Thus, in the present study the larvicidal and oviposition deterrent activities of extracts of *T. avellanedae* (Bignoniaceae) wood residues against 3rd instar larvae of *A. aegypti* were investigated.

MATERIALS AND METHODS

Sample materials

Wood residues from *T. avellanedae* were derived from trees planted in Gurupi, Tocantins, Brazil (11°44'27.05"S latitude, 49°3'52.70"W longitude). Branches containing leaves and flowers from *T. avellanedae* were collected for taxonomic identification. Taxonomic identification of dried samples was confirmed by teacher of Botany, Dr. Rodney Vianna, where it was deposited in the Herbarium of the Nucleus of Environmental Studies of the Federal University of Tocantins (Campus of Porto Nacional), where the specimen voucher was deposited under the code HTO-10000707.

Extraction

Extracts were obtained using maceration and Soxhlet methods, as described below. The classical maceration method (Vieitez et al., 2018) was as follows: Samples of wood residue from *T. avellanedae* (20 g) were placed in a flask and 200 ml of 70% ethanol was added. The solution was then stirred for 24 h. In similar fashion, acetone and ethyl acetate were used as solvents. The Soxhlet method (Silva et al., 2014) was as follows: plant material (wood residue) was milled with Willey-type knives. After pulverization, the material (500 g) was subjected to polarity increasing extraction with three different solvents, first with the apolar solvents ethyl acetate PA (500 ml) and acetone PA (500 ml), and finally the solvent polar ethanol PA (500 ml), separately in a Soxhlet extractor (Marconi, modelMA-487/6/25, Brazil). It should also be noted that, in the present study, solvent selection was based on polarity differences in order to verify and compare its toxic effects on *A. aegypti* larvae. The extracts obtained by the two methods were filtered using 125 mm, n°. 3 filter paper (Whatman), and were concentrated under low pressure in a rotary evaporator (Marconi, model MA120) to remove the solvent. Standard stock solutions were prepared at 1% by dissolving the residues in dimethyl sulfoxide (DMSO). From these stock solutions, various concentrations were prepared and the solutions were used for larvicidal, residual activity, time response and mosquito ovipositor-deterrence bioassays.

Qualitative phytochemical analysis

The phytochemical screening of the acetone, ethanol and ethyl acetate extracts obtained by the Soxhlet method was carried out as described by Matos (2009) and Abate and Mengistu (2018). Extracts (1 mg/ml) were used to detect phytochemicals through qualitative tests, involving precipitation reactions or color development.

Alkaloids

The determination of alkaloids from extracts obtained from the residue of *T. avellanedae* wood was performed according to Abate and Mengistu (2018) with some modifications. For this reaction, 2 ml of alkaline extract were mixed with 1 ml of hydrochloric acid (HCl) and 4 drops of oil in a test tube. Two milliliters of extract, 1 ml of HCl and 4 drops of drag oil were added to second tube, and 2 ml of extract, 1 ml of HCl and 4 drops of Mayer reactive were added to a third tube. The formation of insoluble and flocculent precipitates confirmed the presence of alkaloids.

Anthraquinones

The determination of anthraquinones from extracts obtained from the residue of *T. avellanedae* wood was performed according to Abate and Mengistu (2018) with some modifications. For each extract solution, a 2.0 ml methanolic solution was placed in a test tube and chloroform (5.0 ml) was added and stirred. It was allowed to stand for 15 min. The chloroform phase was collected and divided into two test tubes. In the first tube, 1 ml of 5% aqueous NaOH solution was placed. The purple coloration in the aqueous phase indicated the presence of anthraquinones (Borntraeger Reaction). In the second tube, 1 ml of 5% magnesium acetate solution in methanol was added. Purple staining indicated the presence of free anthraquinones.

Coumarins

The determination of coumarins from extracts obtained from the residue of *T. avellanedae* wood was performed according to Abate and Mengistu (2018) with some modifications. For this reaction, 2 ml of methanolic solution was placed in a test tube with filter solution dissolved with 10% NaOH solution and brought to a 100°C water bath for a few minutes. The filter papers were removed and examined under UV light. Yellow fluorescence indicated the presence of coumarins.

Phenols and tannins

The determination of phenols and tannins from extracts obtained from the residue of *T. avellanedae* wood was performed according to Matos et al. (2009). They were placed in a test tube with 2 ml of alcohol extract and 3 drops of an alcohol solution of Fe_2Cl_3. Precipitates with blue hue indicated the presence of hydrolysable tannins, and green indicated the presence of condensed tannins.

Flavonoids

The determination of flavonoids from extracts obtained from the residue of *T. avellanedae* wood was performed according to Matos et al. (2009).The cyanidin or Shinoda test (concentrated HCl and magnesium) was performed: 2 ml of extract, a piece of metallic magnesium and 2 ml of HCl were added. The end of the reaction was determined by the cessation of effervescence. Red coloration indicated the presence of flavonoids in the extract.

Quantitative determinations of phytochemicals

Total phenolic content and total tannin content

The quantification of the total phenols was performed by the Folin–Ciocalteu method, as described by Amorim et al. (2008) using gallic acid as a standard. Methanolic solutions (0.2 ml) of the extract (1 mg/ml, w/v) or standard (0.1-1.0 µg/ml w/v) aqueous solutions were mixed with the Folin–Ciocalteu reagent (0.5 ml of 10%, v/v), sodium carbonate (1 ml of 75%, w/v) and 8.3 ml of Milli-Q water, gently agitated and maintained for 30 min in the dark. The absorbance was measured at 760 nm in a UV–vis Spectrophotometer (Biospectro® SP-220) equipped with quartz cells of 1 cm path length, calibrated with Milli-Q water. Total phenols were determined by interpolation of the absorbance of the samples against a calibration curve (GAE mg/g) ($y = 1.3644x + 0.0212$; $R^2 = 0.9617$) generated with concentrations of gallic acid standard, expressed as mg gallic acid equivalents (GAE) per gram of extract. To quantify the tannin content, the extract solution (10 ml) was mixed with an equal volume of casein solution (0.1 g/ml). This mixture was stirred for one hour and was then centrifuged at 1358 × g for 10 min at 10°C. In the clear supernatants, the non-tannin phenolics were determined in the way similar to that of the total phenolics. Tannin content was calculated as the difference between total phenolic and non-tannin phenolic content in the extracts. The total tannin content was expressed as milligrams of gallic acid equivalents per gram of *T. avellanedae* extract (mg GAE/g).

