

ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS OF *Origanum vulgare* AND *Ocimum basilicum* AGAINST *Vibrio parahaemolyticus* AND *Vibrio vulnificus* AND ADDITION OF THESE OILS ON *Mugil platanus* FILLETS

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ABSTRACT

Species of the *Vibrio* genus are commonly reported as agents of food poisoning outbreaks associated with fish consumption. The objective of this study was to assess the antimicrobial activity of the essential oils of *Origanum vulgare* and *Ocimum basilicum* against *Vibrio parahaemolyticus* and *Vibrio vulnificus*, as well as to assess their addition to fillets of *Mugil platanus* and sensorial acceptance among consumers. The antimicrobial activity was analyzed by the disc diffusion test and minimal bactericidal concentration (MBC). *M. platanus* fillets, experimentally contaminated, were marinated for 24 hours in a solution containing 1.0% and 1.5% of essential oil of *O. vulgare*. For the sensory analysis, samples marinated in 1.5% of *O. vulgare* oil were prepared and offered to 100 testers. The oil of *O. basilicum* shows no antimicrobial activity, so its application would be ineffective; for such reason the subsequent tests were not performed. However, the essential oil of *O. vulgare* produced inhibition halo diameters ranging from 24.6 to 34.1 mm, on average, and the MBC ranging from 3.9% to 15.6%. When added to the product, the essential oil of *O. vulgare* eliminated the microorganisms that were experimentally inoculated. In the sensorial analysis, samples marinated in 1.5% of *O. vulgare* essential oil obtained an average of 6.82 of acceptance note among consumers.

Keywords: antimicrobial activity; *Vibrio*; mullet; natural additives; oregano; sensory analysis.

ATIVIDADE ANTIMICROBIANA DOS ÓLEOS ESSENCIAIS DE *Origanum vulgare* E *Ocimum basilicum* FRENTE A *Vibrio parahaemolyticus* E *Vibrio vulnificus* E SUA APLICAÇÃO EM FILÉS DE *Mugil platanus*

RESUMO

Espécies do gênero *Vibrio* são comumente reportadas como agentes causadores de surtos alimentares associados ao consumo de pescados. O objetivo desse trabalho foi verificar a atividade antimicrobiana dos óleos essenciais de *Origanum vulgare* e *Ocimum basilicum* frente a *Vibrio parahaemolyticus* e *Vibrio vulnificus*, assim como verificar sua aplicabilidade em filés de *Mugil platanus* e aceitação sensorial. O efeito antimicrobiano foi analisado pelo teste de disco-difusão e concentração bactericida mínima (CBM). Filés de *M. platanus*, experimentalmente contaminados, foram marinados por 24 horas em solução com 1,0% e 1,5% de óleo essencial de *O. vulgare*. Para análise sensorial foram preparadas amostras marinadas em solução de 1,5% de óleo de *O. vulgare* e oferecidas a 100 avaliadores. O óleo de *O. basilicum* não apresentou atividade antimicrobiana; logo, a aplicação seria ineficaz e, por esse motivo não foram realizados os testes seguintes. Em contraste, o óleo essencial de *O. vulgare* apresentou halos em que as médias variaram entre 24,6 e 34,1 mm e a CBM ficou entre 3,9 e 15,6 µL mL⁻¹. Quando aplicado no produto, o óleo essencial de *O. vulgare* eliminou os microrganismos que foram inoculados experimentalmente. Na análise sensorial, amostras marinadas em 1,5% de óleo essencial de *O. vulgare* obtiveram nota média de 6,82 de aceitação entre os consumidores. Sendo considerado alternativa no controle de microrganismos.

Palavras-chave: atividade antimicrobiana; *Vibrio*; tainha; aditivos naturais; orégano análise sensorial.

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INTRODUCTION

Vibrio is a bacterial genus typically found in marine and estuarine environments. Bacteria of this genus are isolated from fish and crustaceans and are capable of multiplying freely in marine waters (Lima, 1997). Among the species that are pathogenic to humans, the most important are *Vibrio parahaemolyticus* and *Vibrio vulnificus*.

