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The potential effect of Trivir® (10% carvacrol) as an alternative antibacterial agent for controlling bacterial infections in the African catfish (*Clarias gariepinus*).

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ABSTRACT

Production and control of fish health safely together with living in harmony with the nature, natural substances considered as an important area for future developments in aquaculture. In this respect, the current study aimed to investigate the antibacterial activities of Trivir® (10% carvacrol), one of the major essential oils of oregano, against some fish pathogenic bacteria isolated from diseased catfish (*C. gariepinus*). Trivir® solution was designated to be used as broad-spectrum disinfectant for aquaculture according to the manufacture instructions. The antibacterial effects of the Trivir® were tested on clinical isolates of *Aeromonas hydrophila*, *Enterococcus faecalis and Shewanella putrificans*. The minimum inhibitory concentrations (MICs) of carvacrol against *Aeromonas hydrophila* were estimated as 62 µg/ ml, 125µg/ml for *Shewanella putrificans* and 250µg/ml for *Enterococcus faecalis*. At all tested levels, Trivir® showed no bactericidal activities against all tested clinical bacterial isolates. *C. gariepinus* exposed to 32, 62 and 125 µg/ml of Trivir® showed survival rates of 100% at all exposure times tested. On contrast, Trivir® showed its toxic effects at concentrations of 1000, 500, 250 µg/ml after exposure times of 15, 30 and 60 min causing 100% mortalities in catfish. In conclusion, carvacrol can be considered as an effective alternative for antibiotics usage in aquaculture with an ultimate competent health and safe environment.

INTRODUCTION

The alarming increase in antibiotic-resistant bacteria has revived the interest in plant derived products as alternative/adjunct antimicrobial agents to control pathogenic micro-organisms **(Cowan, 1999; Hemaiswarya *et al.*, 2008; Hyldgaard *et al.*, 2012; Silva *et al.*, 2021).** Carvacrol has been classified as generally recognized

as safe (GRAS) bio-product of thyme and approved for food use. The bacteriostatic effects

found in oregano are due to its high content of phenolic compounds, i.e. the efficiency of clove, thyme and oregano essential oils was assigned to eugenol, thymol and carvacrol, respectively (**Moleyar and Narasimham, 1992; Kim *et al*., 1995a; Kim JTP *et al*., 1995b; Tsao and Zhou, 2000; Lambert *et al*., 2001).** Recent studies have shown that oregano essential oils have great antimicrobial properties (**Hammer *et al*., 1999; Dorman and Deans, 2000).** In addition to its anti-inflammatory, antioxidant, antitumor, analgesic, anti-hepatotoxic, and insecticidal properties, several studies have demonstrated that carvacrol has antimicrobial properties (**Hammer *et al*., 1999; Dorman and Deans 2000; Langeveld *et al*., 2014 Firmino *et al*., 2021).** Carvacrol (isoprenyl phenol) is known to have one of the strongest antimicrobial activity **(Roller and Sheedhar, 2000).**

Carvacrol has extensively been tested as an antimicrobial agent in food to control Gram- positive and Gram-negative pathogens, including *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *Vibrio cholerae*, and *V. vulnificus* **(Langeveld *et al*., 2014; Coelho *et al*., 2021).**

The antibacterial action of carvacrol principally relies on bacterial membrane damage, which is stronger against Gram-positive than Gram-negative bacteria. Obviously, it results in dissolution of the proton motive force and subsequent reduction in ATP synthesis that lead to reduction in other energy-dependent cell processes, including synthesis of enzymes and toxins **(Nostro and Papalia, 2012).** The ability of carvacrol to exert synergistic effects in combination with a number of antibiotics, including macrolides, has been recently reported **Langeveld *et al.* (2014).** The antibiotic resistance can be transmitted throughout aquatic environment to humans and animals. For these reasons, researchers have been profoundly investigating the use of antimicrobial safer compounds that cause no bacterial resistance and/or impact on fish, consumers and ecosystems.

The current study was conducted to evaluate the antibacterial activities of Trivir®, a patent product containing 10% carvacrol, which is designated for use as antibacterial product in aquaculture.

