ISAAC FILIPE MOREIRA KONIG

COMPARATIVE TOXICOLOGICAL PROSPECTION OF CARVACROL AND ITS ACETYLATED DERIVATIVE IN THE ZEBRAFISH (*Danio rerio*) AND HUMAN BLOOD MODELS

LAVRAS-MG
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PROSPECÇÃO TOXICOLÓGICA COMPARATIVA DO CARVACROL E SEU DERIVADO ACETILADO NOS MODELOS ZEBRAFISH (*Danio rerio*) E SANGUE HUMANO

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Obrig

RESUMO

A busca por compostos naturais que apresentem atividade pesticida intensificou-se na última década. No entanto, a falta de estudos toxicológicos comparativos é um fator limitante para sua comercialização. O objetivo deste trabalho foi comparar a toxicidade do carvacrol (natural), acetilcarvacrol (semi-sintético) e fipronil (sintético) empregando-se dois modelos: sangue humano e zebrafish (*Danio rerio*). Uma série de concentrações foi utilizada para avaliar os efeitos desses produtos químicos na atividade hemolítica, morfologia eritrocitária, viabilidade leucocitária e fragmentação do DNA. No modelo zebrafish, avaliamos a sobrevivência, eclodibilidade, malformações, morfometria, comportamento, bioenergética mitocondrial, apoptose, espécies reativas de oxigênio e expressão gênica. Nossos resultados sugerem que o fipronil apresenta toxicidade relativamente alta para as células do sangue humano em comparação com os outros produtos químicos. O carvacrol em altas concentrações também foi tóxico, mas seu uso em baixas concentrações, bem como seu derivado acetilado, causou danos reduzidos às células sanguíneas. Em zebrafish, o fipronil exibiu alta toxicidade aguda após sete dias de exposição contínua. Também afetou a expressão de genes relacionados à atividade mitocondrial, diminuiu a área de superfície da bexiga natatória, induziu espécies reativas de oxigênio bem como hipoatividade no teste de resposta motora visual. O carvacrol e o acetilcarvacrol foram consideravelmente menos tóxicos para o zebrafish em comparação com o fipronil. A acetilação do carvacrol também foi responsável pela redução da letalidade. Em conjunto, nossos resultados demonstram que carvacrol e acetilcarvacrol apresentam toxicidade consideravelmente reduzida quando comparados ao fipronil, nos dois modelos avaliados. Além disso, a modificação química de um composto natural como o carvacrol parece ser uma alternativa promissora para melhorar sua atividade biológica e reduzir sua toxicidade para organismos não-alvo.

Palavras- chave: Acetato de carvacrol. Pesticida. Sangue humano. Toxicidade. Zebrafish.

ABSTRACT

The search for natural-based compounds presenting pesticide activity has intensified in the last decade. However, the lack of comparative toxicological studies is a limiting factor for their commercialization. The aim of this study was to compare the toxicity of carvacrol (natural), acetylcarvacrol (semi-synthetic), and fipronil (synthetic) employing two models: human blood and developing zebrafish (*Danio rerio*). A series of concentrations was used to evaluate the effects of these chemicals on hemolytic activity, erythrocyte morphology, leucocyte viability, and DNA fragmentation. In the zebrafish model, we assessed survivability, hatchability, malformations, morphometrics, behavior, mitochondrial bioenergetics, apoptosis, reactive oxygen species as well as gene expression. Our findings suggest that fipronil presents relatively high toxicity to human blood cells compared to the other chemicals. Carvacrol at high concentrations was also toxic, but its use at low concentrations as well as its acetylated derivative caused reduced damage to blood cells. In the zebrafish model, fipronil exhibited high acute toxicity following seven days of continuous exposure. It also affected the expression of genes related to mitochondrial activity, decreased swim bladder surface area, induced reactive oxygen species, and hypoactivity in the visual motor response test. Carvacrol and acetylcarvacrol were considerably less toxic to developing zebrafish compared to fipronil. Acetylation of carvacrol was also responsible for a reduction in lethality. Taken together, our results demonstrate that carvacrol and acetylcarvacrol have considerably reduced toxicity when compared to fipronil in the two models evaluated. Moreover, chemical modification of a natural compound such as carvacrol would seem to be a promising alternative to improve its biological activity and reduce its toxicity to non-target organisms.

Keywords: Carvacrol acetate. Human blood. Pesticide. Toxicity. Zebrafish.

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Supplemental Figure 2. Expression levels of transcripts related to lipid metabolism of zebrafish larvae exposed continuously to either 0.1 μM fipronil (F), carvacrol (C) or acetylcarvacrol (A) for 7 days. (A) acaca, Acetyl-CoA Carboxylase Alpha; (B) acs, Acetyl-coenzyme A synthetase; (C) cpt1, carnitine palmitoyltransferase 1A; (D) dgat1, Diacylglycerol O-Acyltransferase 1; (E) dgat2, Diacylglycerol O-Acyltransferase 2; (F) elovl2, Fatty Acid Elongase 2; (G) elovl6, Fatty Acid Elongase 6; (H) fad2, Delta(12)-fatty-acid desaturase; (I) mogat3, Monoacylglycerol O-Acyltransferase 3; (J) pgc1a, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; (K) pparg, Peroxisome Proliferator-Activated Receptor Gamma. Horizontal line represents the group's median value (Kruskal Wallis followed by Dunn's multiple comparison test, ns = nonsignificant, $p > 0.05$, n = 4-8 biological replicates/treatment).

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PART I

1. INTRODUCTION

Ticks are blood-sucking ectoparasites of great importance to human and veterinary medicine. Their control is mainly based on the use of synthetic pesticides. Fipronil is a vastly used pesticide employed to control ticks and insect pests. Many countries have restricted or even banned the use of fipronil due to its high acute toxicity to non-target organisms (Kathage et al., 2018). In this sense, there is a need to replace this chemical with environmentally safer pesticides to meet the growing demand for increased food production and cause less harm to the environment. Many studies have suggested that natural-based substances are promising substitutes for synthetic pesticides. Among a multitude of substances present in plant essential oils, carvacrol stand out for its well-studied pesticide activity besides other biological actions attributed to this chemical (Magierowicz et al., 2019; Costa-Júnior et al., 2016; Senra et al., 2013). Interestingly, studies have shown that acetylation of carvacrol can enhance its biological activity while decreasing its toxicity to non-target organisms. However, these studies were conducted without compassion to a commercial pesticide such as fipronil, which is possibly the main reason why natural-based chemicals are not commonly seen in commercially available pesticide formulations (Pavela and Benelli, 2016).

This study aimed to compare the toxicity of fipronil (synthetic) with carvacrol (natural) and acetylcarvacrol (semi-synthetic) employing two well-established toxicity models: human blood cells and zebrafish. In the first paper (part II), we investigated the effects of exposure to these chemicals on erythrocytes by hemolytic assay and by light microscopy. Additionally, the cytotoxicity of these molecules to leucocytes was also evaluated. Finally, their effects on DNA molecules were studied through comet and DNA fragmentation assays.

The following paper shows the effects of exposure to these chemicals on early staged zebrafish (*Danio rerio*) (part III). We conducted a series of toxicity assays and measured endpoints related to survivability, hatchability, malformations, morphometrics, mitochondrial bioenergetics, apoptosis, reactive oxygen species as well as locomotor and behavioral activities. Additionally, we analyzed the expression of genes related to swim bladder inflation, oxidative stress, lipid metabolism, and mitochondrial activity. Finally, we concluded in part IV four presenting the final remarks of this work.

2. OBJECTIVES OF THE STUDY

2.1 Main objective

The main goal of this study is to assess the toxicity of carvacrol (natural compound), acetylcarvacrol (semisynthetic compound), and fipronil (synthetic pesticide), which are commonly used for tick control. We employed two toxicity models in our assays: human blood and developing zebrafish (*Danio rerio*).

2.2 Specific objectives

Assess the effects of these chemicals on hemolytic activity and erythrocyte morphology.

Evaluate the action of these chemicals on leucocyte viability and DNA fragmentation.

Expose zebrafish embryos to these three chemicals and compare their toxicity by measuring endpoints related to survivability, hatchability, malformations, morphometrics, mitochondrial bioenergetics, apoptosis, reactive oxygen species as well as locomotor and behavioral activities.

Analyze the expression of genes related to swim bladder inflation, oxidative stress, lipid metabolism, and mitochondrial activity in zebrafish larvae exposed to carvacrol, acetylcarvacrol, or fipronil.

3. LITERATURE REVIEW

3.1 Ticks

Ticks are blood-sucking ectoparasites in at least one stage of their life (NAVA et al., 2010). These parasites are widely distributed and can infest mammals, birds, reptiles, and amphibians (ZAJAC et al., 2022; MENDOZA-ROLDAN et al., 2020). Ticks belong to the subclass Acari and are divided into three families: Argasidae, Ixodidae, and Nuttalliellidae.

Ixodid ticks are also known as 'hard ticks' due to the presence of a shield that fully covers, in the case of males, and partially, in the case of females, the dorsal part of their bodies (SONENSHINE; ROE, 2014). Ixodid ticks have a life cycle divided into three stages (larva, nymph, and adults), all with a single instar (SAUER, 1995). These parasites stand out as

important transmitters of pathogens, such as viruses, bacteria, fungi, and protozoa (PERVEEN et al., 2021; DANTAS-TORRES et al., 2012). Some characteristics of the life cycle of ixodid ticks place them in a prominent position in the transmission of pathogens, such as the slow metabolism, with the exception of the engorged female; the possibility of remaining long periods without feeding; the ingestion of large amounts of blood; and the production of large amounts of eggs (SONENSHINE; ROE, 2014).

The main tick species that affect cattle production in Brazil is *Rhipicephalus microplus*. This species causes discomfort to animals, in addition to affecting the development of the herd, and the production of meat, milk, and leather (JONSSON, 2006). Thus, it is estimated that infestation by this species accounts for an economic loss of US$ 968 million dollars per year in Brazil (RODRIGUES; LEITE, 2013). In this sense, this and other tick species are subjected to control methods, which are mainly based on the use of synthetic chemicals.

3.2 Tick control

Tick control is accomplished through various ways such as vaccination (CONTRERAS et al., 2017), biological control (WASSERMANN et al., 2016), chemical control through synthetic acaricides (MILLER et al., 2011), or plant extracts (TAK; ISMAN, 2017), among others. The use of synthetic acaricides is still the most widespread tick control method even though it should occur in an integrated manner (DE LA FUENTE, 2018).

