DOI: https://doi.org/10.17921/1415-6938.2021v25n5-espp776-782

Eucalyptus Wood Vinegar: Chemical Profiling, Evaluation of Acute Toxicity to *Artemia salina* and Effect on the Hatching of *Betta splendens* Eggs

Extrato Pirolenhoso de Eucalipto: Composição Química, Avaliação da Toxicidade Aguda em *Artemia salina* e Efeito na Eclosão de Ovos de *Betta splendens*

Yanka Beatriz Costa Lourenço[a]; Alexandre Santos Pimenta[b]; Leoclécio Luís de Paiva[c]; Francisco Marlon Carneiro Feijó[d]; Maíra Fasciotti[e]; Renato Vinicius Oliveira Castro[f]; Elias Costa de Souza[g]

[a]Universidade Federal de Lavras, Graduate Program in Forest Sciences. MG, Brazil.
[b]Universidade Federal do Rio Grande do Norte, Graduate Program in Forest Sciences. RN, Brazil.
[c]Instituto de Desenvolvimento Sustentável e Meio Ambiente. RN, Brazil.
[d]Universidade Federal Rural do Semiárido, Laboratory of Veterinary Microbiology. RN, Brazil.
[e]Instituto de Desenvolvimento Sustentável e Meio Ambiente, Laboratory of Analytical Chemistry. RJ, Brazil.
[f]Universidade Federal de São João Del Rei, Department of Agricultural Sciences. MG, Brazil.
[g]Universidade Estadual de São Paulo, Escola Superior de Agricultura Luiz de Queiroz, Graduate Program in Forest Sciences. SP, Brazil.
*E-mail: eliasrem@hotmail.com

Abstract

In Brazil nowadays, eucalyptus wood vinegar (WV) is an important input in food industry and for agriculture and veterinary uses. The main objectives of the present research work were to characterize the phenolic fraction in the industrial WV by gas chromatography and mass spectrometry, evaluate the acute toxicity of industrial WV to the brine shrimp *Artemia salina*, and assess the effect of the product on the hatching of *Betta splendens* eggs. Two types of WV were employed in the experiments, raw and bi-distilled WV. Both were profiled by gas chromatography and mass spectrometry (GC/MS). Bioassays with *Artemia salina* nauplii were performed to determine the acute toxicity of WV. To assess the effect of WV on the hatching of *B. splendens* eggs, five concentrations of bi-distilled WV were employed encompassing one control and four experimental treatments, 0% (T0), 0.025% (T1), 0.050% (T2), 0.075% (T3) and 0.100% (T4) with ten replicates per treatment. Probit and regression analysis were applied to the experimental data of the brine shrimp bioassay and the experiment with the bettas, respectively. Phenols reported in the literature as having biological activity were identified in the WV by GC/MS. Median lethal doses (LD_{50}) equal to 272 and 298 mg mL^{1} were determined for the raw and bi-distilled WV, respectively. Regarding the experiment with *B. splendens* eggs, a concentration of 0.05% was found ideal to reach maximum of hatching and minimize the fungal infection in the fingerlings. The bi-distilled WV has potential to be applied in the commercial production of this fish species, with significant decrease in mortality rates.

Keywords: Eucalyptus Wood Vinegar. Eucalyptus Pyroligneous Extract. Phenolic Compounds. Acute Toxicity. *Artemia salina.* Antifungal Activity. *Betta splendens*

Resumo

Atualmente no Brasil, o extrato pirolenhoso de eucalipto (EP) é um importante insumo na indústria de alimentos e também para uso na agricultura e veterinária. Os principais objetivos do presente trabalho de pesquisa foram avaliar a toxicidade aguda do EP industrial para em Artemia salina e o efeito do produto na eclosão de ovos de Betta splendens. Dois tipos de EP foram empregados nos experimentos, EP bruto e bidestilado. Ambos foram analisados por cromatografia gasosa e espectrometria de massa (GC/MS). Bioensaios com náuplios de A. salina foram realizados para determinar a toxicidade aguda do EP. Para avaliar o efeito do EP na eclosão de ovos de B. splendens, cinco concentrações de EP bidestilado foram empregadas perfazendo 5 tratamentos, 0% (T0), 0,025% (T1), 0,050% (T2), 0,075% (T3) e 0,100% (T4) com dez repetições por tratamento. Os dados experimentais do bioensaio com A. salina e do experimento com B. splendens foram submetidos à análise Probit e de regressão, respectivamente. Todos os fenóis relatados na literatura como tendo atividade biológica foram identificados no EP por GC/MS. Doses letais medianas (DL_{50}) iguais a 272 e 298 mg mL^{-1} foram determinadas para o EP bruto e bidestilado, respectivamente. Em relação ao experimento B. splendens, a concentração de 0,05% foi considerada ideal para atingir o máximo de eclosão e minimizar a infecção fúngica nos alevinos. O EP bidestilado tem potencial para ser aplicado na produção comercial dessa espécie de peixe, com queda significativa nas taxas de mortalidade.

