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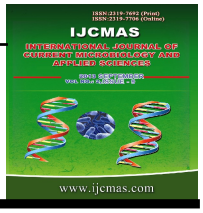

Original Research Article

Investigating the use of *Enaphalodes rufulus* (red oak borer) as an antimicrobial agent

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Keywords

Antibacterial; *Enaphalodes rufulus*; Red Oak Borer; Kirby-Brauer Disc Diffusion method; cross-streaking method.

A B S T R A C T

Enaphalodes rufulus (red oak borer) are insects that dwell within the bark of Red Oak trees and cause the indwelled trees to die. The relatively warm, moist environment in which they live is a perfect environment for bacteria to grow as well. It was hypothesized that in order for these insects to survive in such conditions, they must possess antimicrobial resistance. This antimicrobial resistance in insects has been shown to inhibit other types of bacteria, which often cause many health problems for humans. It was decided to apply this concept in researching the *Enaphalodes rufulus* as a potential inhibitor. The inhibiting nature of the bacteria from these insects was determined based on the Kirby-Brauer Disc Diffusion Method and the cross-streaking method. Results show that these insects do not possess inhibiting properties; however, the bacteria of the Red Oak borers adapted and grew along with the tester strains. Further investigation could lead to the development of an antibiotic using Quorum signaling to create a Quorum quenching mechanism.

Introduction

As diseases continue to advance and develop resistance to antibiotics, the development and discovery of new medical solutions is necessary. Insects have provided solutions to many people groups throughout the world for many centuries (Costa-Neto, 2002). The first formal investigation of this concept, however, was done in 1981 with the Cecropia moth, or *Hyalophora cecropia* (Steiner, et al 1981). This has led to other successful investigations, which have resulted in several new antibiotics. For example, in 2000, an investigation was completed so as to determine the function of peptides in antimicrobial compounds in

order to (Otvos, 2000). In 2003, the flesh fly *Neobellieria bullata* was investigated and found to inhibit the growth of several tester strains (Meylears, *et al.*, 2003). Another similar investigation was done using the American cockroach, *Periplaneta americana*, and the desert locust, *Schistocerca gregaria* (Ehrenberg, 2010). It was decided to further the concepts from this previous research by applying them to *Enaphalodes rufulus*, a species previously not researched.

Enaphalodes rufulus, commonly known as the Red Oak Borer, are a growing threat to oak forests (Meyers, *et al.*, 2007). They dwell within and damage Red Oak trees by feeding on the infrastructure of the trees. Methods for reducing the effects of their infestations have been researched (Meyers, *et al.*, 2007); however, these methods do not provide practical uses for the species after they have been removed from the tree. One of the purposes in choosing this species was to find a productive use for this species, rather than simply exterminating the borers. This species, if left alone, causes millions of dollars of damage to the quality of the trees. This also poses danger to campers, hikers, and loggers as limbs and branches are weakened (Meyers, *et al.*, 2007). Another justification for the use of this species is that of its habitat. Because of its dwelling in conditions which are also ideal for bacteria growth, these insects may well possess antimicrobial compounds, which allow them to survive. These antimicrobial compounds, it was hypothesized, would be able to inhibit other tester strains which are pathogenic to humans.

Materials and Methods

Preparation of Samples

Samples of the Red Oak borer were collected at the Missouri Department of Conservation Office in Camdenton, MO, United States with the assistance of forester, Paul Johnson. Mr. Johnson was contacted regarding the presence of the oak borer in the local area. As the oak tree adjacent to the office had been infested with the borer, it was decided to collect samples from that tree. Samples of larvae, pupae, and adults were stored individually in sterile, plastic containers. These samples were taken immediately to the Camdenton High School Research laboratory to be crushed and streaked onto LB Agar plates (C Waters, personal communication, September 29, 2012). It was decided to crush the insects in their entirety and use the resulting emulsion for the streaking of plates. This was performed because the internal components of the insects would also possess the antimicrobial compounds, if present (Ehrenberg 2010). This was verified also through conversations with Dr. Chris Waters (University of Michigan), who acted as a second-opinion throughout the duration of this experiment. The insects were crushed using a mortar and pestle, which were sterilized thoroughly between each use with ethyl alcohol. Upon being crushed, a sterile inoculating loop was used to streak the insects onto the LB Agar plates.