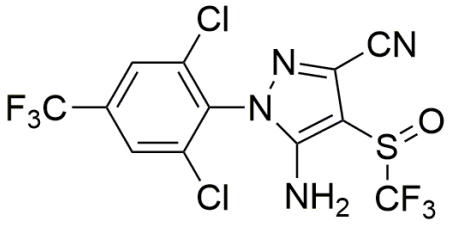
The frequent use of synthetic acaricides can result in the selection of resistant ticks, requiring higher dosages and applications at shorter intervals (KLAFKE et al., 2017). Additionally, the indiscriminate use of these chemicals can generate toxic effects on animals, plants, and humans, as well as contaminate soil and water (TSABOULA et al., 2016).

3.3 Fipronil

Fipronil [5-amino-3-cyano-1-(2, 6-dichloro 4-trifluoromethylphenyl)-4-trifluoromethyl sulfinyl pyrazole] is a commercial insecticide released to the market in 1993 (Figure 1). It belongs to the phenyl pyrazoles or fiproles, a small class of pesticides. It is highly effective against a variety of insect pests and ticks and acts through contact and stomach exposure (TINGLE et al., 2003). It inhibits the gamma-aminobutyric acid (GABA)-gated chloride channel leading to hyper-excitation, convulsions, paralysis, and death of insects. This chemical

has a long persistence in the environment and cultivated areas end up contaminating land surfaces and water bodies by leaching and surface runoff (SINGH et al., 2021).

Figure 1. Chemical structure of fipronil.



Source: From the author (2023).

Brazil is one of the world leaders in the use of fipronil and other pesticides. It has been used in this country to control pest infestations affecting various crops such as sugarcane, corn, and rice as well as in the cattle production to control ticks (GONÇALVES et al., 2022). However, this chemical is known to cause harmful effects to pollinators, bedbugs, mosquitoes, fleas, and other non-pest insects (FARDER-GOMES et al., 2021; CASTILHOS et al., 2019). Additionally, this chemical has a huge impact on aquatic organisms. Indeed, a study that investigated the effects of fipronil exposure (0.05-0.2 mg/L) on rainbow trout (*Oncorhynchus mykiss*) adults demonstrated that fipronil is dangerous environmental pollutant for aquatic organisms, even at low concentrations, inducing oxidative stress, damaging the brain tissue of fish and stimulating apoptosis (UÇAR et al., 2021).

Fipronil exposure is also a concern for humans and other animals. For instance, fipronil exposure to pregnant Wistar rats decrease their levels of thyroid hormones, which was also observed in the offspring at 30 days of postnatal life (TUKHTAEV et al., 2013). In China, 3467 breast milk samples of lactating women were collected and fipronil was detected in 63% of the samples at concentrations ranging from 178 to 2947 ng/L (median: 921 ng/L) (LIU et al., 2022). This exposure can lead to genotoxic and mutagenic effects on humans promoting the development of a series of diseases (ÇELIK et al., 2014). Considering its toxicity, the use of fipronil has been totally banned in some countries, while it is still used in restricted fields in others ones (LI et al., 2019). In this sense, there is an urgent need to replace this pesticide with environmentally safer ones.

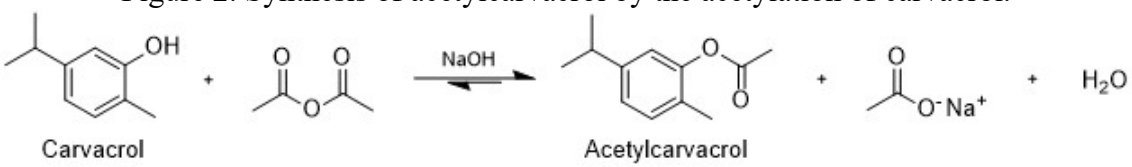
3.4 Carvacrol and its derivative

Carvacrol (2-methyl-5-(1-methylethyl)phenol) is a phenolic monoterpene found in essential oils from plants of the Lamiaceae family. It is one of the major constituents of the essential oils of *Origanum* sp. and *Thymus* sp. (SANTORO et al., 2007). Carvacrol stands out for its wide range of biological activities such as antimicrobial, antioxidant, anticancer, immunomodulatory, insecticidal, and antifungal (RATHOD et al., 2021).

Carvacrol is also known for its acaricidal activity against various tick species. For instance, fasting larvae of *Rhipicephalus sanguineus* (ARAÚJO et al., 2016), nymphs and fasting adults of *Ixodes scapularis* and *Amblyomma americanum* (DOLAN et al., 2009), fasting larvae of *Amblyomma sculptum* and *Dermacentor nitens* (NOVATO et al., 2015), among others. Other studies evaluated the acaricidal activity of essential oils in which carvacrol was one of the major constituents, in addition to evaluating the activity of the isolated compound for comparison purposes. Thus, they demonstrated that carvacrol is the main responsible for the acaricidal activity against fasting adults of *Hyalomma marginatum* (CETIN et al., 2010), fasting larvae of *Rhipicephalus microplus* (CRUZ et al., 2013), fasting adults of *Rhipicephalus turanicus* (KOC et al., 2013), among others.

The number of commercially available pesticides, particularly based on carvacrol, remains low even though this chemical presents high acaricidal activity. There are a few limitations that make it difficult to use these compounds in field applications (PAVELA; BENELLI, 2016). For instance, factors such as low stability against oxidation make the practical use of these substances difficult. Carvacrol, for example, has a hydroxyl group in its structure that can be easily oxidized (KONIG et al., 2021). Therefore, the synthesis of semi-synthetic analogues would seem to be an alternative to overcome this and other issues. Semi-synthetic chemicals have the aim of increasing their stability and biological activity, in addition to reducing toxicity to non-target organisms (CACCIATORE et al., 2015). Among these modifications, the acetylation of carvacrol stands out (Figure 2) (NOVATO et al., 2018).

Figure 2. Synthesis of acetylcarvacrol by the acetylation of carvacrol.



Source: KONIG et al. (2019).

Acetylcarvacrol has an ester group in its structure, replacing the hydroxyl group of carvacrol, which gives greater stability to the compound (KONIG et al., 2021). Furthermore, acetylation has been shown to increase acaricidal activity in fasting larvae of *R. microplus*. Compared to carvacrol, the mean larval mortality rate increased from 35.85 to 67.83% when the acetylated compound was used (RAMÍREZ et al., 2013). The repellent activity against *R. sanguineus* adults also showed a greater effect over time of acetylcarvacrol compared to carvacrol (KONIG et al., 2021). Moreover, acetylcarvacrol also presents various biological activities such as anxiolytic effect in rats (PIRES et al., 2013), bactericidal activity (CACCIATORE et al., 2015), anti-inflammatory action (DAMASCENO et al., 2014), anthelmintic activity *in vitro* against *Schistosoma mansoni* (DE MORAES et al., 2013), among others.

Regarding the toxicity of these chemicals, carvacrol is used as a food additive due to its antimicrobial and antioxidant properties. It also possesses the Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration (GUARDA et al., 2011). In addition, it is considered a safe flavoring compound by the Council of Europe's Department of Health and Food Safety. The addition of this compound is allowed in Europe at a concentration of up to 25 ppm (=mg/L), in the case of flavorings for sweets (DE VINCENZI et al., 2004). However, Suntres et al. (2015) argue that the *in vivo* toxicity of carvacrol is not fully understood since most studies were conducted *in vitro*.

A study that compared the toxicity of carvacrol and acetylcarvacrol showed that the LD_{50} was 1544.5 mg/kg for acetylcarvacrol and 919 mg/kg for carvacrol following oral administration to female Swiss albino mice (*Mus musculus*) (ANDRE et al., 2016). Compounds that have an LD_{50} greater than 1,000 mg/kg when administered orally are considered safe or of low toxicity (LEI; SUN, 2018). Another study that assessed the toxicity of acetylcarvacrol following oral and intraperitoneal administration to male and female Swiss mice found no differences in behavior, body weight, water intake, food consumption, excreta production, biochemical and hematological parameters in treated animals up to 1000 mg/kg (OLIVEIRA et al., 2020). However, the use of these chemicals as a replacement for synthetic pesticides would increase the concentration at which humans and other animals are exposed, requiring further toxicity assessment. Finally, a comparative toxicological study between these chemicals and a commercial pesticide is also needed to assess the safety of these chemicals.

3.5 Human blood model

The traditional toxicity testing approaches date back some 30–60 years. These tests were mainly based on the observation of adverse health responses in laboratory animals treated with high doses of these agents, which are not commonly present in the environment at those concentrations (ANDERSEN; KREWSKI, 2009). In this sense, high-throughput tests were developed to assess the toxicity of various chemicals at different concentrations simultaneously (KREWSKI et al., 2020). The cellular (e.g. erythrocytes and leucocytes) and acellular (clotting factors, immunoglobulin, and electrolytes) components of the blood stand out as interesting biomarkers for toxicity evaluations (BOJARSKI and WITESKA, 2020).

The erythrocytes, for instance, are the main cells in circulation and possess a series of advantages for screening of xenobiotics toxicity. Their plasma membrane is a multi-component structure that keeps the cell morphology, elasticity, flexibility and deformability. Alteration of membrane structure upon exposure to xenobiotics could induce various cellular abnormalities and release of intracellular components. Therefore, the morphological changes and extracellular release of hemoglobin (hemolysis) and increased content of extracellular adenosine triphosphate (ATP), as signs of membrane stability, could be used to evaluate the cytotoxic effects of various molecules. Additionally, the nucleated erythrocytes from birds, fish, and amphibians as well as leucocytes from humans can be used to evaluate the genotoxicity of different xenobiotics using comet, DNA fragmentation, and micronucleus assays. Moreover, erythrocytes are vulnerable to peroxidation making it a good biological membrane model for analyzing the oxidative stress and lipid peroxidation of various xenobiotics (FARAG; ALAGAWANY, 2018; INTRANUOVO et al., 2018).