Palavras-chave: *Extrato Pirolenhoso de Eucalipto. Compostos Fenólicos. Toxicidade Aguda. Artemia salina. Atividade Antifúngica. Betta splendens*

1 Introduction

Wood vinegar (WV) or pyroligneous extract is the aqueous fraction resultant from the carbonization of lignocellulosic biomass, such as wood, forest and crop wastes and other types of similar raw materials (YANG *et al.,* 2016; PIMENTA *et al.*, 2018; ZHANG *et al*., 2019). The main components of WV are organic acids, phenols, ketones, furans, and pyrans, along with other compounds in low concentrations. WV has a broad range of applications in agriculture, pharmacy and biomedicine, veterinary and animal production, food processing and, as wood preservative (THEAPPARAT *et al*., 2018). Antibacterial and antifungal properties of the WV have been consistently demonstrated (WU *et al*., 2015; ARAÚJO *et al*., 2018; SOUZA *et al*., 2018) along with anti-inflammatory (KIM *et al*., 2011; HO *et al*., 2013) and even antiviral actions (LI *et al*., 2018). The herbicide effect of WV also has been proven when used alone or in mixture with conventional products (TIILIKKALA *et al*., 2010; SEO *et al*., 2015). On the other hand, WV is cited as a plant growth stimulator even in low concentrations around 0.1% (BORTOLETTO *et al*., 2009; WANG *et al*., 2019). Another application of WV is as feed additive for animals like poultry, swine and cattle on which either ruminal and intestinal flora are favored resulting in an improvement of digestibility and absorption of nutrients, as highlighted by Araújo et al. (2018), also citing other authors to support the affirmation. Therefore, real potential antimicrobial uses of WV exists along with its other effects such as antioxidant, antiseptic, anti-inflammatory, anti-allergic and detoxifying (YIN *et al*., 2008; YANG *et al*., 2016; SOUZA *et al*., 2018). The research into natural products with such properties, especially as antimicrobials, is important, but as mentioned by Souza et al. (2018), the high variability of the sources from where WV can be obtained and also variations in the processes and equipment used to prepare the product are issues that have been preventing effective standardization and spread of its use in a larger scale.

While mainly used as flavor agent for meat (beef, pork, poultry, etc.), fish and cheese, WV can be extended to a wide variety of other foods due to its great versatility, such as soups, canned vegetables, condiments and other seasonings (CADWALLADER, 2007; BUDARAGA *et al*., 2016). In the context presented above, efforts should be directed to use and certify the properties of WV produced in industrial-scale and also from biomass grown in large-scale plantations under sustainable management, as is the case of eucalyptus forests in Brazil. Therefore, as long as the chemical profile and biological properties WV produced in industrial scale are tested and certified, the greater commercial use of the product is possible since a product with reproducible and constant quality can be marketed. Another point to address is the shortage of objective information about the acute toxicity of WV to living organisms since natural products can be toxic at high doses. In this sense, acute toxicity bioassays conducted with *Artemia salina* are useful tools to assess WV toxicity, since the method is rapid, inexpensive, can be performed in laboratory conditions and provides reliable information on the toxicity of a large range of natural products and chemicals (MEYER *et al*., 1982; LIMA *et al*., 2009; FERRAZ FILHO *et al*., 2012; DUARTE *et al*., 2019).

Along with the studies concerning the use of industrial WV as an additive in bird feed (DIÓGENES *et al*., 2019), our research efforts also encompass assessment of using the same type of product for the management of ornamental fishes. *Betta splendens*, known simply as betta or Siamese fighting fish, is among the five species of ornamental fish most imported by the USA, for instance (FARIA *et al*., 2006). These fish are reared in several tropical countries, in which their natural habitat is composed of areas flooded with stagnant oxygen-poor water, such as rice paddies (CHAPMAN *et al*., 1997). In Brazil, the commercial rearing of betta has been expanding in various regions, particularly the forest zone of the state of Minas Gerais and states of the Northeast. Besides the attractive appearance, the species has good adaptation, few requirements and rusticity, facilitating positive breeding results, making it a good source of income for fish farmers (SANTOS *et al*., 2013). However, one of the most common problems of fish farming is the occurrence of diseases during the fingerling stage, in which high mortality rates are associated with hatching from eggs that did not receive any type of fungicidal treatment. In the breeding of bettas, the incidence of saprolegniosis (infection by *Saprolegnia* sp.) is high, mainly during spawning, affecting the eggs. In response, some breeders are already using fungicides in the reproduction water to prevent this problem (FARIA et al., 2006). A large number of pathogens causing diseases in fingerlings are present in the water, or even the substrate, of aquariums and tanks. Most of the time, the fish are resistant to these pathogens, but this resistance declines when they are subjected to stressful conditions, as occurs in aquaculture. Therefore, the antimicrobial properties of WV might enable its use as an antifungal agent in o the initial phase of the *Betta splendens* life cycle in aquaculture, specifically egg hatching. In addition, eucalyptus WV is increasingly being used as a flavoring agent in Brazil and a problem arises from this fact. Despite being a food industry additive, the issue lies on what type of damage could be caused by spillage of product into the aquatic environment since everyday larger and larger amounts of WV are being transported by railways and roads and there is no study dealing with the toxicity of WV to aquatic organisms. Other point to be considered is the complex chemical composition of the product and its acidity with pH in the range of 2.8 to 3.0. So, there is a major concern about its toxicity in case of spilling in eventual road or railroad crashes. The idea of the present research work was starting to fill the gaps of information cited above.