Once the organisms were plated, they were stored at room temperature for six days. On the seventh day, they were taken to the Lake Regional hospital in Osage Beach, MO for the remainder of the experimentation process. They were treated as potential BSL Level II organisms because it was unknown if they were pathogenic. Individual colonies were isolated, using sterile techniques, and placed into sterile test tubes with LB broth. These test tubes remained at room temperature until the tester strain lawns could be set up.

Tester Strains

Four gram-positive (*Bacillus thuringiensis*, *Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus aureus*), three gram-negative (*Shigella sonnei*, *Salmonella enteritidis*, and *Escherichia coli*), and one fungus (*Candida albicans*) were selected as tester strains. These strains were selected based on the research conducted by Meylears, *et al.*, 2003 and because they are human pathogens and would accurately reveal the antimicrobial properties of *E. rufulus*. Each strain was grown on Remel Blood Agar plates according to the instructions given by the manufacturer.

Kirby-Brauer Disc Diffusion Method (Olson, 2012)

After 24 hours of incubation at 35°C, colonies from the tester strains were isolated and placed into test tubes with distilled water. Sterile swabs were used to streak the solutions onto three plates each. The control for this experimentation was a plate with only the tester strain lawn. The other two plates were divided into fourths, which allowed for two replications of each generation of the insect. A sterile forceps was used to soak small discs of filter paper in the *E. rufulus* bacteria solution (Olson 2012) and then place the disc in the center of the divided area (Figure 1).

These plates were incubated at 35°C for 24 hours after which time the amount of growth was observed. Due to shipping issues, this method could not be carried out using *Escherichia coli* as a tester strain. Because there was no inhibition of growth to be measured quantitatively, qualitative descriptions regarding the appearance of the growth were recorded Table1).

Cross-Streaking Method (Velho-Pereira, *et al.*, 2011)

Three Remel Blood Agar plates were used for each tester strain; separate plates were used for the adults, pupae, and larvae. The tester strains were streaked in one vertical stripe directly from the Kwikstick solution. Sterile swabs were soaked in the *E. rufulus* bacteria solution and then streaked in one horizontal stripe, perpendicular to the tester strain stripe. These plates were then incubated at 35°C for 24 hours and examined for any inhibition of growth. Again, qualitative descriptions regarding the growth were recorded (Table 2).

Result and Discussion

Despite what was hypothesized, the bacteria of *E. rufulus* did not inhibit the growth of the tester strains; however, the bacteria from the insects often grew with the tester strains. The growth shown using the Kirby-Brauer Disc Diffusion method was fairly uniform. The results of the cross-streaking method generally supported the results of the disc diffusion method by once again showing that the insects' bacteria were able to grow with the tester strain. However, the tester strains were generally more dominant and did not allow for very much growth of the hypothesized inhibitor. While the bacteria did not act as was originally hypothesized, it was shown that the bacteria from the Red Oak Borer were able to adapt and grow with the tester strains.

Data was organized using Microsoft Excel 2010. In order to perform statistical analysis, numerical values were assigned to the qualitative analyses. For the Kirby-Brauer Disc Diffusion method, no growth surrounding the disc was classified as zero. Little distinct growth was classified as one, and uniform growth

Table.1 Qualitative analyses of the growth exhibited by the bacteria of *Enaphalodes rufulus* in conjunction with the tester strains