Condensed tannin content (CTC)

The determination of the condensed tannin content of the *T. avellanedae* was performed according to the vanillin method of Burns (1971) with some adaptations. Briefly, 2 ml of the extract were mixed with 3 ml of methanol and 2.5 ml of hydrochloric acid (10% in methanol). After 10 min, 2.5 ml of vanillin (1% in methanol) were added. The mixture was heated to 60°C for 10 min, and absorbance was measured at 500 nm. Total condensed tannins were determined by interpolation of the absorbance of the samples against a calibration curve (y = standard, expressed as mg catechin equivalents (CE) per gram of extract (CE mg/g).

Insect culture

A. aegypti mosquitoes were raised in the Entomology Laboratory of the Federal University of Tocantins, Gurupi campus, according to the methodology of Aguiar et al. (2015). Adult mosquitoes were maintained in a 10% aqueous sucrose solution and the blood of live Wistar rats (*Rattus norvegicus albinus*). The larvae were raised in plastic containers (35 cm × 5 cm) and were fed a sterilized diet (80/20 mix of chick chow powder/yeast). All bioassays were conducted at 26 ± 1°C, 60.0 ± 5% RH, with 12 h light-dark photoperiod. All applicable international, national, and institutional guidelines for the care and use of animals were considered.

Larvicidal bioassay

Standard methods for assaying larvicidal activity, as recommended by the World Health Organization (2005), were followed in all experiments with acetone, ethyl acetate and ethanol extracts against the 3rd instar larvae of *A. aegypti*to determine the LC_{50} and LC_{95}. Briefly, a stock solution (1000 µg/ml) of each extract was prepared with 0.5% dimethyl sulfoxide (DMSO) and was then diluted with dechlorinated water to obtain the desired concentrations. The concentrations used were for chloroform and hexane extracts were 33.3, 166.7 and 333.3 µg/ml. For each extract, 0.1 g was weighed and solubilized in 500 µl DMSO and diluted to 29.5 ml distilled water in 200 ml disposable cups. The concentrations used were between 33.3 and 333.3 µg/ml extract for the toxicity bioassay. Assays were carried out in triplicate using 25 larvae for each replicate assay. Mortality was verified after 24 and 48 h exposure of the 3rd instar larvae to extracts. After obtaining the

Table 1. Results of the phytochemical assay of three different extracts obtained from the wood residue of *T. avellanedae* by the maceration and Soxhlet method.

Phytochemicals	Types of extracts		
	Ethyl acetate extract	Ethanol extract	Acetone extract
Phenols	+	+	+
Flavonoids	-	-	-
Flavonones	-	-	-
Alkaloids	-	-	-
Anthraquinones	-	-	-
Coumarins	-	-	-

(+) sign indicates the presence of specific phytochemicals whereas (−) sign indicates the absence.

results, selective bioassays were performed to determine the LC_{50} and LC_{95}. The concentrations used for determination of LC were as follows: ethyl acetate extract: 33.3, 66.7, 100, 166.7, 173.3, 233.3 and 300 µg/ml; Acetone extract: 33.3, 100, 166.7, 200 and 333.3 µg/ml; ethanolic extract: 100, 166.7, 186.7, 333.3, 400, 466 and 500 µg/ml. The LC_{50} and LC_{95} were obtained for each extract and were carried out according to the Probit method (Finney, 1971).

Residual activity of extracts

The measurement of residual activity of acetone, ethyl acetate and ethanol extracts obtained by Soxhlet extraction was performed using a solution corresponding to LC_{95}. Bioassays were made in triplicate with 30 ml solution containing 25 *A. aegypti* 3rd instar larvae in each replicate. To determine residual activity the number of dead larvae, all were removed from the solution after 24 h, and 25 new larvae were added in each recipient with each extract. This process was repeated for seven days. The same procedure was repeated for the control group, with only water and water + DMSO. The percentage mortality was corrected by Abbott's (1925) formula according to Leong et al. (2018):

$$\text{Corrected \%mortality} = [(T - C) / (100 - C)] \times 100$$

Where, T = % mortality in test concentration. C = % mortality in control.

Time response

For each of extract, larvae mortality was initially estimated by LC_{95} response curves. Mortality was recorded after 26-119 min of exposure to extracts of *T. avellanedae* and the lethal response time (LT) activity was reported as LT_{50} and LT_{95}, in minutes. To obtain the LT_{50} and LT_{95} of each extract, we used concentrations of acetone extract at 222.3 µg/ml, ethanol extract at 620 µg/ml and ethyl acetate extract at 319.3 µg/ml.

Mosquito oviposition deterrence

The effect of acetone, ethyl acetate and ethanol extracts of *T. avellanedae* on the oviposition response of female *A. aegypti* was carried out according to previously described methodology (Aguiar et al., 2015). A total of 25 *A. aegypti* females (3 days after blood feeding) were transferred to a cage containing four plastic vessels (10 cm diameter), one containing 30 ml of 0.5% (v/v) DMSO solution in distilled water (control) and the others containing 30 ml of extract solution at concentrations of 33.3, 166.7 or 333.3 µg/ml. These concentrations were determined from the toxicity test. The vessels were placed at diagonally opposite corners of the cage. A piece of filter paper was placed in each vessel as a support for oviposition. The females were maintained at 27 ± 0.5°C with 70 ± 10% relative humidity for 14 h in the dark. After this period, the eggs deposited in each vessel were manually counted with the aid of a stereomicroscope. The test was repeated three times. The eggs were counted 24 and 48 h after treatment. The oviposition-inhibition percentage was calculated as described by Mulla et al. (1974).

Statistical analysis

From the mortality data, each value of LC_{50} and LC_{95} and LT_{50} and LT_{95} was estimated according to Finney's probit method, using the program Polo Plus. The repellency and emergence data were corrected using the Abbott formula (Abbott, 1925). The mean values and standards deviations were calculated from replication data. One-way analysis of variance (ANOVA) was used to determine the significance of the treatments and means were determined by Tukey's test comparisons using SISVAR 4.6 (Ferreira, 2011). Significant differences were considered when $p< 0.05$.

RESULTS

Phytochemical screening of *T. avellanedae* wood residue extracts revealed the presence of tannins and phenols (Table 1). We found no other metabolites such as alkaloids, flavonoids, steroids, anthraquinones or coumarins. After phytochemical screening performed quantitative analysis of phenolic compounds in acetone, ethanol and ethyl acetate extracts.

Total phenolic content (TPC)

The quantification of total phenols in the extracts obtained from the wood residue of *T. avellanedae* showed that phenolic compounds were present in significant quantities. For the acetone, ethanol and ethyl acetate extracts by the Soxhlet method, the values ranged from 237.272 ± 6.770 to 57.461 ± 5.863 mg

Table 2. Analysis content of phenolic compounds and tannins present in wood *T. avellanedae* by the Soxhlet method.