The present research work had the goals of characterizing the phenolic fraction present in the industrial eucalyptus WV by gas chromatography and mass spectrometry, assess the acute toxicity of eucalyptus WV to *Artemia salina*, and evaluate the action of eucalyptus WV on the hatching of *Betta splendens* eggs and to ascertain its antifungal potential in fingerlings.

2 Material and Methods

Eucalyptus WV used in the present experiment was provided by Ibiré Negócios Sustentáveis Ltda., São Paulo, SP, Brazil (www.ibire.com.br). Two types of WV were provided: (1) raw WV, which corresponds to a fraction obtained by simple four-week settling from crude pyrolysis liquids from eucalyptus wood, and (2) purified WV, obtained by vacuum bi-distillation of the raw WV.

Initially, 1.5 mL of concentrated ammonium hydroxide solution was added to 5 mL aliquots of bi-distilled WV samples to increase the pH to about 5. Then extractions of the organic fraction were carried out by using 3 mL of ethyl acetate. All reagents employed for sample preparation were HPLC grade. After the liquid-liquid extraction, 1 mL of the organic portion was analyzed to get the chemical profile. The chemical profiling was carried out by using a Shimadzu QP 2010 gas-chromatography coupled to a mass spectrometer. Chromatographic separation was done with a CP-Wax column (Restek 52 DB with 30 m length, 0.25 mm diameter and 0.25 μm film thickness). Injector temperature was kept at 250 °C. The sample (1 μL) was injected in a split ratio of 1:10, and the oven temperature was held at 50 °C during 2 minutes, after which a heating rate of 2 °C min^{-1} was applied from 50 to 240 °C, concluded by holding the final temperature for 2 min. The carrier gas was helium, used at a constant flow of 1 mL min^{1}. Compounds were identified having their typical mass spectra as the basis and comparing them with data from NIST library with similarity equal to or over 85%.

Artemia salina cysts were acquired from the collection of the Department of Veterinary Microbiology of Federal University of the Semiarid (UFERSA). The experiment was properly approved by ethics committee of the UFERSA, nº 574.490. Acute toxicity of the eucalyptus WV to *A. salina* was assessed according the methodology from the work of Meyer et al. (1982). Dried cysts were placed in a flask containing artificial seawater prepared by dissolving 35 g of commercial marine salt (Cisne®, Salinas, RN, Brazil) in 1,000 mL of distilled water. After 36 – 48 h of incubation at room temperature (25 °C) under strong aeration and continuous lighting, the nauplii hatched. Then raw and bidistilled WV were separately diluted in distilled water in the gravimetric proportion of 1:1 (m/m) and the resulting mixtures were used in serial dilutions to obtain the following concentrations in mg mL^{-1} or %, as follows, 250 mg mL^{-1} (25%), 125 (12.5), 62.5 (6.25), 31.25 (3.125), 15.63 (1.563), 7.81 (0.781), 3.91 (0.391), 1.95 (0.195), 0.975 (0.975), 0.487 (0.0487), 0.243 (0.0243) and 0.121 (0.0121). By using disposable pipettes, ten nauplii at a time were placed in test tubes containing 5 mL of saltwater solutions containing the two types of WV in the descending concentrations identified above, with four tubes for each concentration. Four control tubes containing only salt water were prepared and ten nauplii were added to them as well. All tubes were kept under continuous illumination and observed hourly. After 24 hours of exposure, using a 5X magnifying glass, surviving nauplii were counted and the percentage of death at each WV concentration was determined. For estimating the lethal dosage of the eucalyptus WV, Probit analysis was employed by following the steps described by Finney (1971) and Gujarati (2006). For data analysis, the *gml* function of the software R (version 3.5.2) was used. Statistical models were adjusted and the best models were selected by significance of parameters (p = 0.05) and the Akaike information criterion (AIC). The lethal dosage able to kill 50% of the nauplii was calculated with the expression $LD_{50} = [- \beta_1/\beta_2]$ (GUJARATI, 2006).

Pairs of bettas were placed in aquariums and left to reproduce for 2 or 3 days. Then 10 eggs from each female were collected and used in the experiment. 300 mL aquariums were filled with 200 mL of filtered water and bi-distilled WV was added to reach the different concentrations displayed in Table 1.