The search for natural foods has become a major concern for the food industry, especially in order to decrease the use of chemical preservatives that could be replaced by natural alternatives less harmful to human health (Viuda-Martos et al., 2008). Foods that have high levels of preservatives to reduce the microbial load are undesirable, and there is pressure from consumers towards a greater production of fresh food containing natural preservatives that guarantee its safety (Forsythe, 2002).

According to Barbosa (2010), essential oils with antimicrobial activity have the potential to be used in foods as natural additives, but their use must be driven according to the levels tolerated by consumers. According to Sivropoulou et al. (1996), the essential oil of *Origanum vulgare* is rich in carvacrol, which has shown antimicrobial action against pathogenic bacteria. *Ocimum basilicum* (basil) has been used in for medical purposes, since it presents beneficial properties for health (Al Abbasy et al., 2015). According to Silveira (2012), the essential oil of *O. basilicum* shows action against bacteria. Both linalool, 1,8-cineole and eugenol are listed as substances with antibacterial activity present in oil of *O. basilicum* (Soković et al., 2008). Hence, studies on the addition of essential oils from condiments in foods are necessary and the consumers' acceptance must be tested.

Based on the hypothesis that the essential oils of *O. vulgare* and *O. basilicum* have antimicrobial activity, and are promising natural additives for fish, the objective of this study was to assess the antimicrobial activity of these oils against *V. parahaemolyticus* and *V. vulnificus*, as well as to assess their addition to *M. platanus* fillets, and sensory acceptance by consumers.

MATERIAL E METHODS

Dry leaves of *O. vulgare* and *O. basilicum* were purchased and their essential oils were extracted using the methodology established by the Brazilian Pharmacopeia (Brasil, 2010).

The antimicrobial activity of the oils was tested against the microorganisms *Vibrio vulnificus* and *Vibrio parahaemolyticus*, using a reference strain (ATCC 8001 e ATCC 27562, respectively), as well as two wild isolates of each microorganism. The wild isolates of *V. parahaemolyticus* (V3 and V6) were previously isolated from *M. platanus* captured in the Patos Lagoon estuary in the study carried out by Rosa et al. (2016). The *Vibrio vulnificus* isolates (37 e 38) were isolated from *Paralichthys orbignyanus* that was also captured in the Patos Lagoon estuary, for an unpublished work by Silveira et al. The antimicrobial activity was assessed using the disc-diffusion test and the minimum bactericidal concentration (MBC).

The disk-diffusion analysis was performed according to the National Committee for Clinical Laboratory Standards (NCCLS, 2008), with some modifications. 0.1 mL of the culture with approximately 10^7 CFU mL⁻¹ (concentration measured through optical density and plate count) was distributed evenly, with the aid of a Drigalsky loop, on the Mueller Hinton agar surface (Kasvi, Roseto Degli Abruzzi, Italy) in which 1% sodium chloride was added. Six-millimeter diameter sterile filter paper discs were deposited on the inoculated-medium, and then they were impregnated with 5 µL of the oils to be tested, being them *O. vulgare* oil, *O. basilicum* oil and the blend of both oils in equal parts. The plates were incubated at 37°C for 24 hours. Discs without addition of oil were analyzed as multiplication control. The averages of inhibition halo measurements were classified according to the scale stipulated by Carović-Stanko et al. (2010). It was considered a strong inhibitory activity when inhibition halos were larger than 15 mm; moderate inhibitory activity when halos ranged from 10 to 15 mm and non-inhibitory activity when halo sizes were inferior to 10 mm. The normality was checked with a Shapiro Wilk test, then the analysis of variance test was performed to check the effect of oil treatments and the differences between the averages was checked with a Tukey comparison test, using a significance level of $p < 0.05$.