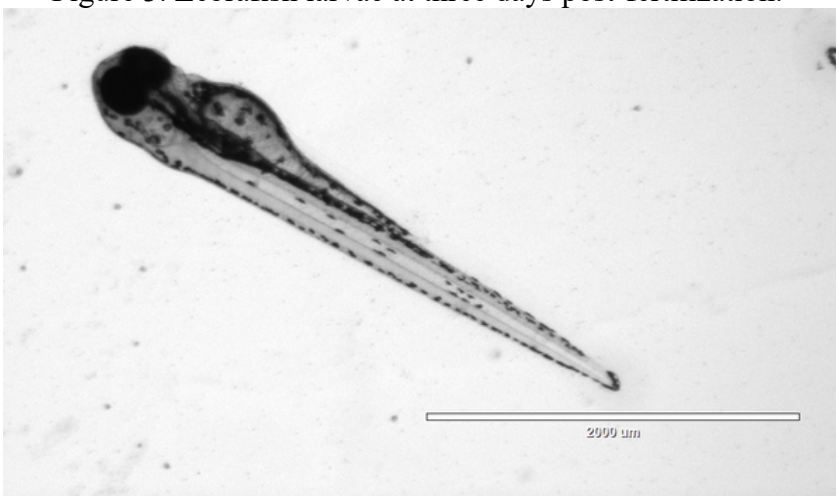
Thus, blood cells can be used to measure a series of endpoints following exposure to a variety of chemicals, particularly pesticides. For instance, genotoxic and mutagenic effects (KAPELEKA et al., 2021), enzymatic activity related to toxicity and oxidative stress (ASSIS et al., 2018), and effects on morphology (MAHESHWARI et al., 2019) are commonly investigated using this model. Consequently, this model contributes to elucidating the toxic effects of chemicals on humans and other animals.

3.6 Zebra fish model

The embryonic zebrafish (*Danio rerio*) is a powerful toxicity model that offers the power of whole-animal investigations such as intact organisms, functional homeostatic feedback mechanisms, and intercellular signaling. Additionally, it possesses the convenience of cell culture, presenting advantages such as cost- and time-efficiency, minimal infrastructure and small quantities of chemical solutions required (TRUONG; TANGUAY, 2017). Interestingly, numerous studies have confirmed that zebrafish and mammals are similar in their genome, physiology, development, and metabolic pathways. Indeed, the zebrafish genome presents approximately 70% similarity with human genes (HOWE et al., 2013). Moreover, zebrafish responses to toxic substances are highly predictive of mammalian responses (HE et al., 2014).

The description of the embryo development of zebrafish by Kimmel et al. (1995) has enormously contributed to establishing it as a toxicity model. It undergoes a series of morphological changes during its development until three days post-fertilization (dpf) (Figure 1), including the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. By 3 dpf, the hatched larva has completed most of its morphogenesis, and it continues to grow rapidly. After this period, inflation of the swim bladder, beginning of the swimming and protrusion of the mouth are processes normally seen (KIMMEL et al., 1995).

Figure 3. Zebrafish larvae at three days post-fertilization.



Source: From the author (2023).

A series of endpoints can be measured in zebrafish embryos/larvae following exposure to a variety of chemicals, particularly pesticides. Locomotor and anxiolytic activities, survivability, deformities, oxidative stress, mitochondrial bioenergetics, gene expression,

morphometrics, and biochemical parameters are commonly assessed in these experiments (YANG et al., 2021; EADIE et al., 2020; WANG et al., 2018). Thus, contributing to elucidating the toxic effects of chemicals on these organisms in an attempt to predict what would happen with humans.

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PART II – Paper I

Comparative toxicological evaluation of carvacrol, acetylcarvacrol and a fipronil-based pesticide in human blood cells

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Abstract

Plant-derived chemicals are promising substances to control arthropod pests, although synthetic ones are still the most frequently used. Thus, comparative toxicological studies are needed to determine if natural substances are safe alternatives to replace the use of synthetic chemicals. This study aimed to compare the toxicity of carvacrol (natural origin), acetylcarvacrol (semi-synthetic), and a fipronil-based pesticide (synthetic). We assessed the effects of these chemicals on hemolytic activity, erythrocyte morphology, and leucocyte viability using whole blood from human subjects. Additionally, DNA damage was evaluated through comet and DNA fragmentation assays. Fipronil and carvacrol caused hemolysis at concentrations ranging from 0.5 to 2.0%, whereas acetylcarvacrol did not cause hemolysis at 0.5 and 0.75%. Fipronil and carvacrol caused severe alterations in erythrocytes' morphology at 2%, such as ghost erythrocytes, elliptocyte-like shape, and rouleau-like shape, presenting only 3.3 and 8.3% normal cells, respectively, at this concentration. However, 73.3% of erythrocytes incubated with 2% acetylcarvacrol exhibited normal morphology. Fipronil considerably reduced leucocyte viability, decreasing it to 78% at 2%. Carvacrol and acetylcarvacrol showed no differences in leucocyte viability for 0.5 to 1.0%, but a decrease was observed for 2% carvacrol. The comet assay showed similar DNA damage for fipronil and carvacrol, but it was significantly lower for 1 and 2% acetylcarvacrol. Incubation with genomic DNA showed that only fipronil caused the fragmentation of this molecule. Thus, we conclude that carvacrol and fipronil can present similar toxicity at higher concentrations. However, acetylation of carvacrol significantly reduced its toxicity to human blood cells compared with the other chemicals.

Keywords: carvacrol; acetylcarvacrol; fipronil; human blood; genotoxicity; erythrocytes; leucocytes.

1. INTRODUCTION

Pesticides are agrochemicals used to protect crops against harmful pests, weeds or diseases as well as humans and other animals from vector-borne diseases. An average of 2 million tons of these chemicals are used globally each year in agricultural lands, public health programs, and urban green areas (Syafrudin et al., 2021). Among various pesticides, fipronil stands out for being one of the most widely used, systemic and broad-spectrum insecticide (Singh et al., 2021). However, exposure to this chemical can trigger adverse health outcomes, which is mainly due to its ability to cross the blood brain barrier inducing neurotoxicity in rodent models (Cravedi et al., 2013). Additionally, this chemical can cause cell cycle arrest, DNA damage, and apoptosis (Park et al., 2021). Moreover, fipronil is highly toxic to aquatic organisms (Eadie et al., 2020) as well as non-target insects, including pollinators (Farder-Gomes et al., 2021). Thus, its use has been prohibited in some countries due to its high toxicity.

Plant secondary metabolites emerge as promising alternatives to fipronil-based pesticides for their effectiveness and high abundance (Ntalli et al., 2019). In this sense, carvacrol, 5-isopropyl-2-methylphenol, a phenolic monoterpene found in essential oils of plants of the family Lamiaceae, such as *Origanum* sp. and *Thymus* sp., attracted the attention of many researchers globally (Rathod et al., 2021; Vinciguerra et al., 2018). Indeed, this natural agent is recognized for its acaricidal action against various tick species (Pereira-Junior et al., 2019; Tabari et al., 2017; Novato et al., 2015; Senra et al., 2013; Dolan et al., 2009). Additionally, carvacrol is well-known for its insecticidal (Magierowicz et al., 2019; Park et al., 2017) and nematicidal properties (Trailovic et al., 2021). However, the number of commercially available biopesticides, particularly based on carvacrol, remains low. This is mainly because of the reduced stability of phenolic compounds to oxidation and the lack of toxicological studies (Pavela and Benelli, 2016).

The possibility to chemically modify carvacrol aiming at increased stability and biological activity as well as reduced toxicity to non-target organisms was first exploited in an *in silico* study (Ramírez et al., 2013). These authors evaluated 754 possible modifications for carvacrol and demonstrated that the acetylation of this chemical would lead to a promising candidate for tick control. Indeed, several studies have later confirmed that acetylcarvacrol exhibits greater acaricidal activity (Konig et al., 2020; Oliveira et al., 2020b; Gonçalves et al., 2019; Ramírez et al., 2016). Moreover, the replacement of a phenolic hydroxyl group in the structure of carvacrol by an ester group in acetylcarvacrol is likely to increase its stability to

oxidation (Konig et al., 2021). Despite this, field applications of these chemicals would require a vast toxicological investigation.

A comparative acute toxicity test was carried out in female Swiss albino mice by esophageal gavage administration of carvacrol and acetylcarvacrol (Andre et al., 2016). In this study, the dose able to induce 50% mortality in the animals (LD) was considerably greater for acetylcarvacrol (LD_{50} = 1544.5mg/kg) than carvacrol (LD_{50} = 919 mg/kg), demonstrating reduced acute toxicity of the acetylated derivative. Nonetheless, toxicological studies comparing these chemicals with commercially available pesticides were not yet performed, to the best of our knowledge. In addition to mortality, the random and inappropriate use of pesticides, even natural-based ones, may cause mutational events, carcinogenic effects and genetic damages to non-target organisms (Sabarwal et al., 2018; Sinha et al., 2014). In this sense, we hypothesized that carvacrol and its acetylated derivative would present reduced toxicity compared to fipronil to human blood cells, which are highly sensitive toxicity models (Intranuovo et al., 2018).

Thus, the present study was undertaken to investigate the toxicity of carvacrol, acetylcarvacrol and a fipronil-based commercial pesticide to human blood cells. Effects on erythrocytes were assessed by hemolytic assay and by light microscopy. Additionally, the cytotoxicity of these molecules to leucocytes was also evaluated. Finally, their effects on DNA molecules were studied through comet and DNA fragmentation assays.

2. MATERIALS AND METHODS

2.1 Obtaining human blood

Human peripheral blood was collected by vein puncture from healthy volunteers (n= 5, 20–30 years old, non-smokers, non-alcohol consuming and not undergoing any medication 30 days prior to collection). All experiments were carried out according to protocols previously approved by the Human Research Ethics Committee of the Federal University of Lavras (COEP/UFLA), under registration number 4.684.242.

2.2 Chemical compounds

Carvacrol (CAS 499–75-2, 98% purity) was purchased from Sigma–Aldrich Fine Chemicals, St. Louis, MO, USA. The commercial pesticide (Regente ® 800 WG – BASF) (fipronil 800 mg/g), with valid registration in the Brazilian Ministry of Agriculture, Livestock and Food Supply (registration number: 005794), was purchased in Lavras, Minas Gerais, Brazil.

The crystallized venom of *Bothrops atrox* was purchased from the Bioagents Serpentarium (Batatais, São Paulo, Brazil). This snake venom was used as a positive control in the experiments involving erythrocytes based on the capacity of this venom to induce damage in these cells (Cesar et al. 2020). The venom was dissolved in a phosphate-buffered saline (PBS) solution, pH 7.4, to reach a concentration of 0.01% (w/v). The solution was kept in the dark at −12°C until experimental analysis. Normal melting point agarose (CAS 9012-36-6), low melting point agarose (CAS 39346-81-1), and propidium iodide (CAS 25535-16-4) were obtained from Sigma Chemicals Inc. (St. Louis, MO, USA).