MATERIALS AND METHODS

2.1. Trivir® (Indiana, USA) was obtained from Tripharma Company (Cairo, Egypt). Trivir® contains 10% carvacrol as main basic components of the patent product (carvacrol 10%, thymol 3.9%, 1-8 cineol 1.6%, pinene 0.64%, pinene beta 0.03%, propylene glycol 1.5% and distilled water up to 1000 mL).

2.2. Bacterial strains:

Aeromonas hydrophila, *Enterococcus Faecalis* and *Shewanella putrificans* were isolated from characteristic lesions of clinically diseased freshwater fish. Retrieved

bacterial isolates were presumptively identified using API*20NE (BIO-Merieux) and kept in 18% glycerol/brain heart infusion broth (GBHIB) at −80°C till used **(Table 1.).** All tested bacterial strains were exposed to pathogenicity test before being used in this study. They were grown and maintained on brain- heart infusion agar plates (BHIA, Oxoid) and incubated at 25°C.

2.3. Trivir® bacteriostatic activity:

The bacteriostatic effect of the Trivir® solution was performed by agar plate dilution method through incorporating the solution directly into the melted BHIA medium. Briefly, Trivir® solution was prepared at 10 times the desired final concentration and 1 mL of this solution was placed in sterile Petri dishes (20 mm x 80 mm). Nine ml of Melted medium was added with gentle shaking in order to distribute the Trivir® solution within the medium evenly. To determine the minimum inhibitory concentration (MIC), plates with serial dilutions of Trivir® solution were prepared beginning with the greatest concentration of 1000, 500, 250, 125, 62 and 32µg/mL. The prepared plates were inoculated with each tested bacterial strain, and at 25°C.The growth of each bacterial strain was checked after 24 and 48 hours.

2.4. Trivir® bactericidal activity:

The bactericidal effect of Trivir® was determined using broth micro-dilution assay. The 96-well sterile micro-titer plates with U-bottom wells were prepared by dispensing 160 µl of BHI broth into each well. The first column of wells in micro-titer plate received a 20 µl of original solution at 10 times the desired final concentration starting with 1000 µg/ml. The mixtures (90 µl) were transferred to the next column of wells in a process of double fold serial dilution until column number 10 to obtain final concentrations of 1000, 500, 250, 125, 63, 32, 16, 8, 4 and 2 µg/ml. Columns number 11 and 12 were designated as controls with the former containing BHI broth with DMSO and the later contained only BHI broth. A suspension of each bacterial isolates in sterile saline was prepared and adjusted to $1.5x10^8$ CFU/ml; according to McFarland tube No. 0.5. Further, 10 µl of each selected bacterial isolate were inoculated into each well of the micro-plate rows and incubated at 25ºC for 24 and 48 hours. The viability of the tested bacterial isolates was determined by observing the morphological characteristics of the tested bacterial colonies in comparison with the control ones and confirmed by plating 10 µl from each well onto BHI agar medium and observed over a period of 24 and 48 hours.

2.5. Experimental Fish:

A total of 100 apparently healthy *C. gariepinus* with an average weight of 150.0 ± 2.0g and an average length of 25±2 Cm were obtained from a private catfish fish farm from EL- Fayoum Governorate, Egypt. Fish were acclimatized up to two weeks in well aerated 600-liter fiberglass tanks (three tanks). During acclimatization period, they were fed a commercial feed pellets (Geo-trade, 10^{th} of Ramadan City, Egypt) at rate of 2% of their body weight, then stopped 3 days prior to the experiment. Daily monitoring of water parameters was carried out to maintain the advisable limits for keeping catfish **Noor El Deen *al el.* (2014).** After elapsing of acclimation period, the remaining fish were divided into seven groups (each of 10 individuals) namely, A,

B, C, D, E, F and G. Fish groups A, B, C, D, E, F were exposed to 1000, 500, 250, 125, 62 and 32 µg/ml of Trivir®, respectively, for 2, 4, 6, 12, 24, 48 and 96 hours. Fish group G was designated as a control and exposed to distilled water (DW) instead of Trivir®. Results of tolerance bioassay were recorded in situ on a daily basis with removal of dead individuals.