The MBC was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012), with minor modifications. Ninety-six-well microplates were used. In each well, 50 µL of alkaline peptone water (APW, Himedia, Mumbai, India) was dispensed, with 1% Tween 80, to decrease the surface tension in the contact between the oil (nonpolar) and the culture medium (polar). In the first well, 50 µL of the essential oil in test (*O. vulgare* or *O. basilicum* essential oil or the mixture of the two oils in equal parts) was dispensed and from this, consecutive serial dilutions were made, transferring 50 µL from the well of highest concentration to the lowest concentration, reaching a total of eight dilutions and the minimum concentration of 0.195%. In the end, the last 50 µL were discarded. Afterwards, 50 µL of the APW bacterial culture was added with approximately 5.0×10^3 bacterial cells in each well.

Wells in which the oil was not added, and those in which no inoculum was added, were used for multiplication and sterility controls, respectively. The plates were incubated at 36°C for 48 hours. After incubation, aliquots of 5 µL were removed from each well, and plated on standard plate count to counting (Acumedia, Baltimore, Maryland).

The absence of bacterial growth in the culture medium was considered as an indication that the essential oil tested showed bactericidal activity. The lowest oil concentration in which no growth of colonies was observed on the surface of the culture medium was considered the MBC.

For the analysis of the addition of the essential oils on fillets, whole units of fresh fish of the species *Mugil platanus* were purchased and aseptically filleted for later preparation of the samples, according to the methodology used by Van Haute et al. (2016), with minor modifications. Samples consisted of 10 g of *M. platanus* fillet, which were experimentally contaminated with 0.1 mL of bacterial inoculum at a concentration of 10^3 CFU mL⁻¹.

Then, samples were marinated (immersed for 24 hours at 4°C) in an aqueous solution of essential oil of *O. vulgare* in concentrations 1% and 1.5%.

After 24 hours, the samples were removed from the contact with the solution, allowing the excess to drain. To assess whether the oil inactivated the APW *Vibrio* cultures that were inoculated in the samples, the assay was carried out as recommended by U. S. Food and Drug Administration (Kaysner et al., 2004). The non-growth of typical colonies was considered as bactericidal activity of the oil against the microorganism. The analysis was performed in triplicate.

Sensory analysis was performed according to Silveira (2012), with modifications. The fillets of *M. platanus* were marinated in a solution containing 1.5% essential oil of *O. vulgare* for 24 hours, under refrigeration. Then, the fillets were breaded according to Gonçalves and Gomes (2008), deep-fried and offered to 100 untrained testers older than ten years old. For interpretation, following the norms of the Associação Brasileira de Normas Técnicas (ABNT, 1998), the mean and standard deviation were calculated using the structured 9-point hedonic scale, in which 9 represents the maximum score “Like Extremely”, and 1 represents the minimum score “Dislike Extremely”.

RESULTS

In the disk-diffusion analysis, the essential oils of *O. vulgare* and *O. basilicum* demonstrated different effects (Table 1). In the results, the inhibition halos of *O. vulgare* were larger than 15 mm, which is considered a strong inhibitory activity. However, the inhibition halos of *O. basilicum* oils were null, indicating absence of antimicrobial activity.

For the MBC test (Table 2), the *O. basilicum* essential oil also had no antibacterial effect against all tested microorganisms. Even at the highest concentration tested (250 µL mL⁻¹) there was no inhibition.

When the fillets were marinated in a solution with a concentration of 1% of *O. vulgare* essential oil, no bactericidal effect was observed. Only when fillets were marinated in a 1.5% solution, a bactericidal effect was observed on the *Vibrio* strains that had been experimentally inoculated. The positive control exhibited bacterial growth in all strains tested, while no growth of these bacteria was observed in the negative control.

In the sensory analysis, a market survey form was applied to consumers, in which 62% of them reported the regular habit of consuming fish, mainly for cultural reasons and for being a healthy food. Those that responded that were not used to eating fish, claimed it to be due to difficulty on purchasing the product, due to the high prices or lack of cultural habits. Six percent of the consumers said they had issues in consuming food that contained condiments, mainly because they disliked the food presentation as of ready-made products available on the market.