2.3 Acetylation of carvacrol

Acetylcarvacrol was obtained by acetylation of carvacrol according to the methods proposed by Gonçalves et al., (2019). Briefly, 5 mL of carvacrol was added to a flask containing 25 mL of 10% sodium hydroxide solution at 25 °C. Subsequently, 5.5 mL of acetic anhydride was added to the flask at 4 °C. The mixture was stirred for 15 min. The oil obtained was separated from the solution and characterized by infrared spectroscopy. An Affinity-1 Shimadzu IR Infrared spectrometer was used on the structural characterization, operating in a spectral range of 400–4000 cm^{-1}.

2.4 Effects on erythrocytes

Human blood was collected in heparin tubes and centrifuged for 5 min at 25°C and 700 G (Fanem Baby I Model 206-BL). Plasma was removed from the tube, and the erythrocytes were suspended in 5 mmol/L of PBS (pH 7.4) and centrifuged under the same conditions. This washing step was repeated two times, as described by Preté et al. (2011). The concentrate of human erythrocytes was used to assess hemolytic activity in a solid medium. To assess the effects on erythrocytes' morphology exposed to the chemicals, the erythrocytes concentrate was

similarly obtained. However, EDTA was used as an anticoagulant and calcium-free PBS was used in the washing step.

2.4.1 Hemolytic activity

The hemolytic activity in a solid medium was assessed according to Gutiérrez et al. (1988). A gel (3 mL of 0.01M $CaCl_2$, 4.4 mL of erythrocytes concentrate, 300 mL of PBS, pH 7.4, 3.3 g bacteriological agar, and 0.1g sodium azide) was poured into Petri dishes at 50°C. After gel solidification, 30 µL of each chemical in different concentrations were applied in holes, and the Petri dishes were maintained in a cell culture chamber for 24h at 37°C. The concentrations used were 0.5 % (w/v) (= 5 µg/mL), 0.75 % (w/v) (= 7.5 µg/mL), 1.0 % (w/v) (= 10 µg/mL), and 2.0 % (w/v) (= 20 µg/mL) for carvacrol, acetylcarvacrol and the commercial pesticide diluted in 1% (w/v) DMSO in PBS, pH 7.4. These concentrations were chosen based on pilot assays to define the range at which comparisons among the treatments would be feasible. Additionally, these concentrations fall within the range commonly tested to evaluate the pesticide activity of chemicals (Konig et al., 2020, 2021). The positive control was composed of 0.01% (w/v) *B. atrox* venom dissolved in PBS. Negative control I was composed of PBS and negative control II was composed of 1% (w/v) DMSO diluted in PBS. The hemolytic activities were evaluated by measuring (mm) the translucent halo formed around the holes in the gels where the samples were applied. A minimum of three halos were measured for each treatment in two independent experiments.

2.4.2 Erythrocytes morphology

The overall shape morphology of the erythrocytes was evaluated by conventional light microscopy after previous incubation with the chemicals, as adapted from Wang et al. (2010). Briefly, 100 µL of the erythrocytes concentrate was incubated with an equal volume of the chemicals at concentrations of 0.5, 0.75, 1.0 and 2.0% (w/v) for carvacrol, acetylcarvacrol and the commercial pesticide diluted in 1% DMSO in calcium-free PBS. The positive and negative controls had the same composition as previously described for the hemolytic activity. The samples were incubated at 37 °C for 30 min under gentle shaking. Then, 25 µL of each sample was applied to microscope slides in triplicate. After air drying, the glass slides were fixed in methanol and stained with Panotico (Laborclin, Ribeirão Preto, Brazil) according to the

manufacturer's instructions. Then, ten fields in different areas of each replicate were examined in conventional light microscopy (Olympus CX31 microscope) in two independent experiments. Finally, the main morphological alterations found in the erythrocytes, as well as the percentage of cells showing altered morphology, were evaluated.

2.5 Effects on leucocytes

2.5.1 Cell viability

Analysis of the viability of peripheral blood leucocytes exposed to the chemicals was adapted from Avelar-Freitas et al. (2015). Briefly, 20 mL of EDTA-anticoagulated whole blood was incubated for 20 min in the presence of 10 mL of red blood cell lysis solution (NH_4Cl 8.3 mg/mL, $NaHCO_3$ 1 mg/mL, EDTA 1 mg/mL) followed by two washing steps and centrifugation (250 × g for 10 min) of the leucocytes in PBS. Cellular concentration of leucocytes was adjusted to 1×10^5 cells/mL in RPMI-1640 medium (Sigma-Aldrich). Then, a 450 µL aliquot of the cell suspension was incubated with 50 µL of carvacrol, acetylcarvacrol and the commercial pesticide, in concentrations of 0.5, 0.75, 1.0 and 2.0 % (w/v), diluted in 1% DMSO. Negative control I was composed of PBS and negative control II was composed of 1% DMSO diluted in PBS. After incubation for 3 h at 37 °C (Sinha et al., 2014), the leucocytes were washed by centrifugation and fresh media was added. The leucocytes were stained with trypan blue dye (0.4% w/v) and the number of viable and dead cells was scored under light microscopy (Olympus CX31 microscope) with Neubauer's hemocytometer. A minimum of 300 cells were analyzed for each treatment in three independent experiments.

2.6 Effects on DNA fragmentation

2.6.1 Single cell gel electrophoresis with leucocytes (comet assay)

The comet assay was used to detect fragmentation in DNA molecules of human lymphocytes. In this analysis, 300 µL of citrated blood was incubated in a cell culture chamber for 4h at 37 °C, with 290 µL of PBS and 10 µL of treatments. They consisted of carvacrol, acetylcarvacrol and the commercial pesticide, in concentrations of 1.0 and 2.0 % (w/v), diluted in 1% DMSO in PBS. Negative controls consisted of PBS and 1% DMSO, and the positive

control was composed of 1% cisplatin (w/v). The slides were previously covered with one layer of normal melting point agarose. From the incubated, 75 µL aliquots were transferred to tubes containing 225 µL of low melting point agarose. Three slides (75 por 25 mm) per treatment, 100 µL per slide, were prepared (Nandhakumar et al., 2011), covered with coverslips, subjected to a temperature of 4ºC for 15 minutes with subsequent removal of the coverslips. The slides were submitted to osmotic lysis (0.25 M NaCl, 100 mM EDTA, 1% Triton X-100, 5% DMSO, pH 10), for 2 hours. Then, the slides were kept in the electrophoresis solution (1 mM EDTA, 30 mM NaOH, pH 13) for 20 min at 4 °C followed by electrophoresis run at 30V (1 V/cm) and 300mA for 30 min. The temperature of the electrophoresis solution was 20 ± 2°C, and circulation of the buffer was done as an attempt to keep the temperature constant during the run. After that, the nucleoids remained in a neutralization solution (0.4 M Tris-HCl, pH 7.4) for 25 min and then were fixed with ethanol, dried at room temperature and visualized, after staining with 50 µL of propidium iodide in the concentration of 20 µg/mL, using fluorescence microscopy (Zeiss Axio Observer Z1). The readings were carried out in the center of the glass slides, avoiding to use the edges for the nucleoids count. Analysis of nucleoid fragmentation levels was performed according to classes described by Collins et al. (1997), with some adaptations: class 0, damages ≤ 5%; class 1, damages between 5 and 20%; class 2, damages between 20 and 40%; class 3, damages between 40 and 85%; and class 4, damages ≥ 85%. A minimum of 600 nucleoids were analyzed for each treatment in two independent experiments, and the number of nucleoids observed in each class was converted into a percentage. Arbitrary units were calculated according to Collins (2004), (0- 400, in which 0 = no damage and 400 = 100% damage), using the equation: (1 x number of nucleoids in class 1) + (2 x number of nucleoids in class 2) + (3 x number of nucleoids in class 3) + (4 x number of nucleoids in class 4).

2.6.2 DNA fragmentation assay

Peripheral blood leukocytes were isolated from whole blood as previously described in section 2.4.1. Then, genomic DNA was extracted from these cells using the QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA). The concentration of DNA was adjusted to 10 ng/µL using a NanoDrop-2000 (Thermo Scientific, Wilmington, DE); 260/280 ratio was examined to confirm sample purity. The DNA solution was incubated at 37 °C for 3h with carvacrol, acetylcarvacrol and the commercial pesticide, in concentrations of 1.0 and 2.0 % (w/v), diluted

in 1% DMSO. Negative control I was composed of PBS and negative control II was composed of 1% DMSO diluted in PBS. This assay was carried out in duplicate using proportions of 1:5, 1:10 and 1:20 (chemicals/DNA). After incubation, the solutions were electrophoresed on 0.5% agarose gels for 1h at 80V, stained with SYBR Green and visualized under UV light, as adapted from Ansari et al. (2017).

2.7 Statistical analysis

Results were presented as mean ± standard deviation (SD). All data were first analyzed for normality (Shapiro–Wilk). Then, data were subjected to two-way ANOVA followed by Tukey's multiple comparison *post hoc* test ($p < 0.05$) using Minitab software (version 17.1).

3. RESULTS

3.1 Acetylation of Carvacrol

The infrared spectrum graph of acetylcarvacrol and carvacrol is shown in figure 1. The absence of intense broadband between 3000 to 4000 cm^{-1} indicates no residual phenol from the reactant. An intense band at 1753 cm^{-1} is the confirmation of the ester carbonyl structure.

3.2 Effects on erythrocytes

The effects of carvacrol, acetylcarvacrol and the commercial pesticide on hemolytic activity are shown in Table 1. The negative controls presented no hemolytic activity, whereas the positive control exhibited the greatest halo diameter. All chemicals showed an increase in hemolysis as the concentration raised ($p < 0.001$). The hemolysis caused by the commercial pesticide was statistically equal to carvacrol at all concentrations ($p = 0.086$). However, the halo diameter for acetylcarvacrol was significantly lower compared with the other chemicals ($p < 0.001$). At concentrations of 0.5% and 0.75%, acetylcarvacrol did not cause any hemolysis.

The effects of the chemicals on erythrocyte morphology are presented in Table 2 and Figure 2. The solvent (1% DMSO) did not affect the morphology of the erythrocytes, whereas 0.01% (w/v) *B. atrox* venom caused severe alterations in these cells. Erythrocytes in the positive control exhibited a damaged plasma membrane, which is visualized as a circular region in the

central part of the cells without reactivity to the dyes. Additionally, erythrocytes exhibited an acanthocyte-like shape, showing spikes of different lengths and widths unevenly positioned on the cell surface.