Table 1 - Experimental treatments to assess the effect of bi-distilled WV on the hatching of *Betta splendens* eggs

Treatments	Eucalyptus WV Concentration (%) *
T0	0.000
T1	0.025
T2	0.050
T3	0.075
T4	0.100

*Values calculated to the present experiment.
Source: Research data.

For each treatment, ten aquariums were employed to compose ten replicates per experimental treatment. Ten aquariums with pure water were used as control. To each aquarium, ten eggs were added and observed daily until all had hatched. During observation, eggs infected with fungus were removed and those hatched were counted. The experimental design was entirely randomized. Regression analysis was applied to experimental data and polynomial regression models were adjusted by using the software Statgraphics (Data Analysis Solutions) for both number of hatched eggs and appearance of infected eggs in function of the concentration of bi-distilled WV. The best models were selected based on the following decision criteria: coefficient of correlation (r) between experimental and estimated values, significance of the parameters, biological realism of the model, Akaike information criterion and root-mean-square error (RMSE), as recommended by Gujarati and Porter (2009). In general, correlation coefficient nearer to 1 and lower RMSE indicate better estimation power of the model.

3 Results and Discussion

In the chemical profile of the bi-distilled WV, 20 biologically active compounds were identified in its organic fraction. This fraction corresponds to 8.98% from the whole mass of the product (DIÓGENES *et al*., 2019). From this total, 57.83% of the total area percent in the TIC are the phenolic compounds, as presented in Table 2. Furfural corresponded to 15.67% of the total area percent and the phenolic compounds added up 42.16%. The remaining chemicals were classified as other compounds and they were not listed in Table 2. In the extractable organic fraction of the WV assessed in the present work, the phenolic compounds are the same identified in WV composition cited by other authors (ACHMADI *et al*., 2013; YANG *et al*., 2016). It is important to highlight at this point that only a qualitative analysis of the phenolic compounds was performed in the present work.

Table 2 - Main compounds identified in the bi-distilled eucalyptus WV

Compound	Molecular Formula	Similarity (%)	Area (%)
Furfural	$C_5H_4O_2$	99	15.67
2-methoxy-phenol (guaiacol)	$C_7H_8O_2$	98	16.31
3-methyl-2-methoxy-phenol	$C_8H_{10}O_2$	96	0.92
2,6-dimethyl-phenol	$C_8H_{10}O$	97	0.19
2-methoxy-5-methyl-phenol	$C_8H_{10}O_2$	97	0.63
4-methyl-2-methoxy-phenol	$C_8H_{10}O_2$	97	4.87
Phenol	C_6H_6O	98	1.43
2-methyl-phenol (o-cresol)	C_7H_8O	97	1.21
4-ethyl-2-methoxy-phenol	$C_9H_{12}O_2$	98	2.61
4-methyl-phenol (p-cresol)	C_7H_8O	97	0.67
2,6-dimethyl-phenol (2,6-xylenol)	$C_8H_{10}O$	95	0.31
3-methyl-phenol (m-cresol)	C_7H_8O	96	0.49
2,5-dimethy-phenol (2,5-xylenol)	$C_8H_{10}O$	87	0.58
3,4-dimethoxy-phenol	$C_8H_{10}O_3$	91	0.17
4-propyl-2-methoxy-phenol	$C_{10}H_{14}O_2$	92	0.37
2,4-dimethyl-phenol (2,4-xylenol)	$C_8H_{10}O$	85	0.14
3-allyl-6-methoxy-phenol	$C_{10}H_{12}O_2$	97	0.15
2,6-dimethoxy-phenol (syringol)	$C_8H_{10}O_3$	97	8.48
4-methyl-2,6-dimethoxy-phenol	$C_9H_{12}O_3$	80	2.52
2,6-dimethoxy-4-allyl-phenol	$C_{11}H_{14}O_3$	88	0.11
Total			57.83
Other Compounds			42.17

Source: Research data.

Since the eucalyptus WV is composed of more than 100 compounds, it would be expensive and time-consuming to perform its quantitative analysis by GC/MS. Another major problem regards the fact of several classes of compounds are present in the WV composition, e.g., alcohols, organic acids, furans, pyrans, phenolic compounds, etc what makes difficult to apply the analytical methods of internal or external standards since it would require a large number of HPCL-grade analytical standards. However, once the bi-distilled product is standard and produced with close reproducibility, the qualitative analysis complies with the aimed objectives of correlating the chemical composition and the acute toxicity. Still, the areas percent presented in this work are close to the quantitative values obtained by Souza et al. (2012), for instance.