Extracts	Phenolic compounds (mg GAE/g)	Phenolic compounds (%)	Total tannins	Total tannins (%)	Condensed tannins (mg CE/g)	Condensed tannins (%)
Ethyl acetate	57.461±5.863	5.75	16.075±5.44	1.61	8.116±0.916	0.81
Acetone	237.272±6.770	23.73	182.69±5.65	18.27	14.196±0.397	1.42
Ethanol	98.505±5.863	9.85	75.442±2.90	7.54	10.099±0.229	1.01

Values were expressed as mean ± S.D. (n = 3); GAE = gallic acid equivalent; CE = catechin equivalent.

Table 3. Results of concentration response ($LC_{50}$and LC_{95}) values of the acetone, ethanol and ethyl acetate extracts of *T. avellanedae* against3rd instar larvae of *A. aegypti*.

Extraction process	Extracts	Slope±SEM	LC_{50} (µg mL^{-1})	CI(LC_{50}) (µg mL^{-1})	LC_{95} (µg mL^{-1})	CI(LC_{95}) (µg mL^{-1})	X^2	P
Soxhlet	Acetone	4.804±0.86	100.1	80.0–118.3	222.3	183.3–312.0	1.43^{ns}	0.0581
	Ethanol	3.13±0.45	185.0	125.3–249.3	620.6	159.9–412.3	5.06^{ns}	0.2750
	Ethyl acetate	4.537±0.70	151.0	132.0–172.7	319.3	262.7–437.3	3.71^{ns}	0.0271
Maceration	Acetone	4.32±0.60	1.499	1.33 – 1.67	3.599	2.99 – 5.33	4.17^{ns}	0.0001
	Ethanol	5.93±1.03	4.633	4.33 – 5.33	8.766	6.99 – 13.33	3.53^{ns}	0.0001
	Ethyl acetate	-	-	-	-	-	-	

SEM: standard error of the mean; LC_{50}: lethal concentration with 50% mortality; LC_{95}: lethal concentration with 95% mortality; CI: Confidence interval the probability of 95%; (1) lower limit of the confidence interval at 95% probability (LCL); (2) upper limit of the confidence interval at 95% probability (UCL); X^2: chi square; Was not significant to the chi-square test ($p < 0.05$). -: Not detected.

GAE/g (Table 2). Thus, the acetone extracts had a higher percentage of total phenolic compounds with 23.73%, followed by the ethanolic extract with 9.85% and the ethyl acetate extract with 5.75%.

Condensed tannin content (CTC)

The acetone extract gave 14.196 mg CE/g ± 0.397, followed by the ethanolic extract with 10.099 mg CE/g ± 0.229 and the ethyl acetate extract with 8.116 mg CE/g ± 0.916. The acetone extracts showed maximal amounts of extractable tannin content as compared to other solvents (Table 2). Among the extracts, acetone presented a larger amount of condensed tannins with 1.42%, followed by ethanol 1.01% and ethyl acetate with 0.81%.

Larvicidal activity

The biological activities of acetone, ethanol and ethyl acetate against the 3rd instar larvae of *A. aegypti* were measured in extracts obtained by maceration and Soxhlet methods. In Table 3 we listed the values of LC_{50} and LC_{95} for all extracts that had larvicidal activity against 3rd instar larvae of *A. aegypti* after 24 h. The extracts obtained by the maceration method showed the following LC_{50} and LC_{95} (Table 3): acetone extract values were 1.499 µg/ml (1.33-1.67) and 3.599 µg/ml (2.99-5.33), respectively; ethanolic extract values were 4.333 µg/ml (4.33-5.33) and 8.766 µg/ml (6.99 –13.33), respectively. The extracts obtained by Soxhlet extraction showed the following LC_{50} and LC_{95} (Table 3): the acetone extract had an LC_{50} value of 100.1 µg/mL (80.0-118.3) and a LC_{95} of 222.3 µg/ml (183.3-312.0). The ethyl acetate extract had promising LC_{50} and LC_{95} values of 151.0 µg/ml (132-172.7) and 319.3 µg/ml (262.7-437.3), respectively, after 24 h against *A. aegypti*.

Time-mortality response

Lethal time (LT_{50} and LT_{95}) of *A. aegypti* exposed to acetone, ethanol and ethyl acetate extracts of *T. avellanedae* obtained by the Soxhlet method were obtained using the LC_{95} concentrations determined for 3rd instar larvae of *A. aegypti* (Table 4). The times required to achieve 50 and 95% mortality of the ethanolic extract were 26.22 and 54.51 min, respectively for of *A. aegypti* 3rd instar larvae; for ethyl acetate extract the values were 38.66 and 66.74 min, respectively; and for acetone extracts the values were 53.47 and 119.96 min, respectively (Table 4).

Residual activity of extracts

Measurement of residual activity of extracts of *T.*

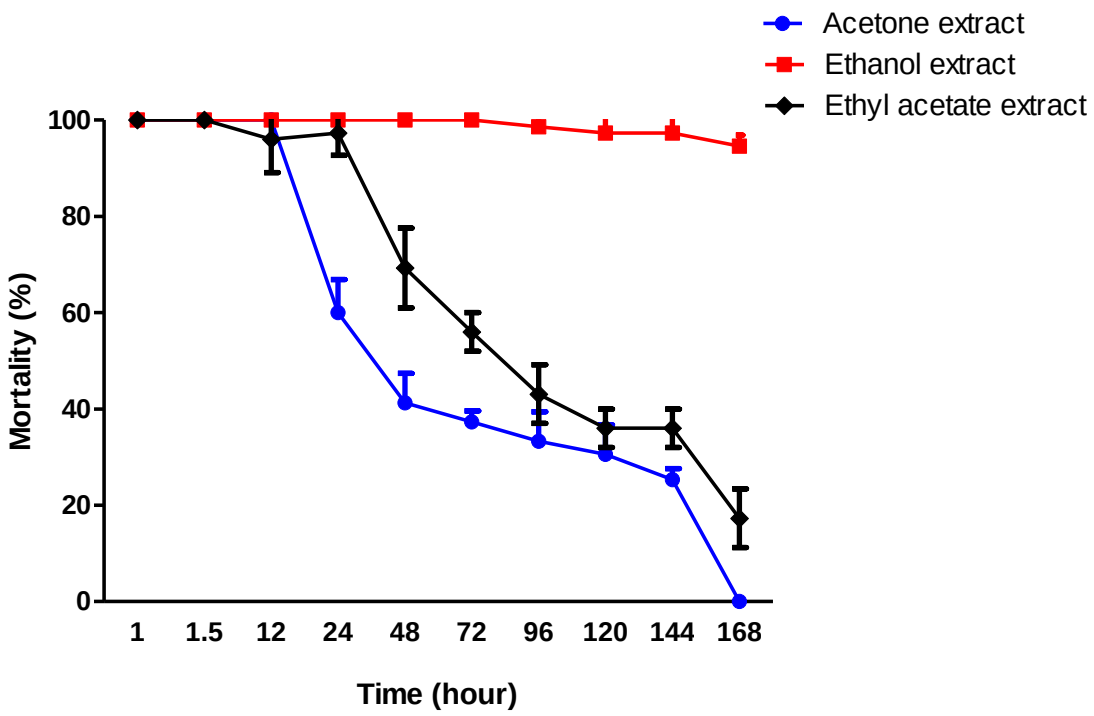


Figure 1. Residual activity of the acetone, ethanol and ethyl acetate extracts from *T. avellanedae* wood residueagainst 3rd instar larvae of *A. aegypti*. Values are mean of three replications ± SE. Treatments followed by the same capital letter in the column did not differ among themselves by the Tukey test (p <0.05).