In treatments, the main morphological alterations found were the presence of ghost erythrocytes, elliptocyte-like shape, as well as aggregations of red blood cells in a rouleau-like shape. The percentage of normal erythrocytes decreased for all chemicals in higher concentrations ($p < 0.001$). In the concentration of 2%, the cells were severely damaged by the action of the commercial pesticide and carvacrol. However, erythrocytes exposed to 2% acetylcarvacrol exhibited normal morphology for most of the cells compared to the other chemicals.

3.3 Effects on leucocytes

The viability of human leucocytes after incubation for 3h with the commercial pesticide, carvacrol and acetylcarvacrol is shown in Figure 3. Cells incubated with PBS and 1% DMSO (negative controls) exhibited viability greater than 97%. Leucocytes exposed to the commercial pesticide and carvacrol presented a decrease in viability at a concentration of 2% ($p < 0.001$), whereas acetylcarvacrol did not affect cell viability as the concentration increased ($p = 0.3376$). Fipronil caused a drop in cell viability compared with the other chemicals at all concentrations ($p < 0.001$). Carvacrol and acetylcarvacrol showed no differences in leucocyte viability at concentrations ranging from 0.5 to 1.0% ($p = 0.3868$). However, cells incubated with 2% acetylcarvacrol exhibited the highest viability (93%) compared to the other chemicals.

3.4 Effects on DNA fragmentation

The arbitrary units for the comet assay are presented in Figure 4. The distribution of nucleoids in different classes according to their respective treatment groups is shown in Table 3 and Figure 5. Fipronil and carvacrol exhibited an increase in arbitrary units as the concentration raised ($p < 0.001$). However, there were no differences in arbitrary units for 1 and 2% acetylcarvacrol ($p = 0.431$). The damage caused by fipronil and carvacrol was similar at a concentration of 2%, whereas for acetylcarvacrol it was significantly smaller ($p = 0.007$). The distribution of nucleoids also shows that cisplatin (positive control), as well as carvacrol and

fipronil, presented class 4 nucleoids at a concentration of 2%. However, most of the nucleoids were distributed in classes 1 and 2 for acetylcarvacrol at the same concentration.

The fipronil-based pesticide partially degraded genomic DNA extracted from human leucocytes after incubation for 3h (Figure 6). Degraded DNA can be seen after incubation in proportions as low as 1:20 up to 1:5 (chemical: DNA). No degradation was observed for the negative controls as well as for carvacrol and acetylcarvacrol in concentrations of 1 and 2%.

Table 1. Hemolytic activity (mm) of carvacrol, acetylcarvacrol and a fipronil-based pesticide.

Chemicals	Concentration % (w/v)	Halo diameter (mm)
PBS (negative control I)	-	0.0 ± 0.0
DMSO (negative control II)	1.0	0.0 ± 0.0
Bothrops atrox venom (positive control)	0.01	20.7 ± 0.5
Commercial pesticide (fipronil 800 mg/g)	0.5	$5.7 \pm 0.6^{A,a}$
	0.75	$7.0 \pm 0.0^{A,a}$
	1.0	$7.7 \pm 0.6^{A,ab}$
	2.0	$10.3 \pm 0.6^{A,b}$
Carvacrol	0.5	$6.0 \pm 0.0^{A,a}$
	0.75	$5.6 \pm 0.6^{A,a}$
	1.0	$6.0 \pm 0.0^{AB,a}$
	2.0	$9.0 \pm 0.0^{A,b}$
Acetylcarvacrol	0.5	$0.0 \pm 0.0^{B,a}$
	0.75	$0.0 \pm 0.0^{B,a}$
	1.0	$4.7 \pm 0.6^{B,b}$
	2.0	$7.0 \pm 0.0^{B,b}$

Capital letters were used to compare the three chemicals in the same concentration. Lowercase letters were used to compare different concentrations of each chemical alone (two-way ANOVA, Tukey's *post hoc* test; $p < 0.05$).

Table 2. Percentage of normal erythrocytes after incubation with carvacrol, acetylcarvacrol and a fipronil-based pesticide.

Chemicals	Concentration % (w/v)	Normal erythrocytes (%)
PBS (negative control I)	-	99.3 ± 1.1
DMSO (negative control II)	1.0	99.6 ± 0.6
Bothrops atrox venom (positive control)	0.01	18.3 ± 2.9
Commercial pesticide (fipronil 800 mg/g)	0.5	$93.3 \pm 2.9^{A,a}$
	0.75	$78.3 \pm 2.9^{A,b}$
	1.0	$71.7 \pm 2.9^{A,b}$
	2.0	$3.3 \pm 5.8^{A,c}$
Carvacrol	0.5	$96.3 \pm 0.6^{A,a}$
	0.75	$91.7 \pm 2.9^{B,a}$
	1.0	$81.7 \pm 2.9^{B,b}$
	2.0	$8.3 \pm 2.9^{A,c}$
Acetylcarvacrol	0.5	$97.3 \pm 1.1^{A,a}$
	0.75	$97.3 \pm 0.6^{B,a}$
	1.0	$96.7 \pm 1.5^{C,a}$
	2.0	$73.3 \pm 5.7^{B,b}$

Capital letters were used to compare the three chemicals in the same concentration. Lowercase letters were used to compare different concentrations of each chemical alone (two-way ANOVA, Tukey's *post hoc* test; $p < 0.05$.

Table 3. Distribution of nucleoids, determined by visual score, obtained from human lymphocytes exposed to carvacrol, acetylcarvacrol and a commercial pesticide (fipronil 800 mg/g).

Treatment	Concentration % (w/v)	Nucleoids (%)				
		class 0	class 1	class 2	class 3	class 4
PBS	-	77.3 ± 2.5	22.7 ± 2.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
DMSO	1.0	78.0 ± 1.0	22.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cisplatin	1.0	1.0 ± 1.0	24.3 ± 5.9	32.0 ± 1.0	31.7 ± 3.2	11.0 ± 3.0
Fipronil	1.0	5.7 ± 3.8	44.7 ± 15.1	39.3 ± 13.0	10 ± 9.5	0.3 ± 0.6
	2.0	1.7 ± 1.5	15.0 ± 3.6	35.7 ± 9.6	43.0 ± 10.8	4.7 ± 2.5
Carvacrol	1.0	12.0 ± 7.8	50.6 ± 7.5	34.3 ± 13.0	3.0 ± 0.0	0.0 ± 0.0
	2.0	0.0 ± 0.0	4.7 ± 4.2	30.3 ± 1.5	61.3 ± 2.3	3.6 ± 2.0
Acetylcarvacrol	1.0	17.7 ± 6.8	57.0 ± 3.6	24.3 ± 9.7	1.0 ± 1.0	0.0 ± 0.0
	2.0	10.0 ± 4.0	58.3 ± 10.0	30.7 ± 10.6	1.0 ± 1.0	0.0 ± 0.0

Negative controls: PBS and 1% DMSO. Positive control: 1% cisplatin. Damage of class 0 (<5%), class 1 (5-20%), class 2 (20-40%), class 3 (40-85%), and class 4 (> 85%), as described by Collins et al. (1997).

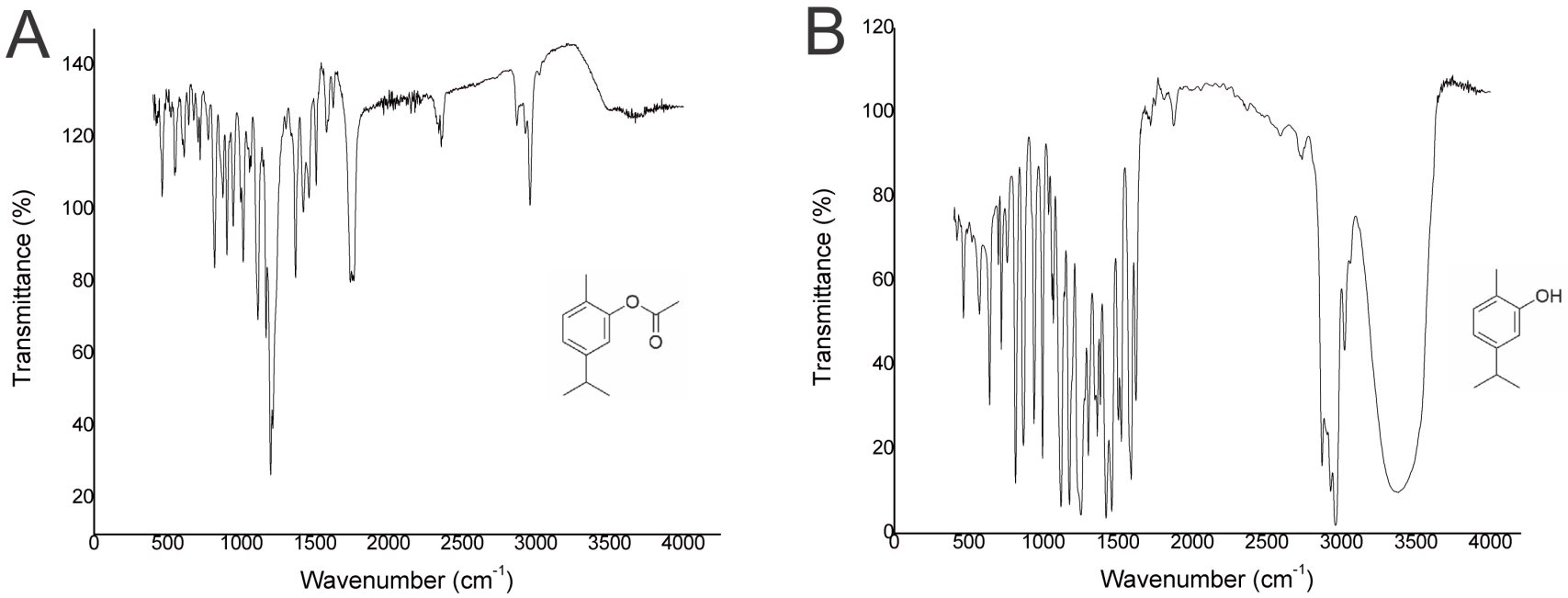

Figure 1. Infrared spectrum of acetylcarvacrol (A) and carvacrol (B), showing that the phenolic -OH group present in the structure of carvacrol has been replaced by an ester carbonyl in the structure of acetylcarvacrol.