As observed in the Table 2, the main group identified in the WV was phenolic compounds, the same major components reported by other researchers (MONTAZERI *et al*., 2013; THEAPPARAT *et al*., 2018). This chemical group is also reported as majority in WV by different sources (YIN *et al*., 2008; YANG *et al*., 2016; SOUZA *et al*., 2018). The preservative properties of WV when applied as input in the food industry, for example, are closely connected to the presence of phenols in WV, as reported by Montazeri et al. (2013) and Budaraga et al. (2016). Among the phenolic compounds present in WV, some are well known for their biological effects and properties. Guaiacol (2-methoxy-phenol), for example, can be used medicinally as expectorant, antiseptic, and even as local anesthetic, according to O'Neil (2013). Phenol presents therapeutic effectiveness as fungicide, antiseptic and disinfectant (O'NEIL, 2013), with activity against a broad range of microorganisms, including even some viruses. The cresols (ortho, meta and para-cresol) are used as local antiseptics, parasiticides, disinfectants and intestinal antiseptics (O'NEIL 2013). Also, xylenols has proven antiseptic properties. Another important component in the WV chemical profile is furfural, which can act as a fungicide, as stated by Abdel-Kahr *et al*. (2015) and the US Environmental Protection Agency (2018). However, as pointed out by Yang et al. (2016), antibacterial and antifungal activities of WV cannot be related simply to a single chemical component, but to a synergistic interaction among several, especially phenolic compounds.

Table 3 presents the results of the statistical analysis to estimate the LD50 of raw and bi-distilled WV to the nauplii of *Artemia salina* with concentrations of 272 and 298 mg mL1 or 27.2 and 29.8%, respectively.

Table 3 - Results of the statistical analysis for the acute toxicity of raw and bi-distilled WV to *Artemia salina*

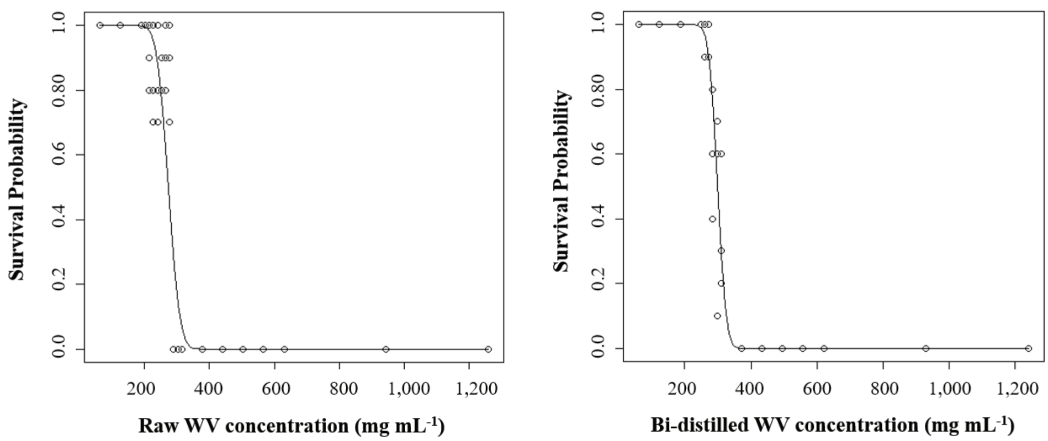
Treatment	Probit model			
	b_1	b_1	AIC	LD_{50} (mg mL^{-1})
Raw WV	9.80193	-0.03602	18.021	
p-value	*(0.00083)*	*(0.000908)*		
Bi-distilled WV	14.51558	-0.04868	16.791	298
Control Treatment				
Saltwater	0% mortality rate			

Source: Research data.

Despite being close, the results of the LD50 are statistically different when the two types of WV are compared, raw and bi-distilled WV, respectively. They are statistically different, which indicates that after bi-distillation, the WV becomes less toxic compared to the product in its raw condition. This is valid based on the Akaike information criterion. The AIC is an outofsample estimator for error prediction, which means that it reflects the quality of statistical models fitted to a determined dataset by estimating the quality of each model relating it to each of the other models adjusted to interpret the same phenomenon (GUJARATI, 2006). This way, if the raw WV is different from its bi-distilled version, the models adjusted for the toxicity of each one to the A. salina nauplii indicate different interpretation of similar but not exactly the same phenomena, i.e., the toxic effect of each WV separately to the living organism assessed here. During the purification of WV by vacuum distillation, residues of oil and vegetable tar are removed (PIMENTA *et al*., 2018), and probably their presence even in low concentrations in the raw product is responsible for the higher toxicity.

Figure 1 presents the curves obtained by plotting the survival rate of the *A. salina* nauplii as function of the raw and bidistilled WV concentrations.