Table 4. LT_{50} and LT_{95} values of extracts of *T. avellanedae* against 3rd instar larvae of *A. aegypti* using the LC_{95}.

Extracts	Slope±SEM	LT_{50} (min)	CI (LT_{50})	LT_{95} (min)	CI (LT_{95})	X^2	P
Acetone	8.38±1.88	53.47	45.87 – 59.02	119.96	95.54 – 209.92	0.25^{ns}	0.0050
Ethanol	9.26±1.96	26.22	23.98 – 29.13	54.51	42.98 – 95.37	0.20^{ns}	0.3953
Ethyl acetate	12.41±2.79	38.66	3.59 – 41.37	66.74	57.44 – 96.0	0.14^{ns}	0.1230

SEM: standard error of the mean; LT50: lethal time of lethal concentration with 50% mortality; LT95: lethal time of lethal concentration with 90% mortality; CI: Confidence interval the probability of 95%; (1) lower limit of the confidence interval at 95% probability; (2) upper limit of the confidence interval at 95% probability; X^2: chi square; Was not significant to the chi-square test (p<0.05).

avellanedae obtained by the Soxhlet method showed that mortality increased with increasing exposure time up to 12 h forall extracts tested (Figure 1). The ethanol extract at LC_{95} of 412.3 µg/ml induced rapid mortality for 3rd instar larvae with residual activity of 100% mortality for a period of 96 h of exposure. The ethyl acetate extract (LC_{95} = 319 µg/ml) residual activity promoted 100% mortality for a period of 24 h. The ethanol solution induced 98.6 ± 2.3% larval mortality rate in the first 120 h. After 168 h, the ethanolic extract was still killing at 82.6% (Figure 1). During the seven-day period, the ethyl acetate extract showed a mortality rate of 64.8 ± 37.1% (Figure 1).

Oviposition deterrent

The oviposition deterrence activity of extracts of *T. avellanedae* obtained by the Soxhlet method against *A. aegypti* is presented in Figure 2. The ethyl acetate extract (EAE) (at 33.3, 166.7 and 333.3 µg/ml) strongly deterred oviposition by *A. aegypti*, with a significantly lower proportion of eggs being laid on ovitraps containing extracts in comparison with controls ($p < 0.01$) (Figure 2). The percentage of effectiveness demonstrated by acetone, ethyl acetate and ethanolic extracts against oviposition were 68.10, 89.04 and 89.89% at 333.3 µg/ml, respectively. The acetone extract (AE) and ethanol extract (EE) were not statistically significant at all concentrations tested for oviposition deterrent activity in comparison with controls.

DISCUSSION

Maceration and Soxhlet extraction were used for wood

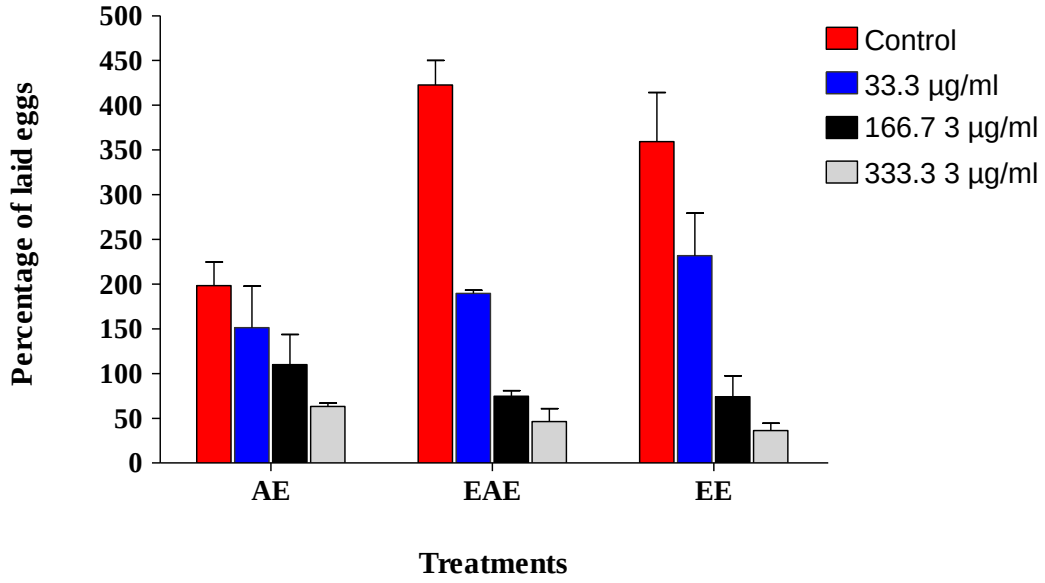


Figure 2. Effect of various concentrations of acetone, ethyl acetate and ethanolic extracts from *T. avellanedae* wood residue on oviposition by adult *A. aegypti* females.

residues of *T. avellanedae*. Solvent extraction is generally used for the preparation of plant material extracts because of its wide applicability, efficiency and ease of use. Most common organic solvents used for the extraction of phenolic compounds include methanol, ethanol, acetone, and ethyl acetate (Ross et al., 2009). Conventional solid liquid extraction techniques such as maceration are mostly used for obtaining bioactive compound extracts from plant material (Belova et al., 2009). However, conventional solvent extraction processes have certain limitations such as lower efficiency, low extraction yield, use of large quantity of solvents and mass transfer resistance (Jadhav et al., 2009).

Conventional Soxhlet extraction has some attractive advantages. The sample is repeatedly brought into contact with fresh portions of extractant, facilitating displacement of the transfer equilibrium. In addition, the system remains at a relatively high temperature by effect of the heat applied to the distillation flask reaching the extraction cavity to some extent. Finally, no filtration is required after leaching and sample throughput can be increased by performing several simultaneous extractions in parallel, facilitated by the low cost of the basic equipment (Castro and Priego-Capote, 2010).