Stage	*Bacillus cereus*	*Bacillus thuringiensis*	*Escherichia coli*	*Micrococcus luteus*	*Salmonella enteritidis*	*Shigella sonnei*	*Staphylococcus aureus*	*Candida albicans*
Adult	Little distinct growth	Uniform growth	-	No growth	Little distinct growth	Little distinct growth	Little distinct growth	Uniform growth
	Little distinct growth	Uniform growth	-	No growth	No growth	No growth	Little distinct growth	Uniform growth
	Slight diminution	Slight diminution	No diminution	No diminution	No diminution	Slight diminution	Slight diminution	No diminution
Pupa	Little distinct growth	No growth	-	Uniform growth	Uniform growth	Little distinct growth	Uniform growth	Uniform growth
	Little distinct growth	No growth	-	Uniform growth	Uniform growth	No growth	No growth	Uniform growth
	Slight diminution	Dominant tester	No diminution	No diminution	No diminution	No diminution	No diminution	No diminution
Larva	Little distinct growth	Uniform growth	-	Uniform growth	No growth	Little distinct growth	No growth	No growth
	Little distinct growth	Uniform growth	-	Uniform growth	No growth	Little distinct growth	No growth	No growth
	Slight diminution	Slight diminution	Slight diminution	Slight diminution	Dominant tester	Slight diminution	Slight diminution	No diminution

Figure.1 Images depicting Kirby-Brauer Disc Diffusion Method

was classified as two. Because there were two samples for each insect generation, the average was taken before adding the results of the three generations. These values for each tester strain were compared with the control group, with values of zero, using a Single Factor ANOVA. The results were found to be statistically significant with a p-value less than .05.

For the cross-streaking method, a plate with a dominant tester strain, or one that grew over the streak of the hypothesized inhibitor was zero. A plate with no diminution of growth was one, and a plate with a slight diminution of growth, meaning that the tester strain did not grow over the point at which the streaks crossed but took over the other half of the hypothesized inhibitor's streak, was 2. Because only one replication of each sample was completed, the average was not taken for each generation. Another replication would have served to further validate the growth and could have the potential to decrease several of the stark differences between the two methods. Despite this, a comparison of these values with the control group, having values of zero, using a Single Factor ANOVA revealed statistically significant results with a p-value less than .05.

The fungi, *C. albicans,* was the most sensitive species to the bacteria from *E.* rufulus, with the gram positive bacteria being the next most sensitive based on the average growth scores, shown in Figure 3. Upon conclusion of this experiment, it was shown that while *Enaphalodes rufulus* (Red Oak Borer) may possess antimicrobial properties, they are not strong enough to inhibit any of the examined tester strains. There has been no research completed previously as to the potential of this species as an antimicrobial agent; this experiment allows the conclusion to be made that this species should not be included in the search for new antimicrobial agents. Despite this, however, these bacteria grew with the tester strains, supported with p-values of less than .05. Because the hypothesized inhibitor did show growth with each of tester strains, it is possible that the bacteria of *Enaphalodes rufulus* may be used in conjunction with other bacteria species in order to provide an antibiotic for previously resistant strains. It is possible that Quorum Sensing between the different species creates an environment in which the bacteria compete with each other and ultimately eliminate the problem by producing virulence factors only possible when associated with each other (Raffa, *et al.,* 2004). Other possibilities for future research could include investigating other species of insects as potential antimicrobial agents. As to this specific study with *Enaphalodes rufulus*, the specific bacteria can be analyzed to determine their exact characteristics. This could lead to a method for effectively eliminating this invasive species from the Red Oak trees (Bulmer, *et al.,* 2009).