2.6. Biosafety measures:

This study applied biosafety measures according to pathogen safety data sheets: Infectious substances- *Aeromonas hydrophila, Enterococcus Faecalis* and *Shewanella putrificans*, Pathogen Regulation Directorate, **Public Health Agency of Canada (2019).**

RESULTS

3.1. Clinical Examination

Clinically, diseased fish showed hemorrhagic skin ulcerations that sometimes expose the underlying musculature together with hyperemic spots at the base and tips of the fins (fig. 1). Internally, congestion/enlargement of kidneys and spleen, distended gall bladder, pale liver and accumulation of yellowish fluid in the abdominal cavity were all common signs (fig. 2).

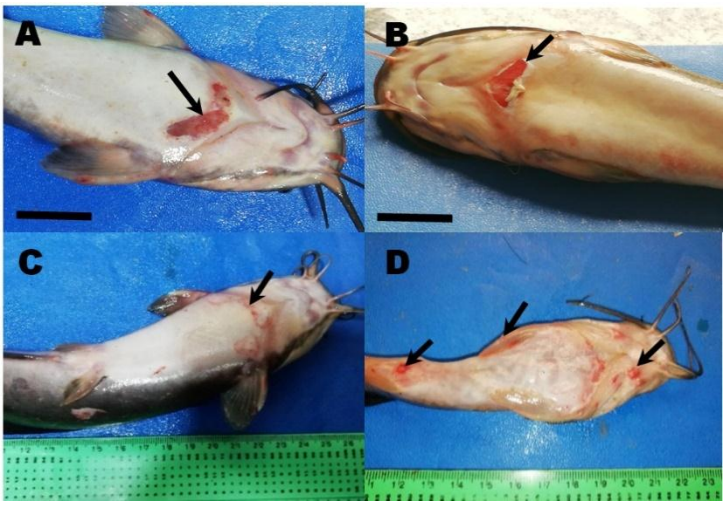

Fig. 1: *C. gariepinus* (a,b); Hemorrhagic ulcers on the skin and congested fins. (c,d) Hemorrhages on the skin, congested fins and around the anal opening.

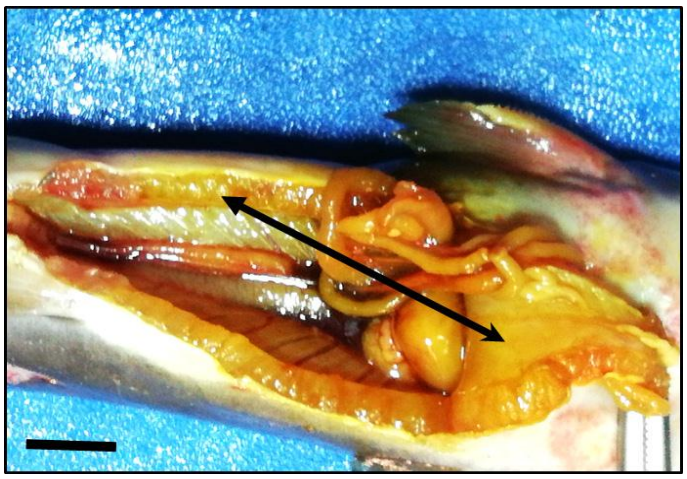

Fig. 2: *C. gariepinus*; showing inflamed empty alimentary tract tanged with sanguineous yellowish fluid. (arrows 5cm)

Fig. 1: *C. gariepinus* showing Hemorrhagic ulcers on the skin and congested fins. Hemorrhages on the skin and around the anal opening. **Fig. 2**: *C. gariepinus* showing inflamed alimentary tract that filled with sanguineous yellowish fluid.

3.2. Biochemical Examination

The presumptive biochemical characterization of the retrieved isolates was consistent with the standard biochemical criteria of *Aeromonas hydrophila*, *Enterococcus Faecalis* and *Shewanella putrificans* reported in (**Austin and Austin, 2016) (Table 1.)**

Table 1. The bacterial strains used for investigation of the antibacterial activity of Trivir®

Isolation	year	Host	Location
BNS 0119	2018	Catfish	Fayoum
BNS 0120	2019	Catfish	Fayoum
BNS 0121	2019	Catfish	Fayoum

3.3. Bacteriostatic activity of Trivir®

The antibacterial inhibitory effects of Trivir® varied considerably. As a result, the MIC of Trivir® for *A. hydrophila* was 62µg/ml; however, it expressed a very faint growth at concentration of 32µg/ml. On the contrary, no growth was expressed at other concentrations. Further, the minimum inhibitory concentration (MICs) of Trivir® for *Shewanella putrificans* and *Enterococcus faecalis* were 125 and 250µg/ml, respectively.