The phytochemical screening of *T. avellanedae* compounds present in our acetone, ethyl acetate and ethanol extracts showed the presence of phytochemicals as well as total phenols and tannin compounds. In another species of the plant (*Tabebuia serratifolia*), Duarte et al. (2015) reported the presence of reducing sugars, organic acids, alkaloids, anthraquinones, depsides and depsidones, catechins, purines, foamy saponins and phenols and tannins in the ethanolic extract of the leaves. Reports in the literature demonstrated that plants of the Bignoniaceae family have a variety of classes of chemical constituents including quinolines, lignans, flavonoids, monoterpenes (mainly iridoids), triterpenes, cinnamic and benzoic acids (Von Poser et al., 2000).

In the present study, the values of total phenolic compounds were 237.27, 98.50 and 57.46 mg GAE/g in acetone, ethanol and ethyl acetate extracts, respectively (Table 3). Similar results were found by Salar and Seasotiya (2011), who investigated the total phenolic content of various extracts of *Butea monosperma* and *Hugoniamystax* and found that total phenolics ranged from 45-141 mg GAE/g in stem bark 262.2 ± 0.96 mg GAE/g in roots, respectively.

In this regard, the amount of phenols found in the wood residues of *T. avellanedae* in this study was higher. According to Romagnoli et al. (2013) this total amount of quinones and phenols was higher. The differences in the amounts of phenolic content may be associated with the agro-climatic conditions that were solely responsible for the occurrence of variable amounts of bioactive components in natural resources (Dhull et al., 2016).

Zarin et al. (2016) reported that of the total phenolic content of crude extracts from *Leucaena leucocephala* was 3.21 mg GAE/g extract, while purified condensed tannins were obtained at 2.06 mg GAE/g extract. Dhull et al. (2016) identified the presence of condensed tannin content in leaf extracts. Three solvents (ethanol, acetone and chloroform) were used to extract condensed tannin content from *Origanum majorana*. The authors found that the extract prepared with acetone gave the maximal

amount of extractable tannin content as compared to other solvents (6.02 mg CE/g). By contrast, in the present study, the maximum amount of extractable CTC was 14.196 mg CE/g (1.42%) in the acetone extract.

The results may differ depending on the type of plant, plant part, extraction temperature, extraction time and extraction phase used for recovery of bioactive compounds as well as the type of solvent used for extraction (Azwanida, 2015). This assertion can be confirmed through our studies with the various extracts (acetone, ethyl acetate and ethanol). Earlier studies reported that saponins, flavonoids and tannins were active insecticidal compounds. Generally, flavonoids and tannins are toxic phenolics that disrupt cellular structure (Julia et al., 2018).

The results we obtained for *T. avellanedae* revealed a chemical composition similar to or different from other studies. Romagnoli et al. (2013) reported an amount of lapachol in the wood extract of *T. serratifolia* of 27% (33.58 mg/g). Some authors reported the presence of lapachol, lapachol, α- and β-lapachone, in addition to furanonaphthoquinones isolated from several species of the genera *Tabebuia*, *Tecoma* and *Handroanthus* (Burnett and Thomson, 1968; Oliveira et al., 1990; Hussain et al., 2007). According to Oliveira et al. (1990), the species *Tabebuia incana*, family Bignoniaceae, there was another chemical constituent, 2-ethyl-5-hydroxy-naphtho [2,3-b] furan-4,9-quinone.

In another species from the same family, *Tabebuia ochracea*, lapachol (0.001%) and seven furanonaphthoquinones including 2- (1'-hydroxyethyl) naphtho [2,3-b] furan-4,9- dione were found in trunks. In the study with *T. serratifolia* using chromatographic fractionation of the ethanolic extracts of the wood, several substances were isolated, includingdehydro-α-lapachone, β-sitosterol, β-sitosterol glycoside, 4-hydroxy-3-methoxy-benzoic acid and lapachol. Our results for lapachol were higher than those of the hexanic and chloroform extracts (33.42 and 31.6%, respectively) in the cited studies.

Sensitivity of mosquitoes to various insecticides are highly variable. Jeon et al. (2011) carried out an insecticidal effect study using the topical active constituent of the *T. avellanedae* bark against *Nilaparvata lugens* and the small brown grasshopper, *Laodelphax striatellus*. In that study, the authors concluded that the constituents 2-hydroxy-3- (3-methyl-2-butenyl) -1,4-naphthoquinone and its derivatives had potential as new agents and that insecticidal activities of *T. avellanedae* methanolic extract against *N. lugens* and *L. striatellus* showed a significant dose-response relationship for toxicity in both insect species.

The extracts obtained by the maceration method showed that the LC_{50} and $LC_{95}$of acetone extract values were 1.49 and 3.59 µg/ml, respectively and for ethanolic extracts the values were 4.33 and 8.76 µg/ml, respectively. All the extracts obtained in the cold gave lower values forLC_{50} and LC_{95} than for extracts obtained by the Soxhlet method. According to Komalamisra et al. (2005), larvicidal activity can classified as follows: an extract is considered efficient when LC_{50} is less than 750 ppm; activity is moderate when LC_{50} is between 50 and 100 ppm and it is high when LC_{50} is less than 50 ppm. Thus, according to our results, the acetone extract obtained from wood residue is considered to have moderate activity, and those obtained from ethanol, methanol and ethyl acetate extracts were efficient. The acetone and ethanol extracts obtained by maceration can be classified as having high larvicidal activity.

Indeed, stirring in the maceration method accelerated the extraction process, minimizing the contact time with the extracting solvent and preserving the bioactivity of the constituents. In addition, extraction at room temperature and the removal of the solvent at low pressure yielded the maximum amounts of compounds (Khoddami et al., 2013). In this context, various plant extracts of *Coleus aromaticus* leaf extract, lichen *Ramalina usnea* (L.) (Ramalinaceae) were tested as potential mosquito larvicides (Baranitharan et al., 2017; Moreira et al., 2016). The larvicidal efficacies of the tested extracts were dose-dependent. The biological activity of plant extracts is generally known to be due to the presence of various bioactive phytomolecules present in the plant, including alkaloids, terpenoids and phenolics (Vindhya et al., 2014; Francine et al., 2016).

This can be compared to our study in that the larvicidal efficacies of our tested extracts were also dose-dependent. The results from our toxicity assays demonstrated that the extracts of *T. avellanedae* had strong toxicity for *A. aegypti* larvae and depended on exposure time and concentrations used. Nevertheless, the differences in toxicity expressed by the various plant species and extracts of the same plant may be due to quantitative and qualitative variations in the chemical composition of the ethanolic extracts of *T. avellanedae* that induced full mortality (98.6%) of *A. aegypti* larvae within 96 h of exposure with a CL_{95} of 706 µg/ml.