Figure 1 - Acute toxicity of raw and bi-distilled eucalyptus WV to *Artemia salina*

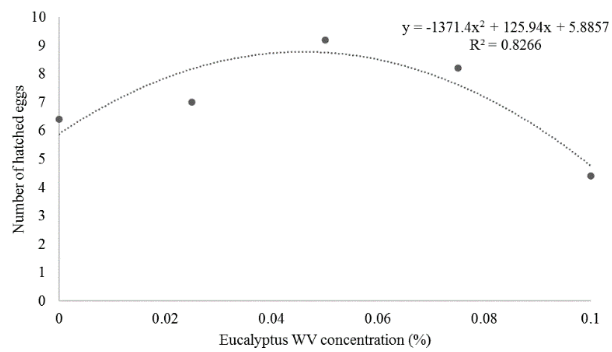


Source: Research data

As can be observed in the curves, concentrations just below 400 mg mL-1 (40%) were able to cause 100% mortality of the nauplii. Most likely, the phenols identified in section 3.1, phenol, guaiacol and cresols, which have antiseptic, disinfectant, fungicidal and antibacterial properties, are responsible for this toxicity. Also, furfural can be responsible for the toxicity found here to the nauplii, since the product has proven toxic effects in low concentrations (from 3.60 to 4.95 mg mL1) on living organisms (Luc and Crow 2013). The acute toxicity of the isolated phenolic fraction to the crustacean Daphnia magna was assessed by Pimenta *et al.* (2000), who determined EC50 of 68 mg L-1, which demonstrates the toxic power of these compounds. When the isolated phenol had its toxic effect assessed by using the bacterium Photobacterium phosporeum, the EC50 was equal to 6 mg L-1. However, the concentrations of the phenolic fraction (composed of phenol and methoxyphenol derivatives) in WV itself are low, which explains the low values of acute toxicity found in that study. Further regarding the concentrations of phenolic compounds in Eucalyptus camaldulensis WV, Theapparat et al. (2014) found values of 10.53 and 12.60 mg mL1 for methoxyphenol derivatives and 13.83 and 18.58 mg mL1 for phenol derivatives, respectively.

Figure 2 shows the best curve fitted for the hatching rate of the *B. splendens* eggs as a function of WV concentration. The best results were obtained at a concentration of 0.05% (T2), with average hatching of 9.2 eggs. The other treatments produced lower results, of 7.0, 8.2 and 4.4 for T1, T3 and T4, respectively. Most likely, below the concentration of 0.05%, the WV was not effective to stimulate the hatching and above this concentration a likely toxic effect occurred.

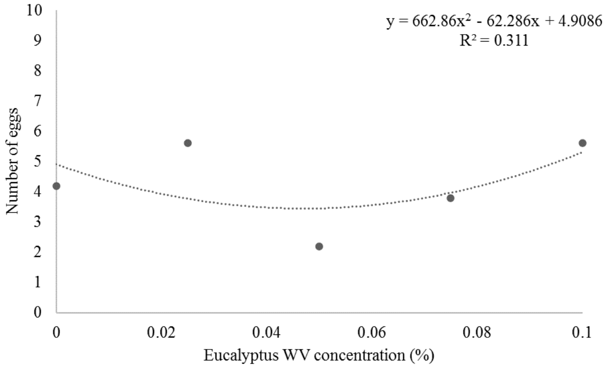
Figure 2. Influence of wood vinegar on the hatching of *Betta splendens* eggs



Source: Research data.

Figure 3 presents the regression curve adjusted from the results obtained for fungal infection of the eggs in response to the WV concentration. Concentrations of 0.012% (T1), (0.050%) T3 and 0.100% (T4) presented negative results regarding the number of fungus-infected eggs, associated with high mortality rates. Otherwise, the concentration of 0.025% (T2) had a positive result, so it can be characterized as an effective treatment against fungal pathogens. The antifungal effect of WV has been reported by several researchers for different types of fungi. For example, Martins (2017) tested WV's action against the fungi Trametes versicolor (white rot) and Neolentinus lepideus (brown rot). He found that at concentrations higher than 0.5%, the WV only inhibited the growth of white rot fungus, while it was 100% effective in controlling brown rot fungus. Similar results were achieved by us, since the treatment with WV concentration of 0.05% was effective in controlling the fungal growth on the eggs.

Figure 3 - Appearance of fungus-infected eggs of *Betta splendens*



Source: Research data

According to Pieta (2017), the use of eucalyptus WV on colonies of Sclerotium rolfsii at concentrations of 1,000, 2,000 and 3,000 ppm favored the fungal development, while at concentrations of 4,000 and 5,000 ppm, the pathogen presented lower mycelial growth, resulting in colonies with smaller diameters. In contrast, in this work, the lowest two WV concentrations (T0 and T1) and the highest two (T3 and T4) were inefficient regarding egg hatching, and also caused deficient development and even death of the eggs. The results found by Pieta (2017) and the results presented here together indicate that the WV can be effective at various concentrations, depending on the target of application. Therefore, the 0.05% WV treatment (T2) can be considered the most satisfactory when evaluated from both hatching and appearance of fungal infection standpoints, in comparison with the other treatments. It can thus be considered an alternative with excellent action to promote the hatching and development of Betta splendens fingerlings. More experiments should be conducted with other fish species to check whether the positive effects of the WV are similar.