When filling in the hedonic form, 70% of the consumers showed some degree of acceptance to the product taste (Figure 1), with the majority indicating “Like Very Much” or “Like Moderately”.

Both people that liked and disliked the product reported that the addition of *O. vulgare* oil ended up attenuating the flavor of the fish, which pleased those that were not used to consuming *M. platanus* fillet. which is a fish that has a remarkable flavor, but, on the other hand, generated less acceptance among people that like the typical flavor of the fish.

When values were assigned to the alternatives of the hedonic scale, with 9 representing the maximum score “Like Extremely” and 1 the minimum score “Dislike Extremely”, the average acceptance score was 6.82, which corresponds to approximately 7 (“Like Moderately”).

In the purchase intention form (Figure 2), more than half of the testers claimed positivity in the purchase intention, with 45% having assigned “Probably Would Buy”.

Table 1. Size (mm) of the bacterial growth inhibition halos produced by essential oils in the disk-diffusion test.

Microorganism	<i>O. vulgare</i> *	<i>O. basilicum</i>	<i>O. vulgare</i> + <i>O. basilicum</i>
<i>V. parahaemolyticus</i>			
ATCC 8001	34.2 ^a (1.7)	0.0 ^c	9.3 ^b (0.8)
V3 (Wild isolate)	25.0 ^a (1.4)	0.0 ^c	13.3 ^b (1.0)
V6 (Wild isolate)	24.6 ^a (1.2)	0.0 ^c	15.0 ^b (1.5)
<i>V. vulnificus</i>			
ATCC 27562	26.6 ^a (0.8)	0.0 ^c	15.3 ^b (1.5)
37 (Wild isolate)	29.6 ^a (1.5)	0.0 ^c	14.0 ^b (1.4)
38 (Wild isolate)	32.0 ^a (1.2)	0.0 ^c	13.6 ^b (1.0)

*Data refer to the average of six repetitions, with standard deviation in parenthesis. Means followed by different letters on the same row are statistically different ($p < 0.05$) according to the Tukey test.

Table 2. Minimum bactericidal concentration (%) of the essential oils of *O. vulgare*, *O. basilicum* and the mixture, in equal parts, of the two oils.

Microorganism	Minimum bactericidal concentration (%)		
	<i>O. vulgare</i> *	<i>O. basilicum</i>	<i>O. vulgare</i> + <i>O. basilicum</i>
<i>V. parahaemolyticus</i>			
ATCC 8001	0.78	NE	3.12
V3 (Wild isolate)	1.56	NE	6.24
V6 (Wild isolate)	1.56	NE	6.24
<i>V. vulnificus</i>			
ATCC 27562	1.56	NE	6.24
37 (Wild isolate)	0.39	NE	0.78
38 (Wild isolate)	0.39	NE	0.78

*The tests were performed in hexuplicate and the values were the same in the six replicates. NE = no antimicrobial effect.

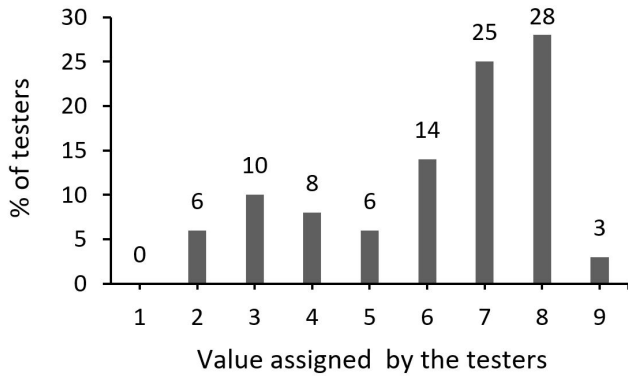


Figure 1. Acceptance test for *Mugil platanus* fillet marinated in *Origanum vulgare* essential oil; 1 = Dislike Extremely, 2 = Dislike Very Much, 3 = Dislike Moderately, 4 = Dislike Slightly, 5 = Neither Like nor Dislike, 6 = Like Slightly, 7 = Like Moderately, 8 = Like Very Much, 9 = Like Extremely.