Table 2. Bacteriostatic effects of the prepared Triver solution on the selected pathogenic bacterial strains of fish.

Bacterial species	Trivir® Concentrations (µg/ml)					
	1000	500	250	125	62	32
Aeromonas hydrophila	–	–	–	–	–	±
Shewanella putrificans	–	–	–	–	+	+
Enterococcus faecalis	–	–	–	+	+	+

Concentration of Triver, (-) negative growth, (+) positive growth, and (±) faint growth

3.4. Bactericidal activity of Trivir®

At all tested levels, Trivir® solution did not kill the tested bacterial strains, including the *A. hydrophila* and the control ones (*E. fecalis and S. putrificans).* These results were evident when a portion of 10 µl from each well concentration was plated onto BHI agar and observed for periods of 24 and 48 hours.

Table 3. Bactericidal effects of the prepared Triver solution on the selected pathogenic bacterial strains of fish.

Bacterial species	Trivir® Concentrations (µg/ml)									
	1000	**500**	**250**	**125**	**63**	**32**	**16**	**8**	**4**	**2**
Aeromonas hydrophila	+	+	++	++	++	++	+++	+++	+++	+++
Enterococcus faecalis	++	++	++	++	+++	+++	+++	+++	+++	+++
Shewanella putrificans	++	++	+++	+++	+++	+++	+++	+++	+++	+++

Concentration of Triver (+) faint growth, c (++) positive growth, and d (+++) High growth

3.5. Bio-assay tolerance test:

Results of Trivir® tolerance bioassay to the fish model used, exihibited different toxicity signs among catfish exposed to concentrations of 1000, 500, 250 µg/ml (A, B and C groups) that terminated with 100% mortalities after 24 hours of exposure. These signs could be summarized as:

1) Excitability and aggressive swimming behaviors;

2) Resting on aquarium bottom raising their heads towards water surface together with fast opercular movements;

3) Convulsions and rolling around their axis;

4) Complete rest on aquarium bottom with low opercular movements before their death. The former three signs started at 15, 30 and 60 min after exposure, however, it

extended to the later sign at 6, 12 and 24 hours, respectively. On the other hand, catfish groups (D, E and F groups) that exposed to125, 64, 32µg/ml for all exposure times were experienced survival rates of 100% accompanied with little changes of normal swimming behavioral signs, which disappeared at the end of the experiment. Control fish group (G group) showed neither toxicity signs nor mortalities.

Table 4. Bio-assay tolerance and mortality rates of *C.s gariepinus* exposed to different concentrations of Trivir® at different times of exposure.

Trivir® concentrations (µg/ml)[a]	Exposure times (min)[b]	Survival rates (%)[c]
1000	15	0
	30	0
	60	0
500	15	0
	30	0
	60	0
250	15	0
	30	0
	60	0
125	15	100
	30	100
	60	100
64	15	100
	30	100
	60	100
32	15	100
	30	100
	60	100
Control	**15**	**100**
	30	**100**
	60	**100**

a, concentration of Trivir; b, exposure time; c, survival rate.

DISCUSSION

Despite its ubiquitous nature in the freshwater aquatic environments, yet, *A. hydrophila* is particularly responsible for MAS outbreaks in aquaculture. The disease causes a serious problem for the fish farming industry in Egypt as well as in other countries [21]. Motile Aeromonads are widely distributed in aquatic environments as well as integral part of the bacterial flora in aquatic animals [22]; [23]. Based on

different clinical symptoms, motile aeromonad infections have been referred to various names, which include motile aeromonad septicemia (MAS), motile aeromonad infection, hemorrhagic septicemia, tail and fin rot and epizootic ulcerative syndrome (**Austin and Adams, 1996; Roberts, 1997)**.