4 Conclusion

Chemical profiling showed that the organic-extractable fraction of the eucalyptus WV carried out through GC/MS is composed mainly of phenolic compounds and furfural, which are responsible for the biological effects of the product. Crude and refined eucalyptus WV presented acute toxicity to *Artemia salina* but only in relatively high concentrations in the range of 27 to 30%. Therefore, in case of spillage of these products in aquatic environments most likely due to natural dilution, the toxic concentrations possibly would not be reached at once what is reassuring. On the other hand, the favorable potential of WV for use in commercial production of *Betta splendens* fingerlings is clearly shown by the positive response on the hatching rate and fungal control when used at the appropriate concentration. The advantage demonstrated in the present work is that the positive effects of WV on both hatching rate and antifungal effect can be achieved at very low concentrations.

References

ABDEL-KAHR, M.M. *et al.* Pesticide alternatives for controlling root rot and root knot of cucumber under plastic house conditions. *Int. J. Innov. Res. Scie. Eng. Technol.*, v.4, n.11, p.25-31, 2015. doi: 10.1590/1806-90882019000400008

ACHMADI, S.S. *et al.* Characterization of redistilled liquid smoke of oil-palm shells and its application as fish preservatives. *J. Appl. Scie.,* v.13, p.401-408, 2013. doi: 10.3923/jas.2013.401.408.

ARAÚJO, E.S. *et al.* Antibacterial and antifungal activities of pyroligneous acid from wood of *Eucalyptus urograndis* and *Mimosa tenuiflora*. *J. Appl. Microbiol.*, v.124, n.1, p.8596, 2018. doi: 10.1111/jam.13626.

BORTOLETTO, M. *et al.* Efeito do extrato pirolenhoso de *Eucalyptus* spp. no desenvolvimento de *Arthobotrys musiforme* in vitro. In: SIMPÓSIO INTERNACIONAL DE INICIAÇÃO CIENTÍFICA DA UNIVERSIDADE DE SÃO PAULO (SIICUSP). São Paulo. Anais..., São Paulo: USP, , 2009.

BUDARAGA, I.K.; ARNIM, Y.M.; BULANIN, U. Analysis of liquid smoke chemical components with GC/MS from different raw materials: variation, production and pyrolysis temperature level. *Int. J. ChemTech Res.*, v.9, n.6, p.694-708, 2016.

CADWALLADER, K.R. Wood smoke flavor. In: NOLLET, L.M.L. Handbook of meat, poultry and seafood quality. Oxford: Blackwell Publishing, 2007.

CHAPMAN, F.A.; FITZ-COY, S.; THUNBERG, J.T. United States of America International Trade in Ornamental Fish. *J. World Aquacul. Soc.,* v.28, n.1, p.1-10, 1997. doi: 10.1146/annurev.physiol.59.1.63

DIÓGENES, G.V. *et al.* Wood vinegar from Eucalyptus as an additive in broiler quail feed. *Int. J. Plant, Anim. Environ. Scie.*, v.9, n.3, p.164-181, 2019. doi: 10.26502/ijpaes.003

DUARTE, G.K. *et al.* Toxicity of *Esenbeckia pumila* Pohl (Rutaceae) on *Artemia salina* and *Atta sexdens rubropilosa*. *Rev. Caatinga*, v.32, n.1, p.101-112, 2019. doi: 10.1590/1983-21252019v32n111rc

ENVIRONMENTAL PROTECTION AGENCY – EPA Office of pesticide programs. OPP pesticide ecotoxicity database. EPA, USA. Available at http://www.ipmcenters.or/ecotoxicity, 2018. Accessed in March 3, 2020.

FARIA, P.M.C. *et al.* Criação, manejo e reprodução do peixe *Betta splendens* (Regan 1910). *Rev. Bras. Reprod. Animal*, v.30, n.4, p.134-149, 2006.

FERRAZ FILHO, Z.S. *et al.* Brine shrimp (*Artemia salina* Leach) bioassay of extracts from *Lychnophoriopsis candelabrum* and different *Lychnophora* species. *Rev. Bras. Prod. Nat.*, v.14, n.2, p.358-361, 2012.

FINNEY, D.J. Probit analysis. London: Cambridge University Press, 1971.

GUJARATI, D.N. Econometria Básica. São Paulo: Elsevier, 2006.

GUJARATI, D.N.; PORTER D.C. Basic econometrics. Boston: McGraw-Hill, 2009.

HO, C.L. *et al.* Bamboo vinegar decreases inflammatory mediator expression and NLRP3 inflammasone activation by inhibiting reactive oxygen species generation and protein kinase C-α/δ activation. *PLOS One*, v.8, n.10, e75738, 2013. doi: 10.1371/

journal.pone.0075738

KIM, S.P. *et al.* Composition of liquid rice hull smoke and anti-inflammatory effects in mice. *J. Agric. Food Chem.*, v.59, n.9, p.457081, 2011. doi: 10.1021/j2003392

LI, R. *et al.* Antiviral activity of phenolic derivatives in pyroligneous acid from hardwood, softwood and bamboo. *Sustainable Chem. Engin.*, v.1, n.6, p.119-126, 2018. doi: 10.121/acssuschemeng.7b01265.