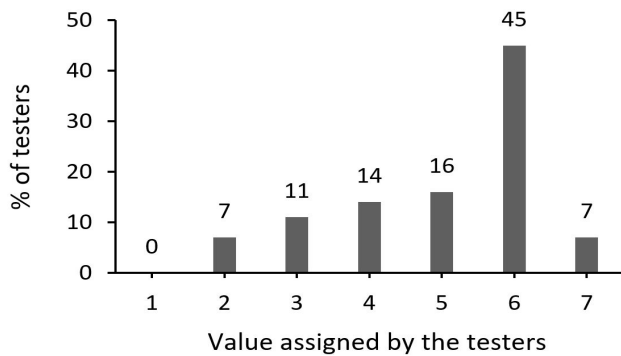


Figure 2. Purchase intention test for fillet of *Mugil platanus* marinated in essential oil of *Origanum vulgare*; 1 = Would Buy Only if Forced, 2 = Would Occasionally Buy, 3 = Would Definitely Not Buy, 4 = Would Probably Not Buy, 5 = Might or Might Not Buy, 6 = Would Probably Buy, 7 = Would Definitely Buy.

DISCUSSION

Koga et al. (1999) tested the effect of the essential oil of *O. basilicum* on *V. parahaemolyticus*, and also on the same strain of *V. vulnificus* (ATCC 27562) that was tested in the present study and found that both were completely eliminated when *O. basilicum* oil was used in the concentration of 0.05%. Hossain et al. (2010) observed that the oil of *O. basilicum* provided the formation of 14.3 mm growth inhibition halos in the disk-diffusion test and a minimum inhibitory concentration (MIC) of 250 $\mu\text{g mL}^{-1}$ for *V. parahaemolyticus*. The difference observed between these data and ours could be due to changes in the methodology, since Koga et al. (1999) used oil purchased from a company, hence, the extraction method could be different. Hossain et al. (2010) used the essential oil diluted in methanol in the disk-diffusion test and the strain of *V. parahaemolyticus* used was from a local isolate, which could present variations in sensitivity. However,

in our study, the oils were extracted using the hydrodistillation process by steam drag methodology.

Variations in the chemical composition of essential oils could also be responsible for the different results, since oil composition could be different according to the part of the plant, the time of plant collection, the environment, degree of development. It could also be genetically determined, varying according to botanical origin, environmental factors, and cultivation procedures (Bandoni and Czepak, 2008). In our study the oil was obtained from the leaves.

The major compounds of the essential oil of *O. basilicum* are biosynthesized by different biosynthetic pathways, such as the shikimate and mevalonate pathways, generating eugenol and linalool, respectively (Simões and Spitzer, 1999). According to Devi et al. (2010), the bactericidal activity of eugenol (major component synthesized by the shikimate pathway) is attributed to its penetrability in the cytoplasmic membrane, promoting its rupture and an increase in non-specific permeability, thus generating an overflow of the cellular content and subsequent death of the bacteria. Hence, the action of the oil could be affected by the fact that these microorganisms are Gram negative, and therefore, there is a restriction on the diffusion of compounds through the external wall.

O. vulgare oil promoted the formation of considerable inhibition halos. These results are in accordance with a study in which the antimicrobial activity of *O. vulgare* was also observed against *V. parahaemolyticus* (Yano et al., 2006). No evidence of synergism between the oils could be observed when the antimicrobial effect of the oil mixture in equal parts was analyzed. The formation of halos was compatible with the concentration of the oil used, that is, the halos formed by the mixture of oils in general were approximately half the size of the halos formed by the action of *O. vulgare* alone.