We evaluated the larvicidal effects of *T. avellanedae* against *A. aegypti* at various concentrations for each extract obtained. The ethyl acetate extract exhibited high larvicidal activity (69.32%) at LC_{95} (319.3 µg/ml) for 48 h post-exposure. The use of quinolines in the treatment of bacterial pathogens, as well as in the defense against pathogens (allopathic and antimicrobial activity), have been shown to play important roles in several living organisms as cofactors of proteins in electron transport (Burnett and Thomson, 1968; McKallip et al., 2010) reviewed the efficacy of quinones that could be attributed to the strongly electrophilic character of the naphthoquinones that promoted their reactivity with thiol groups on proteins. Studies such as that of Pradeep et al. (2015) suggested that isoquinoline alkaloids were natural potential mosquito larvicides. Hussain et al. (20017) reported that lapachol and its enamine showed larvicidal and insecticidal activity against *Artemia salina* and *A.*

aegypti. In this way, we can suggest that the larvicidal activity measured in our extracts was related to lapachol.

The rapid and significant mortality ($p<0.05$) suggested potent insecticidal activity of wood extracts (acetone, ethyl acetate and ethanol) against larvae of *A. aegypti.* Similar time-response results were obtained from essential oils and extracts from other plant species. The essential oil of *S. guyanensis* at LC_{95} concentrations against *A. aegypti* and *Culex quinquefasciatus* required less than 33 min to kill 95% of insects (Aguiar et al., 2015; Soonwera and Phasomkusolsi, 2017). The response time was much lower than that of aqueous extracts of the seed kernel of *Azadirachta indica* A. Juss (Neem) tested on *A. aegypti* larvae that delivered 100% (LC_{100}) mortality at all concentrations used in the present study at 144 h (LT_{100}) (Ndione et al., 2007).

Species of the genus *Tabebuia* have been used empirically as anti-inflammatory agents, anti-cancer agents and antimicrobials in rural areas of the Colombia, Bolivia, Brazil and other countries in Latin America. Additionally, the constituents of several *Tabebuia* spp. have been shown to be potent insecticides (Jeon et al., 2012).

Extracts of *T. avellanedae* proved to be oviposition-deterrents against *A. aegypti*. The ethanol extract reduced oviposition of *A. aegypti* significantly ($p<0.01$). Previously, some investigators reported an oviposition deterrent effect of plant extracts against vector mosquitoes. Coria et al. (2008) reported a 100% oviposition deterrent effect obtained with *Melia azedarach* L. leaf ethanol extract at 1 g/L concentration against *A. aegypti*.

Autran et al. (2009) reported anoviposition deterrent effect of essential oils obtained from leaves, inflorescences, and stems of *Piper marginatum*. Leaves and stems of *P. marginatum* exhibited an oviposition deterrent effect at 50 and 100 ppm in that significantly lower numbers of eggs (<50%) were laid in glass vessels containing test solutions compared with those containing control solution.

The choice of oviposition sites by gravid female mosquitoes is guided by several factors. Initially, visual and olfactory cues are employed to find potential sites, after which the suitability of the location is verified by chemical and physical factors according to appropriate receptors distributed along the body of the mosquito. Clearly, when oviposition deterrents are detected, few, if any eggs are laid at the site (Day, 2016).

The oviposition repellent effect is a relevant property to be considered in the selection of a plant extracts for the control of vector insects. In general, the higher the repellency, the lower the infestation, resulting in reduction or suppression of oviposition, a reduced number of insects emerging and a decrease in cases of dengue.

Further studies on the larvicidal mode of action of *T. avellanedae* wood residue, their effects on non-target organisms and the environment, and formulations for improving the insecticidal potency and stability are needed to support their practical use as naturally occurring mosquito larval control agents. Nevertheless, wood residues of *T. avellanedae* can be an alternative source of mosquito control agents because they are a rich source of bioactive chemicals.

Conclusion

The present study clearly demonstrated that acetone, ethyl acetate and ethanol extracts from *T. avellanedae* had substantial larvicidal and oviposition repellency properties against *A. aegypti*. The presence of bioactive compounds in *T. avellanedae* makes it a potential source for the control of *A. aegypti* vectors, without promoting deforestation of trees.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Council of Scientific and Technological Development (CNPq-MCTI/CNPQ/CAPES/FAPS Nº 16/2014 - PROGRAMAINCT), the State Tocantins Foundation for Research (FAPT/TO- N° - 201420300 0034), SUDAM (project number nº 771756/2011) and the Graduate School, Bionorte Network (Biodiversity and Biotechnology).

REFERENCES

Abate L, Mengistu T (2018). Phytochemical screening and peroxide value determination of methanolic extract of four traditional medicinal plants. Journal of Medicinal Plants Research 12(16):203-208.Journal of Medicinal Plants Research 12(16):203-208.

Aguiar RWS, dos Santos SF, da Silva MF, Ascencio SD, de Mendonça LM, Viana KF, Didonet J, Ribeiro BM (2015). Insecticidal and Repellent Activity of *Siparuna guianensis* Aubl. (Negramina) against *Aedes aegypti* and *Culex quinquefasciatus*. PLoS ONE 10(2):e0116765.

Alonso J (2004). Tratado de Fitofarmacos y Nutraceuticos. Tratado de Fitofármacos y Nutracéuticos P 472.

Amorim ELC, Nascimento JE, Monteiro JM, Peixoto Sobrinho TJS, Araújo TAS, Albuquerque UP (2008). A simple and accurate procedure for the determination of tannin and flavonoid levels and some applications in ethnobotany and ethnopharmacology. Functional Ecosystems and Communities 2(1):88-94.

Autran ES, Neves IA, da Silva CSB, Santos GKN, da Câmara CAG, Navarro DMAF (2009). Chemical composition, oviposition deterrent and larvicidal activities against Aedes aegypti of essential oils from Piper marginatum Jacq.(Piperaceae). Bioresource Technology 100 (7): 2284-2288.

Azwanida NN (2015). A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. Medicinal Aromatic Plants 4(3):3-8.