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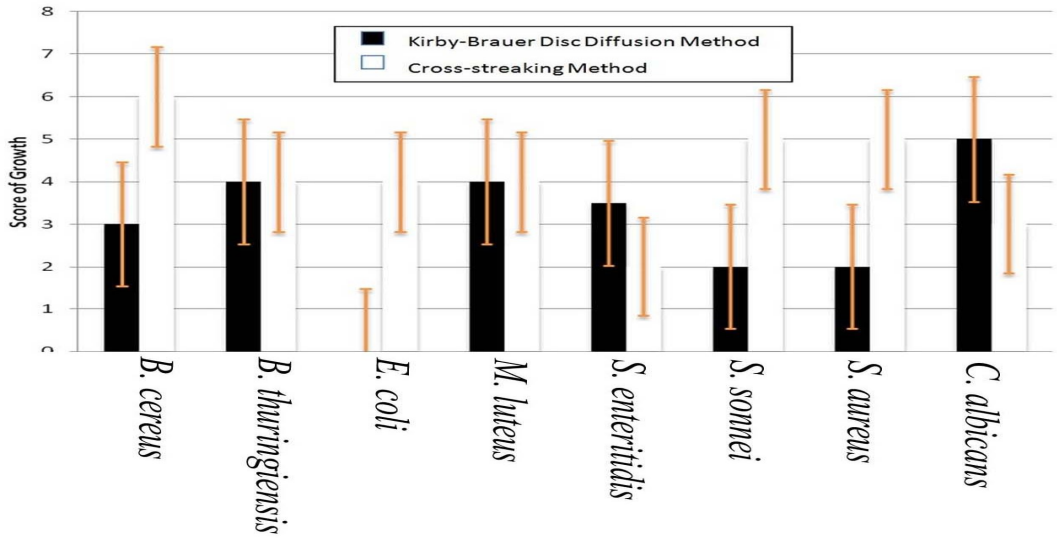
Figure.2 Graph of growth scores for each tester strain. The error bars represent one standard deviation of the average growth scores. There are no results for *E. coli* using the Kirby-Brauer Disc Diffusion method due to shipping issues.



References

Bulmer, M.S., I. Bachelet , R. Raman R.B. Rosengaus, R. Sasisekhara and Wogan, G.N. 2009. Targeting an antimicrobial effector function in insect immunity as a pest control strategy. Proceedings of the National Academy of Sciences of the United States of America. 106: 12652-12657.

Costa-Neto, E.M., 2002. Use of Insects in Folk Medicine in the State of Bahia, Northeastern Brazil, with Notes on Insects Reported Elsewhere in Brazilian Folk Medicine. Human Ecol. 30: 245-263.

Ehrenberg, R., 2010. Cockroach Brains, Coming to a Pharmacy Near You. Retrieved August 31, 2012, from http://www.wired.com/ wiredscience /2010/09/cockroach-brains/.

Lertcanawanichakul, M., and Sawangnop S. 2008. A comparison of two methods used for measuring the antagonistic activity of Bacillus species. Walailak. J. Sci.Technol. 5: 161-171.

Meyers J.M., D.C. Steinkraus, F.M. Stephen and Gold R.E. 2007. Survey for microbial pathogens of the Red Oak Borer (Coleoptera: Cerambycidae) on Northern Red Oak in Northwest Arkansas. J. Agricult.Urban Entomol. 24: 87-94.

Meylears, K., A. Cerstiaens, E. Vierstraete,G. Baggerman ,C.W. Michiels, A. De Loof and Schoofs, L. 2003. Antimicrobial compounds of low molecular mass are constitutively present in insects: Characterization of β-Alanyl-Tyrosine. Curr. Pharma. Design. 9: 99-110.

Olson, A., 2012. The End Zone: Measuring Antimicrobial Effectiveness with Zones of Inhibition. Retrieved December 19, 2012, from http://www.sciencebuddies.org/science-fair-projects/project_ideas/MicroBio_p014.shtml .

Otvos, L., 2000. Antibacterial peptides isolated from insects. J. Peptide Sci. 6: 497-511.

Raffa, R.B., J.R. Iannuzzo, D.R.,Levine, K.K. Saeid, R.C. Schwartz, N.T. Sucic, O.D. Terleckyj and Young J.M. 2004. Bacterial communication ("Quorum Sensing") via ligands and receptors: A novel pharmacologic target for the design of antibiotic drugs. Perspect.Pharmacol. 312: 417-423.

Steiner, H., D. Hultmark, A. Engstrom, H. Bennich and Boman, H.G. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature. 182(11): 6635-6637.

Velho-Pereira, S., and Kamat, N.M. 2011. Antimicrobial screening of Actinobacteria using a modified cross-streak method. Indian. J. Pharma. Sci. 73: 224-228.