Differently from the results found in the current study, Barbosa et al. (2016) used a mixture of oils and found antimicrobial activity when *O. vulgare* oil was combined to *Rosmarinus officinalis* oil (rosemary) in the disk-diffusion test. The oils showed synergistic interaction and were effective against pathogens such as *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella enterica* serovar Enteritidis. However, the essential oil of *O. vulgare* showed no synergistic effect with the essential oil of *O. basilicum*, at least against *V. parahaemolyticus* and *V. vulnificus*.

The differences observed for the MBC values between reference strains and wild strains, possibly occurred due to the first being used in laboratories for many generations, which can affect the characteristics of the microorganism.

The published information on microbiological control in fish using essential oils is limited, especially using a methodology compatible with the present study. Van Haute et al. (2016) marinated fish products in essential oil of *Cinnamomum zeylanicum*, *O. vulgare* and *Thymus vulgaris* and, even at low concentrations (1%), the oils demonstrated a potential to slow bacterial growth (total coliforms, yeasts, lactic acid bacteria, and aerobic psychrotrophic bacteria).

The *M. platanus* fillets prepared for sensory analysis were marinated in a solution containing 1.5% of *O. vulgare* essential oil, as this concentration had an antimicrobial effect against the

Vibrio strains tested in the previous analysis (disc diffusion test and MBC of the present study).

In the comments, those who assigned “Probably Would Buy” reported that the addition of oregano oil generated a good flavor combination, which they would probably buy due to the fact that the oil attenuated the taste of *M. platanus*, and because it was a product with a healthy purpose.

Participants who were indifferent to the product (“Might or Might Not Buy”) reported that the price of the product would be a crucial aspect for choosing to purchase. Minozzo et al. (2008) stated that the general characteristics of meat consumption by the Brazilian population are focused mainly on price, an aspect also observed in the case of fish consumption. In a study carried out in the city of Pelotas-RS by Porto (2011), it was shown that 60% of the interviewees considered fish to be expensive or very expensive. Likewise, Jesus et al. (2014) also observed the same aspect in a study conducted in a municipality of the Amazonas State, in which the interviewees stated that they would increase the frequency of fish consumption should the price be more accessible.

Those who assigned “Probably Would Not Buy” (14%) and “Would Definitely Not Buy” (11%) reported that they would do so for the strong flavor of the essential oil or because they are not used to consuming fish. Those who “Would Occasionally Buy” claimed it to be due to already consuming fish only occasionally and not because of the addition of the oil. Data from Porto (2006) indicated that 64% of the population of Pelotas-RS eventually consumes fish. According to Solomon et al. (2006), religious dates, such as Lent and Holy Week, lead consumers to increase the consumption of fish, and religious orientation is considered a form of consumption influence.

Van Haute et al. (2016) state that the sensory properties of fish are inevitably affected (positively, neutrally, or negatively) when the concentration of essential oils necessary to extend the product’s shelf life is used. The sensory effect resulting from the combination of a certain essential oil in the food is a significant factor, since not all combinations and concentrations will be acceptable for consumption and commercial use. In the present work, when the concentration at which the bactericidal activity was effective was added, the product was accepted, indicating that the sensory characteristics were favorable.

CONCLUSION

The essential oil of *O. vulgare* has antibacterial activity, forming inhibition halos in the disk-diffusion test and presenting a relatively low MBC for all *Vibrio* strains used. The essential oil of *O. vulgare*, when used at 1.5% concentration in *M. platanus* fillets experimentally contaminated with *V. parahaemolyticus* and *V. vulnificus*, was effective to eliminate contamination. Its use, at this concentration, does not harm the palatability of the fish, since once subjected to sensory tests, it was accepted by the consumers.

The use of *O. vulgare* essential oils to control the contamination by *V. parahaemolyticus* and *V. vulnificus* in fish intended for

human consumption is a promising alternative for substitution of synthetic chemical preservatives without reducing food safety.

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