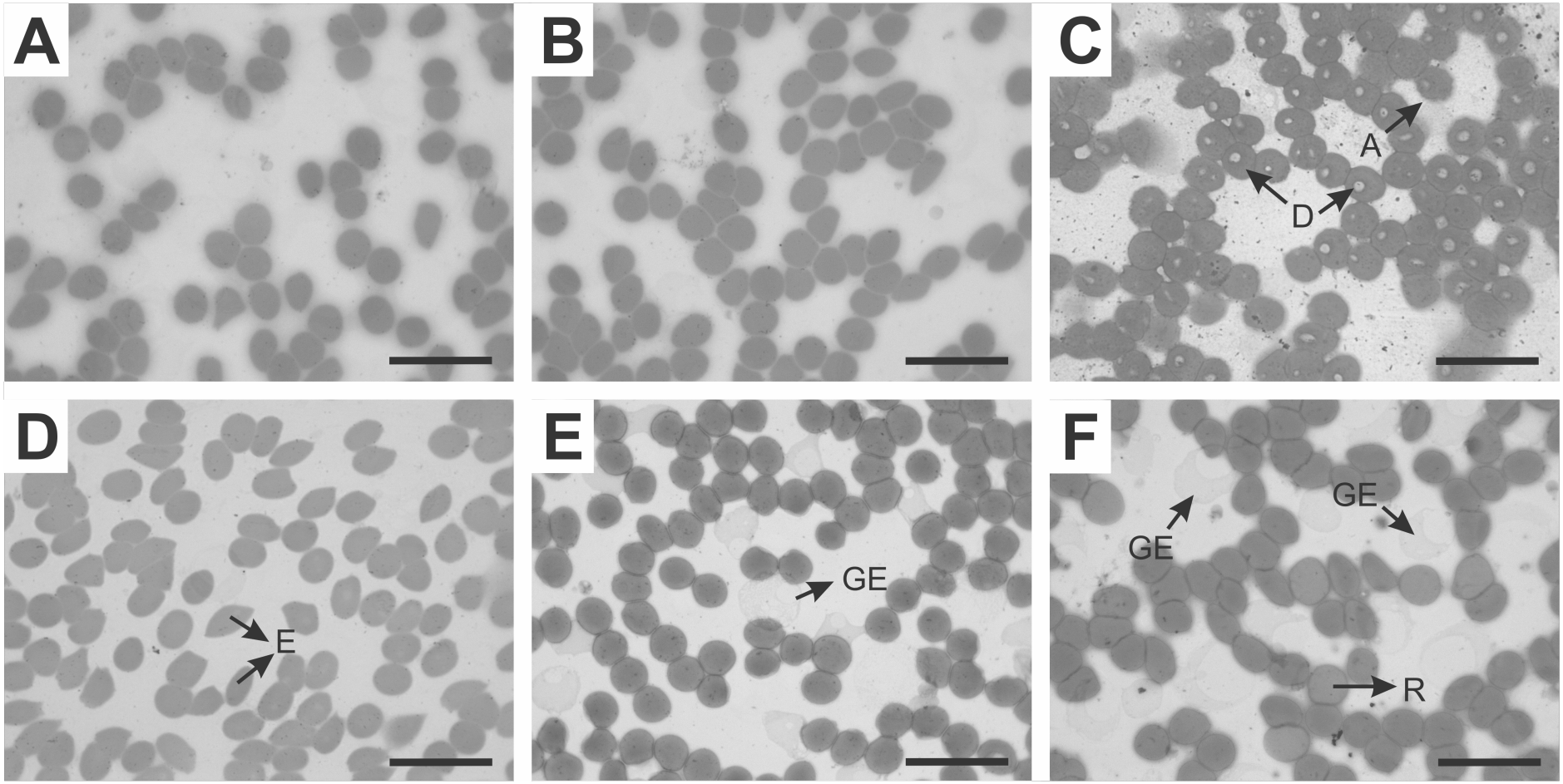

Figure 2. Optical micrographs of human erythrocytes incubated with carvacrol, acetylcarvacrol and a fipronil-based pesticide at 1% (w/v).(A) Negative control I – PBS; (B) Negative control II – 1% DMSO diluted in PBS; (C) Positive control - 0.01% (w/v) *Bothrops atrox* venom dissolved in PBS; (D) 1% acetylcarvacrol; (E) 1% carvacrol; (F) 1% fipronil. Legends: A - acanthocyte-like shape; D - damaged plasma membrane; E - elliptocyte-like shape; GE - ghost erythrocytes; R - rouleau-like shape. Bars: (A–F) 25 μm.

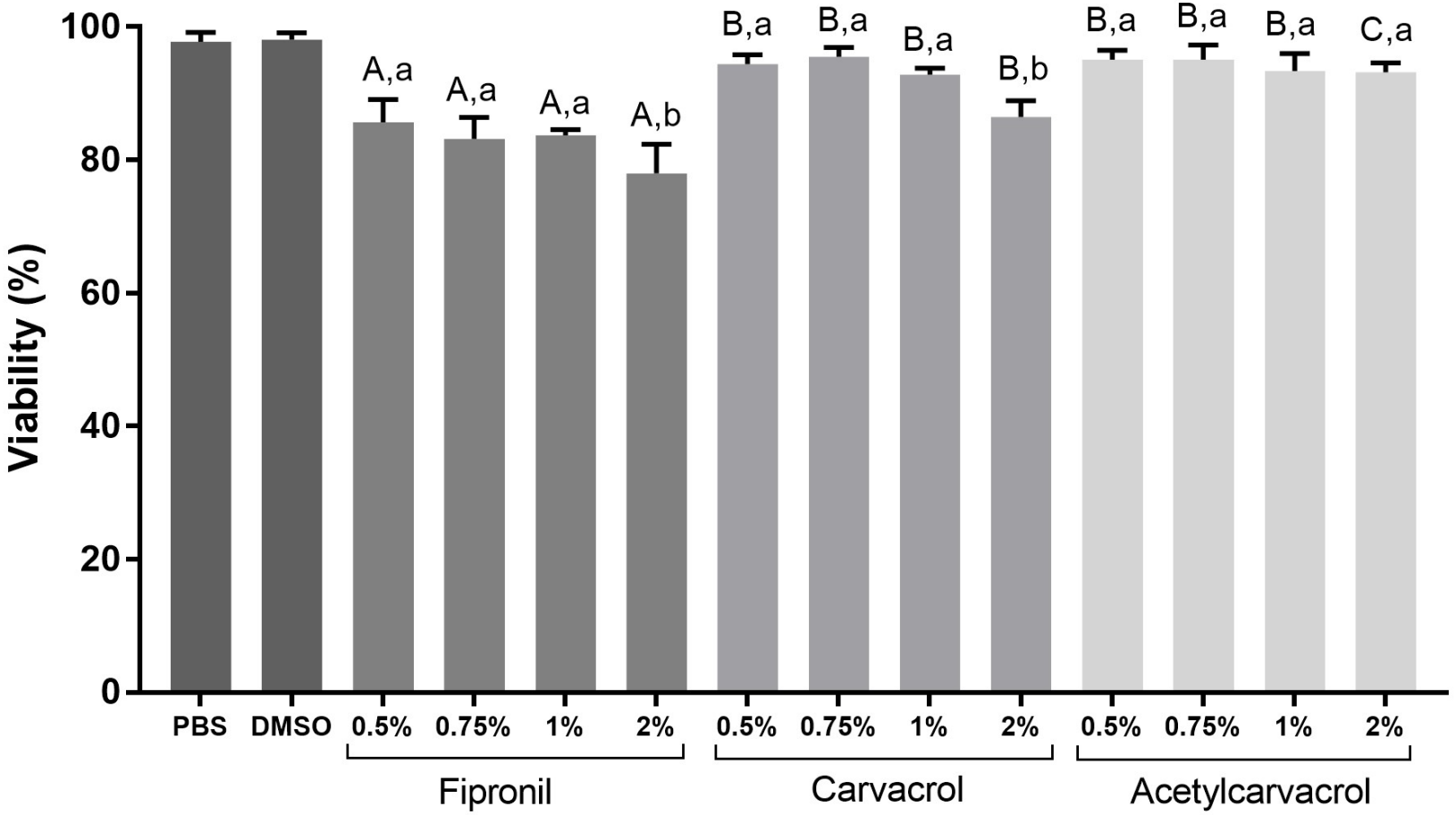

Figure 3. Viability of human leucocytes after incubation for 3h with a fipronil-based pesticide, carvacrol and acetylcarvacrol at different concentrations. Negative controls: PBS and DMSO. Capital letters were used to compare the three chemicals in the same concentration. Lowercase letters were used to compare different concentrations of each chemical alone (two-way ANOVA, Tukey's *post hoc* test; $p < 0.05$).