LIMA, J.M. *et al.* Phytochemical prospecting of *Sonchus oleraceus* and its toxicity to *Artemia salina*. *Planta Daninha*, v.27, n.1, p.711, 2009.

LUC, J.E.; CROW, W.T. Factors affecting furfural as a nematicide on turf. *J. Nematol.*, v.45, n.4, p.260-264, 2013.

MARTINS, V.C. Potencial da ação antifúngica do licor pirolenhoso de *Hovenia dulcis* Thunb. fungos xilófagos *in vitro*. Ponta Grossa: Universidade Tecnológica Federal do Paraná, 2017.

MEYER, B.N. *et al.* Brine shrimp: a convenient general bioassay for active plant constituents. *J. Med. Plant Res.*, v.45, n.3, p.31-34, 1982.

MONTAZERI, N. *et al.* Chemical characterization of commercial liquid smoke products. *Food Scie. Nutr.*, v.1, n.1, p.102-115, 2013.

O'NEIL, M.J. The Merck Index: An encyclopedia of chemicals, drugs and biologicals. Cambridge: RSC Publishing, 2013.

PIETA, S. Eficácia de extratos pirolenhosos de cana-de-açúcar (*Saccharum officinarum* L.) e eucalipto (*Eucalyptus* spp.) no controle in vitro de patógenos da soja. Campo Grande: Univerisade Federal da Grande Dourados, 2017.

PIMENTA, A.S. *et al.* Evaluation of acute toxicity and genotoxicity of liquid products from pyrolysis of *Eucalyptus grandis* wood. *Arch. Environ. Contamination Toxicol.*, v.38, p.169-175, 2000. doi: 10.1007/s002449910022.

PIMENTA, A.S. *et al.* Chemical composition of pyroligneous acid obtained from Eucalyptus GG100 clone. *Molecules*, v.23, n.2, p.426, 2018. doi: 10.3390/molecules23020426.

SANTOS, D.M. *et al.* Uso de extrato aquoso da folha desidratada de amendoeira (*Terminalia catappa*) no cultivo de *Betta splendens*. *PUBVET*, v.7, n.4, p.259-311, 2013.

SEO, P.D. *et al.* Influence of herbicide-pyroligneous acids mixtures on some soil properties, growth and grains quality of paddy rice. *Int. J. Agric. Biol.*, v.17, n.3, p.499-506, 2015. doi: 10.17957/IJAB/17.3.14.349.

SOUZA, J.L.S. *et al.* Antimicrobial potential of pyroligneous extracts – a systematic review and technological prospecting. *Braz. J. Microbiol.*, v.49, n.1, p.128-139, 2018. doi: 10.1016/j.bjm.2018.07.001.

SOUZA, J.B.G.; RÉ-POPPI, N.; RAPOSO, J.L. Characterization of pyroligneous acid used in agriculture by gas chromatography-mass spectrometry. *J. Braz. Chem. Soc.*, v.23, n.4, p.610–617, 2012. doi: 10.1590/S0103-50532012000400005.

TIILIKKALA, K.; FAGERNÄS, L.; TIILIKKAL, J. History and use of wood pyrolysis liquids as biocide and plant protection product. *Open Agric. J.*, v.4, p.111-118, 2010. doi: 10.2174/1874331501004010111

THEAPPARAT, Y.C. *et al.* Physicochemical characteristics of wood vinegars from carbonization of *Leucaena leucocephala*, *Azadirachta indica*, *Eucalyptus camaldulensis*, *Hevea brasiliensis* and *Dendrocalamus asper*. Kasetsart. *J. Nat. Scie.*, v.48, n.6, p.916-928, 2014.

THEAPPARAT, Y.; CHANDUMPAI, A.; FAROONGSANG, D. Physicochemistry and utilization of wood vinegar from carbonization of tropical biomass waste. *Intechopen*, 2018. doi: 10.5772/intechopen.77380.

WANG, Y. *et al.* Root proteomics reveals the effects of wood vinegar on wheat growth and subsequent tolerance to drought stress. *Int. J. Mol. Scie.*, v.20, n.4, p.943, 2019. doi: 10.3390/ijms20040943

YANG, J.F. *et al.* Chemical Composition, Antioxidant, and Antibacterial Activity of Wood Vinegar from *Litchi chinensis*. *Molecules*, v.21, n.9, p.1-10, 2016. doi: 10.3390/molecules21091150

YIN, L.A. Isolation and characterization of antioxidant compounds from pyroligneous acid of *Rhizophora apiculata*. Penang: Universiti Sains Malaysia, 2008.

ZHANG, F. *et al.* Effects of biomass pyrolysis derived wood vinegar (WVG) on extracellular polymeric substances and performances of activated sludge. *Bioresource Technol.*, v.274, p.25-32, 2019. doi: 10.1016/j.biortech.2018.11.064.

WU, Q. *et al.* Study on the preparation of wood vinegar from biomass residues by carbonization process. *Bioresource Technol.*, v.179, p.98-103, 2015. doi:10.1016/j.biortech.2014.12.026.