In the current study, samples from diseased fish with hemorrhagic skin ulcerations together exposed underlying musculature, hyperemic spots at the base and tips of the fins revealed that isolation of *A. hydrophila* which consistently agrees with **(Austin and Adams, 1996; Roberts, 1997; Janda and Abbott, 2007; Noor El Deen *et al.*, 2014; Paul *et al.*, 2015).**

The preceding results show that carvacrol, as one of the major essential oils of oregano, has a significant bacteriostatic activity **(Table 2).** More obviously, the MIC of Trivir® for *A. hydrophila* was 62 µg/ml; however, it expressed a very faint growth at concentration of 32µg/ml and no growth was expressed at other concentrations used. On contrary, the minimum inhibitory concentration (MICs) of Trivir® for *Shewanella putrificans* and *Enterococcus faecalis* were 125 and 250µg/ml, respectively, with the former had a strong bacteriostatic effect. These result conicides with **[10]; [28]** who reported that mixing carvacrol and thymol at proper amounts may exert the total inhibition that is evident by oregano essential oil against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and 19 strains of *Staphylococcus aureus (S. aureus*) of different origins respectively. Also, our results were closely similar to **Hussein *et al.* (2013).** who reported that the minimum inhibitory concentrations (MICs) of allicin in 10% v/v solution in dimethyl sulfoxide (DMSO) against *Aeromonas hydrophila* were found to be 125µg/ml, while ranged between 250-500 µg/ml for each of *A. caviae, A. sobria* and *A. veronii*. Interestingly, the MIC for *Streptococcus iniae* was 63µg/ml. However, at a concentration of 1000, 500, 250, 125, 62µg/ml could inhibit the growth of *A. hydrophila* completely. According to the MIC test and classification of **Rıos and Recio, (2005).** there are several EOs that present strong inhibition against pathogenic bacteria of fish. Among all EOs tested in vitro, the EO of carvacrol has one of the most promising results, being able to inhibit bacterial growth with a MIC as low as 32 ug/ml.

This antibacterial activity evaluated through MIC determined on 96-well microplates has been shown to be effective against a variety of Gram-positive and Gram-negative bacteria, including the major pathogens of aquaculture: *Aeromonas hydrophila* **(Griffin *et al.*, 2013).** Consequently, carvacrol at concentrations of 1000, 500,250, 125, 63, 32, 16, 8, 4 and 2µl/ml could not kill the tested bacterial strains. These differences may be attributed to the character and level of antimicrobial agents resent in carvacrol and its mode of action who examined the mode of action of four herbal essential oils against *Lactococcus garvieae*, and found that *L. garvieae* cells were inhibited by ginger oil and holy basil oil. They have the ability to re-grow after 24 hours of transfer into fresh BHI broth, explaining the bacteriostatic mode of action of the oil. This may explain why the tested bacterial strains re-grow when they were

plated on fresh BHI plates. Further, the current research confirmed the high toxicity of carvacrol to catfish (*C.garipinus*) at concentrations of 1000, 500, 250µg/ml and low toxicity to fish that exposed to125, 64, 32µg/ml. (**Table 4)**. On the basis of the obtained results, carvacrol at high doses of 1000, 500, 250 µg/ml could be used as antimicrobial disinfectant for utensils used in fish farms (e.g. nets, balances, buckets, brushes …. etc). On the other hand, low doses (125, 64, 32µg/ml) could be used in vivo (fish) as a bacteriostatic antiseptic agent, particularly, during farm operational processes (acclimation, vaccination, transportation …. etc). To our knowledge; minimal research has been carried out on the toxic effect of carvacrol on catfish (*C.garipinus*). Therefore, investigations of the factors influencing carvacrol toxicity are needed.

CONCLUSION

The minimum inhibitory concentrations of Trivir® against *Aeromonas hydrophila* was found to be 62µg/ml, while ranged between 125-250 µg/ml for each of *Shewanella putrificans* and *Enterococcus faecalis* respectively. Moreover, Trivir® showed a remarkable toxic effect *to C. garipinus* at concentrations of 1000, 500, 250µg/ml. Yet, catfish could tolerate its toxic effect at concentrations of 125, 64, 32µg/ml. To our knowledge, this is the first Egyptian research to investigate the bacteriostatic effect of Trivir® (10% carvacrol) in infected fish. To sum up, further studies are mandated to unveil all possible toxicities of carvacrol to fish.

Ethical approval

All applicable national and institutional guidelines for the care and use of animals were followed by the authors. This study was carried out by applying Beni-Suef University (BSU-IA CUC) and Use Committee protocol.

Approval number: 021-207

Availability of data and material

The authors confirm that all the data supporting the findings of this study are available within the article.

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