Baranitharan M, Dhanasekaran S, KovendanK, Murugan KJ, Gokulakrishnan J, Giovanni Benelli G (2017).*Coleus aromatic* sleaf extract fractions: A source of novel ovicides, larvicides and repellents against Anopheles, Aedesand Culex mosquito vectors? Process

Safety and Environmental Protection 106:23-33.
Belova V, Voshkin A, Kholkin A, Payrtman A (2009). Solvent extraction of some lanthanides from chloride and nitrate solutions by binary extractants. Hydrometallurgy 97(3-4):198-203.
Bosire CM, Deyou T, Kabaru JM, Kimata DM, Yenesew A (2014).
Larvicidal activities of the stem bark extract and rotenoids of *Millettia usaramensis* subspecies usaramensis on *Aedes aegypti* L. (Diptera: Culicidae). Journal of Asia-Pacific Entomology 17:531-535.
Burnett AR. Thomson RH (1968). Naturally occurring quinones. Part XII. Extractives from *Tabebuia chrysantha* nichols and other bignoniaceae. Journal of the Chemical Society C: Organic pp. 850-853.
Burns RE (1971). Method for estimation of tannin in grain sorghum. Agronomy Journal 63(3):511-512.
Castro MDL, Priego-Capote F (2010). Soxhlet extraction: Past and present panacea. Journal of Chromatography A, 1217:2383-2389.
Cavalcanti MF, Pontes-NetoJN, Kocerginsky POAM, Bezerra-Neto AM, LIMA JLC., Lira-Nogueira MCB, Maciel MAV, Neves RP, Pimentel MF, Santos-Magalhães NS (2015). Antimicrobial activity of β-lapachone encapsulated into liposomes against methicillin-resistant *Staphylococcus aureus* and *Cryptococcus neoformans* clinical strains. Journal of Global Antimicrobial Resistance 3(2):103-108.
Coquillett DW (1898). Report on a collection of Japanese Diptera, presented to the United States National Museum by the Imperial University of Tokyo. US Government Printing Office 21:1146.
Coria C, Almiron W, Valladares G, Carpinella C, Ludueña F, Defago M, Palacios S (2008). Larvicide and oviposition deterrent effects of fruit and leaf extracts from *Melia azedarach* L. on *Aedes aegypti* (L.) (Diptera: Culicidae). Bioresource Technology 99 (8):3066-3070.
Dahlgren G (1989). The last Dahlgrenogram. System of classification of the dicotyledons. The Davis and Hedge Festschrift 249-260.
Day JF (2016). Mosquito oviposition behavior and vector control. Insects 7(4):65.
De Omena MC, Navarro DMAF, De Paula JE, Luna JS, Ferreira de Lima MRF, Sant'Ana AEG (2007). Larvicidal activities against *Aedes aegypti*of some Brazilian medicinal plants. Bioresource Technology 98(13):2549-2556.
Dhull SB, Kaur P, Purewal SS (2016). Phytochemical analysis, phenolic compounds, condensed tannin content and antioxidant potential in Marwa (*Origanum majorana*) seed extracts. Resource-Efficient Technologies 2(4):168-174.
Duarte JL, Mota LJT, De Almeida SSM (201514). Análise fitoquímica das folhas de *Tabebuia serratifolia* (Vahl) Nicholson (Ipê Amarelo). Estação Científica (UNIFAP) 4(1):33-43.
Ferreira DF (2011). Sisvar:um sistema computacional de análise estatística.Ciência e Agrotecnologia 35(6):1039-1042.
Finney DJ (1971). Probit analisis. 3rd. Edition. Cambridge University Press. Great Britain P 333.
Francine TN, Cabral BNP, Constant Anatole PC, Bruno M, Pauline N, Jeanne NY (2016). Larvicidal activities of hydro-ethanolic extracts of three Cameroonian medicinal plants against *Aedes albopictus*. Asian Pacific Journal of Tropical Biomedicine 6(11): 931–936.
Garcez WS, Garcez R, Da Silva LMGE (2009). Hamerski L. Larvicidal activity against *Aedes aegypti* of some plants native to the West-Central regon of Brazil. Bioresource Technology 100(24):6647-6650.
Govindarajan M, Sivakumar R (2014). Larvicidal, ovicidal, and adulticidal efficacy of Erythrina indica (Lam.) (Family: Fabaceae) against *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* (Diptera: Culicidae). Parasitology Research 113(2):777-791.
Hussain H, Krohn K, Ahmad VU, Miana GA, Green IR (2007). Lapachol: Anna overview. Arkivoc 2:145-171.
Jeon JH, Oh MS, Lee HS (2011). Insecticidal Effects of *Tabebuia avellanedae*-derived Constituent and Its Analogues against *Nilaparvata lugens* and *Laodelphax striatellus*. Journal of the Korean Society for Applied Biological Chemistry 54(5):822-826.
Jiménez-González FJ, Veloza LA, Sepúlveda-Arias JC (2013). Anti-infectious activity in plants of the genus Tabebuia. Universit as Scientiarum 18(3):257:267.
Julia AE, Gregory OE, Felicia E, Michael U, Godwin N, Chinelo E (2018). Bioefficacy of *Duranta erecta* leaf extract on yellow fever and dengue vector, *Aedes aegypti i*Linn. in Nigeria. Journal of Medicinal Plants Research 12(11):124-132.
Khoddami A, Wilkes MA, Roberts TH (2013). Techniques for analysis of plant phenolic compounds. Molecules 18: 328-75.
Kim MG, Jeon JH, Lee HS (2013). Larvicidal activity of the active constituent isolated from *Tabebuia avellanedae* bark and structurally related derivatives against three mosquito species. Journal of Agricultural and Food Chemistry 61(45):10741-10745
Komalamisra N, Trongtoki Y, Rongsriyam Y, Apiwathnasorn C (2005). Screening for larvicidal activity in some Thai plants against four mosquito vector species. Southeast Asian Journal Tropical Medicine Public Health 36(6):1412-1422.
Kumar K, Singh PK, Tomar J, Baijal S (2010). Dengue: epidemiology, prevention and pressing need for vaccine development. Asian Pacific Journal of Tropical Medicine 3(12):997-1000.
Leong CS, Vythilingam I, Wong ML, SulaimanW-Y W, LauYL (2018). *Aedes aegypti* (Linnaeus) larvae from dengue outbreak areas in Selangor showing resistance to pyrethroids but susceptible to organophosphates. Acta Tropica 185:115-126.
Matos FJA (2009). Introdução a fitoquímica experimental. 3. ed. Fortaleza: Edições UFC P 150.
Mayer SV, Tesh RB, Vasilakis N (2017).The emergence of arthropod-borne viral diseases: A global prospective on dengue, chikungunya and zika fevers. Acta Tropica 166:155-163.
McKallip RJ, Lombard C, Sun J, Ramakrishnan R (2010). Plumbagin-induced apoptosis in lymphocytes is mediated through increased oxygen species production, upregulation of Fas, and activation of the caspase cascade. Toxicology and Applied Pharmacology 247: 41-52.
Moreira ASN, Fernandes ROS, Lemos FJA, Braz-Filho R, Vieira IJC (2016). Larvicidal activity of *Ramalina usnea* lichen against *Aedes aegypti*. Revista Brasileira de Farmacognosia 26(4):530-532.
Mulla MS, Norland RL, Ikeshoji T, Kramer WL (1974). Insect growth regulators for the control of aquatic midges. Journal of Economic Entomology 67(2):165-170.
Ndione RD, Faye O, Ndiaye M, Dieye A, Afoutou JM (2007). Toxic effects of neem products (*Azadiracht aindica* A. Juss) on *Aedes aegypti* Linnaeus 1762 larvae. African Journal of Biotechnology 6(24):2846-2854.
Olagnier D, Amatore D, Castiello L, Ferrari M, Palermo E, Diamond MS, Palamara AT, Hiscott J (2016). Dengue Virus Immunopathogenesis: Lessons Applicable to the Emergence of Zika Virus. Journal of Molecular Biology 428(17):3429-3448.
Oliveira AB, Raslan DS, Miraglia MCM, Mesquita AAL, Zani CL, Ferreira DT, Maia JGS (1990). Estrutura química e atividade biológica de naftoquinonas de bignoniáceas brasileiras. Química Nova 13(4):302-307.
Pradeepa FS, Palaniswamya M, Ravi S, Thangamani A, Pradeepa BV (2015). Larvicidal activity of a novel isoquinoline type pigment from *Fusarium moniliforme* KUMBF1201 against *Aedes aegypti* and *Anopheles stephensi*. Process Biochemistry 50(9):1479-1486.
Rajeswary M, Govindarajan M (2014). Adulticidal properties of *Pithecellobium dulce*(Roxb.) Benth. (Family: Fabaceae) against dengue vector, *Aedes aegypti* (Linn.) (Diptera: Culicidae). Asian Pacific Journal of Tropical Disease 4(Suppl 1):S449-S452.
Ribeiro KAL, De Carvalho CM, Molina MY, Lima EP, López-Montero E, Reys JRM, De Oliveira MBF, Pinto AV, Santana AEG, Goulart MO (2009). Activities of naphthoquinones against *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae), vector of dengue and *Biomphalaria glabrata* (Say, 1818), intermediate host of *Schistosoma mansoni.* Acta Tropica 111(1):44-50.
Romagnoli M., Segoloni E, Luna M., Margaritelli A, Gatti M., Santamaria U, Vinciguerra V (2013). "Wood colour in Lapacho (*Tabebuia serratifolia*): Chemical composition and industrial implications". Wood Science and Technology 47(4):701-716.
Ross K, Beta T, Arntfield S (2009). A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods. Food Chemistry 113(1):336-344.
Salar RK, Seasotiya L (2011). Free radical scavenging activity, phenolic contents and phytochemical evaluation of different extracts of stem bark of *Butea monosperma* (Lam.) Kuntze., Frontiers in Life Science , 5(3-4):107-116.