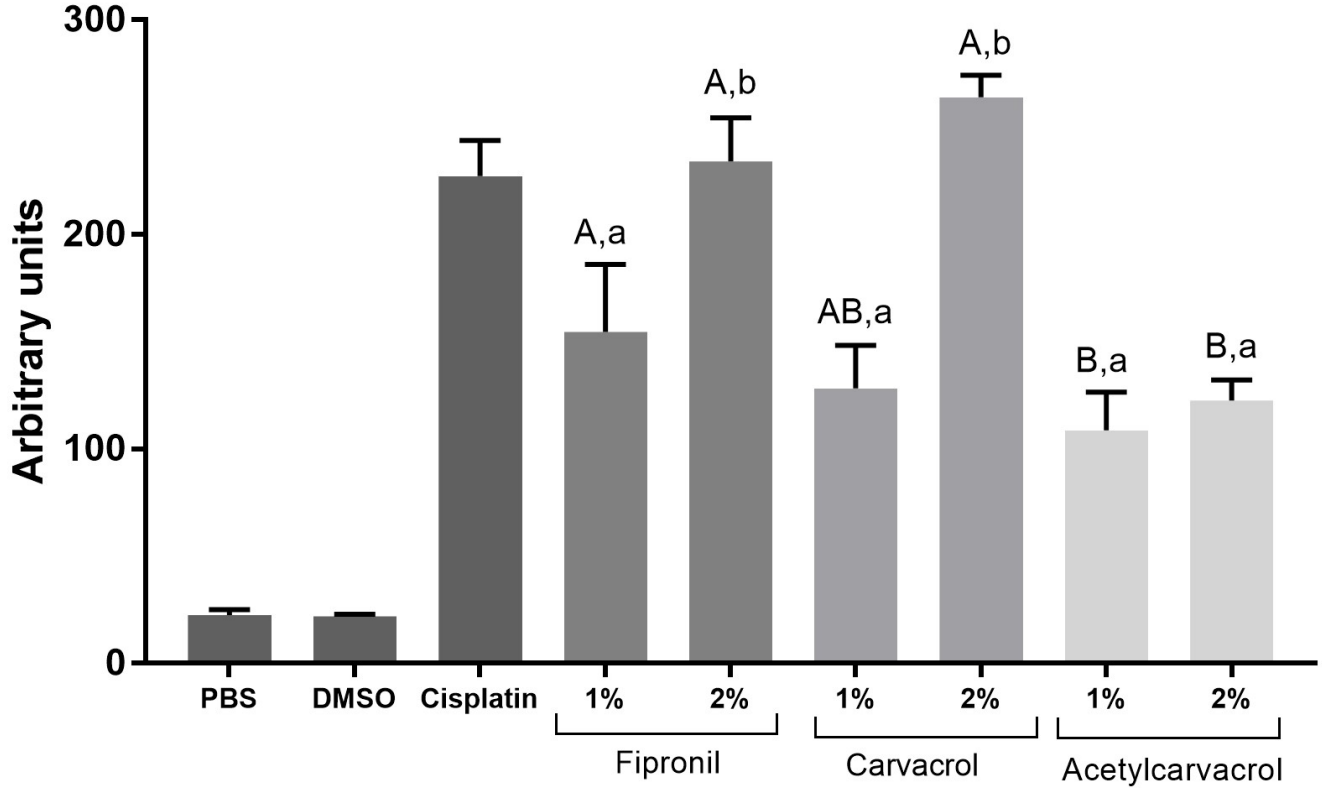

Figure 4. Comet assay to evaluate the effects of a fipronil-based pesticide, carvacrol and acetylcarvacrol on nucleoids obtained from human lymphocytes. Negative controls: PBS and DMSO. Positive control: 1% cisplatin. Arbitrary units (AU) were calculated as described by Collins et al. (2004): (1 x number of nucleoids in class 1) + (2 x number of nucleoids in class 2) + (3 x number of nucleoids in class 3) + (4 x number of nucleoids in class 4). Capital letters were used to compare the three chemicals in the same concentration. Lowercase letters were used to compare different concentrations of each chemical alone (two-way ANOVA, Tukey's *post hoc* test; $p < 0.05$).

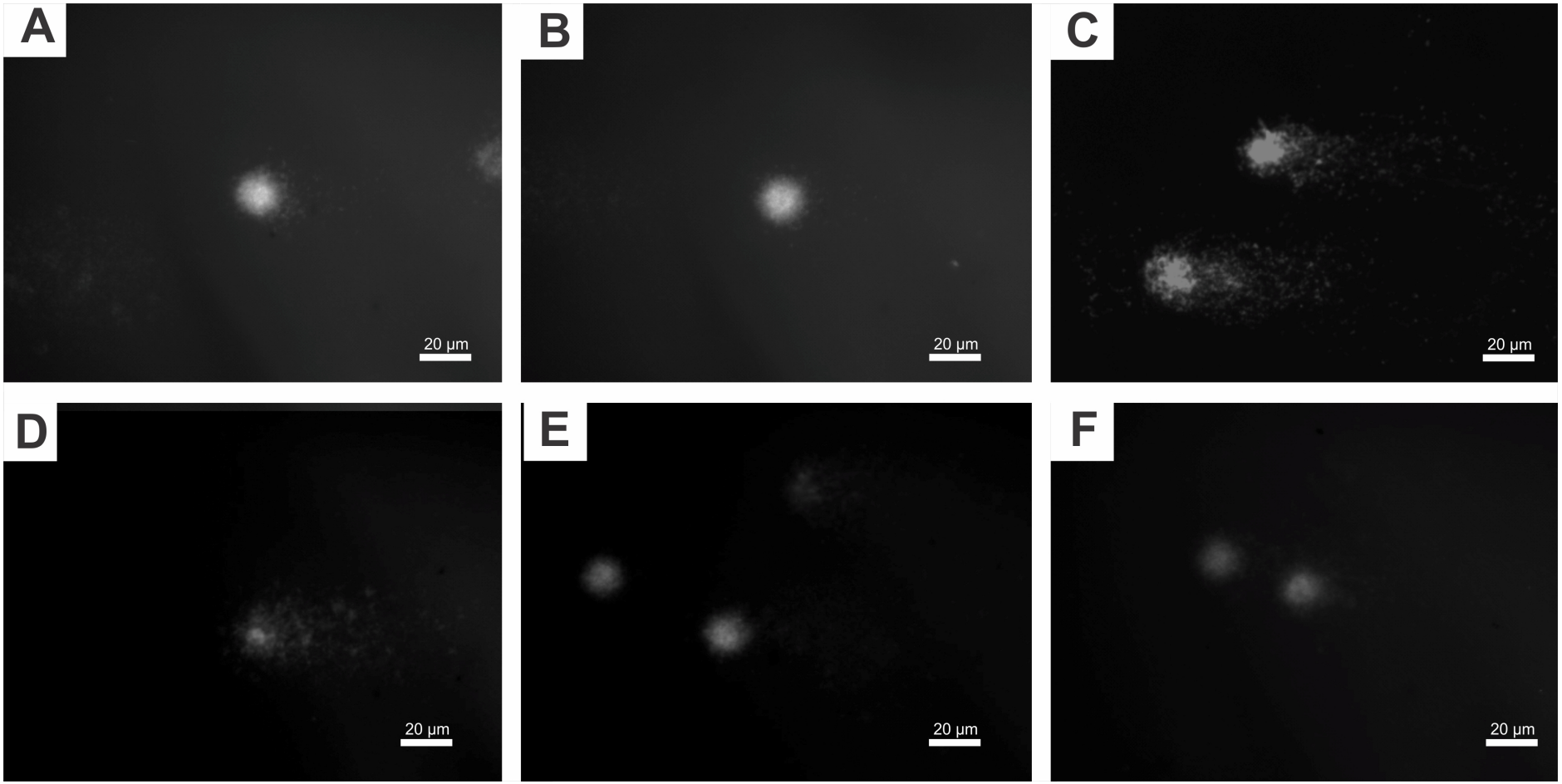

Figure 5. Comet assay on human blood treated with (A) negative control I – PBS; (B) negative control II – 1% DMSO diluted in PBS; (C) positive control - 1% cisplatin; (D) 1% fipronil; (E) 1% carvacrol; (F) 1% acetylcarvacrol. Bars: (A–F) 20 µm.

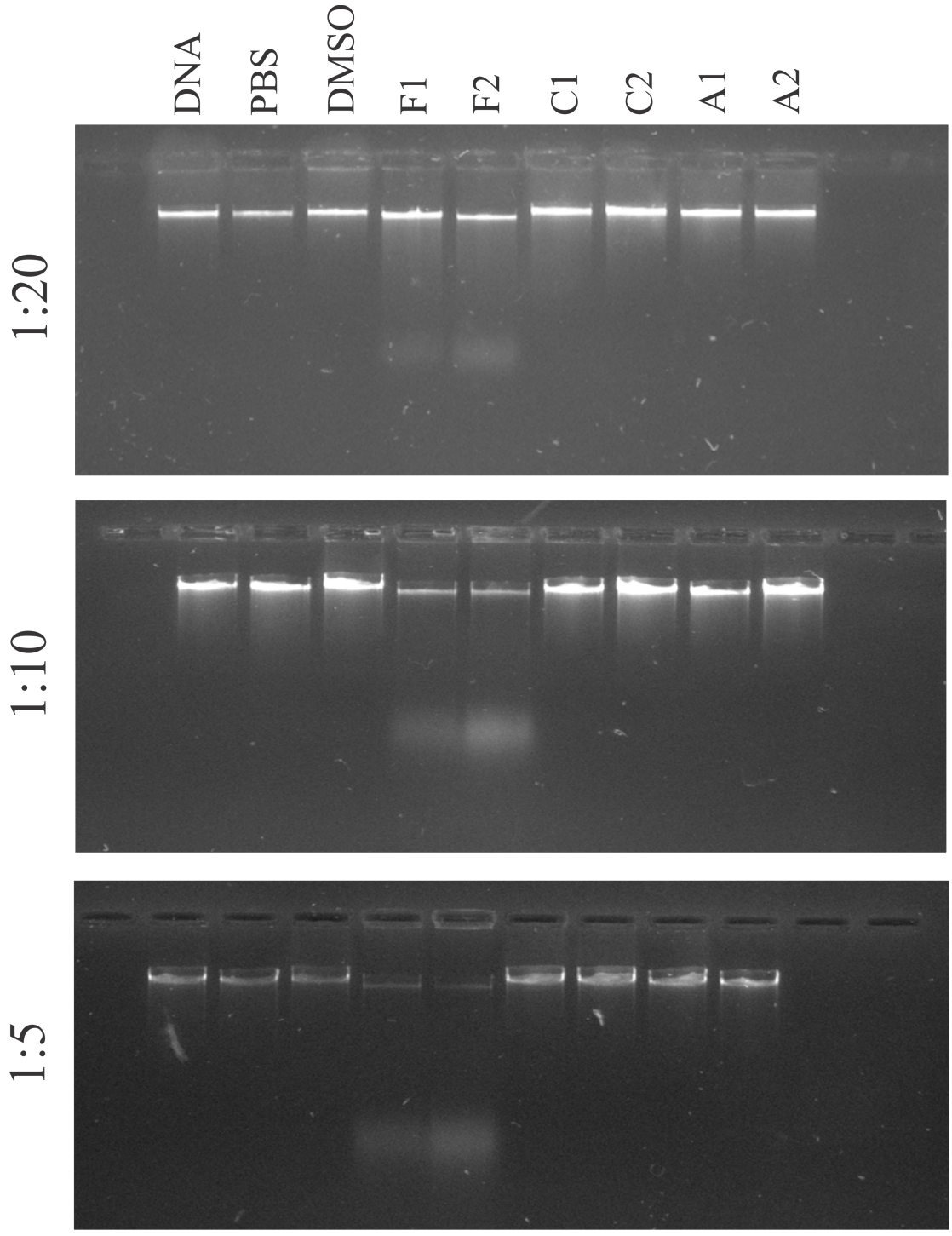

Figure 6. Genomic DNA obtained from blood leukocytes incubated with carvacrol, acetylcarvacrol and a fipronil-based pesticide in the proportions of 1:5, 1:10 and 1:20 (chemicals/DNA) and electrophoresed on 0.5% agarose gels. Legends: (DNA) – genomic DNA; (PBS) – DNA + PBS; (DMSO) - DNA + 1% DMSO; (F1) – 1% fipronil-based pesticide + DNA; (F2) – 2% fipronil based pesticide + DNA; (C1) – 1% carvacrol + DNA; (C2) – 2% carvacrol + DNA; (A1) – 1% acetylcarvacrol + DNA; (A2) – 2% acetylcarvacrol + DNA.