Santos-Mota Mata-Santos T, Pinto NF, Mata-Santos HÁ, Moura KG, Carneiro PF, Carvalho TS, Del Rio KP, Pinto MCFR, Martins LR, Fenalti JM, Da Siva PEA, Scaini CJ (2015). Anthelmintic activity of lapachol, β-lapachone and its derivatives against *Toxocara canis* larvae. Revista do Instituto de Medicina Tropical de São Paulo 57(3):197-204.

Silva CB, Rech KS, Ocampos FMM, Dalarmi L, Jasinski VCG, Dias JFG, Warumbi Zanin SM, Kerber VA, Kulik JD, Fujiwara GM, De Oliveira M, Miguel OG, Miguel MD (2014). Byrsonima intermedia preparations inhibits trypsin and chymotrypsin activities from *Aedes aegypti* larval gut. Journal of Medicinal Plants Research 8(9):416-422.

Soonwera M, Phasomkusolsil S (2017). Adulticidal, larvicidal, pupicidal and oviposition deterrent activities of essential oil from *Zanthoxylum limonella* Alston (Rutaceae) against *Aedes aegypti* (L.) and *Culex quinquefasciatus* (Say) (2017). Asian Pacific Journalof Tropical Biomedicine 7(11):967-978.

Tennyson S, Ravindran J, Eapen A, William J (2015). Activity of *Ageratum houstonianum* Mill. (Asteraceae) leaf extracts against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae). Asian Pacific Journal of Tropical Disease 5(3):199-203

Theobald FV (1907). A Monograph of the Culicidae, Or Mosquitoes: Mainly Compiled from the Collections Received at the British Museum from Various Parts of the World in Connection with the Investigation Into the Cause of Malaria Conducted by the Colonial Office and the Royal Society (Vol. 4). order of the Trustees.

Thongwat D, Lamlertthon S, Pimolsri U, Bunchu N (2017). Larvicidal activity of endocarp and seed crude extracts of *Dracaena loureiri* Gagnep against *Aedes aegypti* (L.) mosquito. Asian Pacific Journal of Tropical Biomedicine 7(3):222-226.

Vieitez I, Maceiras L, Jachmanián I, Alborés S (2018). Antioxidant and antibacterial activity of different extracts from herbs obtained by maceration or supercritical technology. The Journal of Supercritical Fluids 133:58-64.

Vindhya K, Sampath Kumara KK, Neelambika HS, Leelavathi S (2014). Preliminary phytochemical screening of *Gardenia latifolia* Ait. and *Gardenia gummifera* Linn. Research Journal of Pharmaceutical, Biological and Chemical 5(2):527-532.

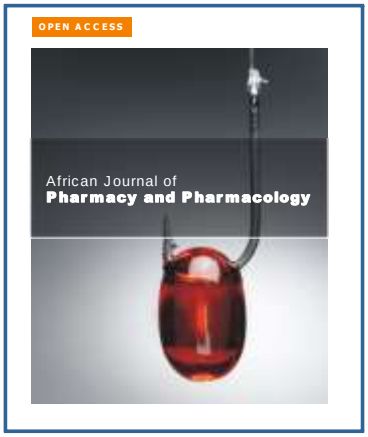
Von Poser GL, Schripsema J, Henriques AT, Jensen SR (2000). The distribution of iridoids in Bignoniaceae. Biochemical Systematics and Ecology 28(4):351-366.

World Health Organization (2005).Guidelines for laboratory and field testing of mosquito larvicides. Geneva: WHO.

Zarin AM., Wan HY, Ishsa A, Armania N (2016). Antioxidant, antimicrobial and cytotoxic potential of condensed tannins from *Leucaena leucocephala* hybrid-Rendang, Food Science and Human Wellness 5(2):65-75.

Zhang L, Tatsuno T, Hasegawa I, Tadano T, Ohta T (2015). Furanonaphthoquinones from *Tabebuia avellanedae* induce cell cycle arrest and apoptosis in the human non-small cell lung cancer cell line A549.Phytochemistry Letters 11:9-17.

Related Journals:



www.academicjournals.org