4. DISCUSSION

Carvacrol and its acetylated derivative present a wide range of biological activities (Marinelli et al., 2018; Suntres et al., 2015; Damasceno et al., 2014). Pesticide activity has also been attributed to these compounds (Gonçalves et al., 2019; Tak and Isman, 2017). However, these chemicals are not yet constituents of commercial pesticides, to the best of our knowledge. The lack of comparative toxicological studies using these chemicals is likely to be one of the main reasons they are not yet used in agriculture (Pavela and Benelli, 2016). In this sense, we decided to compare both chemicals to a fipronil-based commercial pesticide, which is vastly used worldwide (Simon-Delso et al., 2015). We employed in our assays human blood cells, which is a highly sensitive toxicity model (Farag and Alagawany, 2018), avoiding the use of experimental animals.

Evaluating hemolysis is an indispensable initial step to assessing the blood compatibility with chemicals and identifying possible severe acute toxic reactions in erythrocytes *in vivo* (Dobrovolskaia and Mcneil, 2013). The hemolysis assay refers to the disruption of erythrocytes leading to leakage of hemoglobin and other contents. Hemoglobin released through hemolysis has severe consequences as it is vasoactive and redox-active protein, causing toxic effects on vascular, myocardial, renal, and central nervous system tissues (Jeong et al., 2017; Buehler and Agnillo, 2010). Our results showed that both carvacrol and fipronil caused hemolysis in concentrations as low as 0.5 and 0.75% (w/v), but it was not observed for acetylcarvacrol. As the concentration increased to 1 and 2%, hemolysis was observed for all chemicals. However, the values obtained for acetylcarvacrol were significantly lower compared with the other chemicals. Interestingly, incubation of acetylcarvacrol (0.9-7.2 μg/mL) with mice erythrocytes considerably reduced hydrogen peroxide-induced hemolysis, possibly due to the antioxidant properties of this chemical (Oliveira et al., 2020a).

Evaluating erythrocytes morphology is also an alternative to assessing the toxic effects of chemicals on these cells. This assay also presents satisfactory correlations with hemolysis activity (Wang et al., 2010). The presence of ghost erythrocytes indicates irreversible damage to the lipid bilayer, and it is commonly found after hemolysis (Baumann et al., 2000). Our results showed that ghost erythrocytes were frequently found in treatment groups. All three chemicals caused slight alterations at 0.5%. However, as the concentration increased, carvacrol and fipronil considerably damaged these cells, whereas acetylcarvacrol kept the percentage of normal erythrocytes much

higher at 2%. Changes in human erythrocytes morphology were also observed after incubation with bis-(phosphonomethyl)amine, an impurity commonly found in glyphosate formulations (Kwiatkowska and Huras, 2014). These authors stated that the incorporation of xenobiotics into cell membranes and interaction with cytoskeleton proteins may lead to morphological alterations in erythrocytes, ultimately causing hemolysis. The results of these assays, taken together, demonstrate that both carvacrol and fipronil, particularly at higher concentrations, can cause hemolysis and possibly trigger an immune response due to the release of cell contents into the blood plasma.

Leucocytes play a crucial role in immunity, and their total and differential count can be altered in farmers exposed to pesticides (Cattelan et al., 2018; Aroonvilairat et al., 2015). Thus, evaluating the toxicity of new pesticides on these cells is essential to determine their safety for humans. Our results showed that both carvacrol and acetylcarvacrol presented a low effect on leucocyte viability at reduced concentrations. However, carvacrol exhibited a greater cytotoxic effect at 2% compared with acetylcarvacrol. A study that evaluated the effects of carvacrol on human leucocytes also found similar results. The viability of leucocytes exposed to carvacrol was approximately 90% for concentrations ranging from 10 to 100 mg/L, and it was statistically equal compared with the negative control. As the concentration increased to 150 and 200 mg/L, leucocyte viability dropped to approximately 80% (Türkez and Aydın, 2014). Fipronil, on the other hand, considerably reduced leucocyte viability even at low concentrations. Similar results were found after incubation for 12h of rat lymphocytes with organophosphate pesticides, such as chlorpyrifos, methyl parathion and malathion, which resulted in viability values close to 70% (Ojha and Gupta, 2016). These authors also found a dose and time-dependent increases in DNA interstrand cross-links and increase of intracellular active caspase-3 and caspase-9, which are directly related to apoptosis.

The comet assay detects damaged DNA by differential migration of nuclear material when subjected to electrophoresis, being a useful tool for genetic toxicology (Tice et al., 1990). In the present study, carvacrol and fipronil showed a dose-dependent increase in the arbitrary units for the comet assay. Similarly, a study that evaluated the genotoxic effects of fipronil on human lymphocytes also showed that this chemical induced DNA damage in concentrations of 0.1, 0.3 and 0.7 µg/mL (Çelik et al., 2014). Regarding *in vitro* genotoxic studies of carvacrol, contradictory results have been published, as reviewed by Llana-Ruiz-Cabello et al. (2015). Additionally, Rathod

et al. (2021) argue that carvacrol used at higher concentrations may cause genotoxic effects on humans. These authors suggest that nanotechnology and encapsulation can be used to mitigate the possible hazard of this chemical, since circulation time and effectivity at lower concentrations are improved with these technologies.

Acetylcarvacrol, in contrast, caused reduced DNA damage compared with the other chemicals. Moreover, acetylcarvacrol exhibited a greater proportion of nucleoids in classes 1 and 2, and none in class 4. In our study, the comet assay was performed immediately after exposure to treatments, which occurred for 4 h. Thus, several nucleoids were obtained before the action of cell checkpoints, since human lymphocytes complete a cell division cycle in approximately 7h, and others were obtained after the action of cell checkpoints, since the human lymphocytes present in the freshly collected blood were in different stages of cell division (Marcussi et al., 2013, 2011). In this sense, nucleoids classified in levels 1 and possibly 2 could have the damage totally or partially corrected, reducing potential mutagenic effects (Abreu et al., 2021).

Fragmentation of DNA was also evaluated in this study after incubation of genomic DNA with the chemicals. Interestingly, carvacrol did not cause fragmentation of DNA in this assay, although it considerably damaged DNA as evaluated by the comet assay. Our results show that carvacrol does not directly interact and cause fragmentation of DNA as fipronil does. We believe that carvacrol is likely to damage DNA through an indirect mechanism, possibly through increased reactive oxygen species production. For instance, oral administration of thymol, a carvacrol isomer, to adult male Swiss mice considerably increased renal reactive oxygen species and lipid damage levels as well as inhibited antioxidant capacity against peroxyl radicals (Baldissera et al., 2018). Fipronil, on the other hand, caused fragmentation of DNA regardless of the proportion of chemicals used. Thus, these results suggest that this chemical is likely to direct damage DNA as well as cause indirect damage through oxidative stress (Abou-Zeid et al., 2021; Khan et al., 2015).

In conclusion, our data show that acetylation of carvacrol reduced its toxicity to human blood cells. Indeed, acetylcarvacrol was less toxic to erythrocytes and leucocytes compared with the other chemicals. At low concentrations, carvacrol also demonstrated reduced toxicity, particularly to leucocytes. Additionally, carvacrol and its acetylated derivative did not cause fragmentation of DNA in the proportions evaluated. However, fipronil was toxic even at low concentrations and induced DNA damage at all proportions evaluated in this study. Thus, the search for safe alternative pesticides should be intensified and the chemical modification of natural

products presenting pesticide activity would seem to be a promising alternative to reduce their toxicity to non-target organisms.

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PART III - Paper II

Toxicity assessment of carvacrol and its acetylated derivative in early staged zebrafish (*Danio rerio*): safe alternatives to fipronil-based pesticides?

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Abstract

Fipronil is a broad-spectrum pesticide presenting high acute toxicity to non-target organisms, particularly to aquatic species. Natural compounds stand out as promising alternatives to the use of synthetic pesticides such as fipronil. Thus, our study aimed to compare the toxicity of carvacrol (natural), acetylcarvacrol (semisynthetic), and fipronil (synthetic) to early staged zebrafish. We conducted a series of toxicity assays at concentrations ranging from 0.01 μM to 25 μM for fipronil and 0.01 μM to 200 μM for carvacrol and acetylcarvacrol, depending on the assay, after 7-days post-fertilization (dpf). The potency (EC_{50}) of fipronil was ~1 μM for both deformities and mortality at 7 dpf, whereas EC_{50} was > 50 μM for carvacrol and > 70 μM for acetylcarvacrol. Fipronil at 0.1 and 1 μM caused a decrease in body length and swim bladder area of larvae at 7dpf, but no difference was observed for either carvacrol or acetylcarvacrol. Based upon the visual motor response test, fipronil induced hypoactivity in larval zebrafish at 1 μM and acetylcarvacrol induced hyperactivity at 0.1 μM. Anxiolytic-type behaviors were not affected by any of these chemicals. All chemicals increased the production of reactive oxygen species at 7 dpf, but not at 2 dpf. Genes related to swim bladder inflation, oxidative stress, lipid metabolism, and mitochondrial activity were measured; fipronil induced upregulation of *atp5f1c*. However, no changes were observed in oxygen consumption rates and apoptosis. Taken together, our data suggest that carvacrol and its derivative are safe replacements for fipronil due to their low acute toxicity.

Keywords: carvacrol acetate; fipronil; zebrafish; aquatic toxicity; mitochondria.

1. INTRODUCTION

Pesticides are a group of chemical substances used to control or repel insects, weeds, and other pests. Their use is not limited to agriculture and they are used in home gardens and residential areas to control domestic pests as well as disease insect vectors (Rani et al., 2021). Among various pesticides, fipronil stands out for being one the most widely used, systemic, and broad-spectrum insecticides on the market. Fipronil belongs to the phenylpyrazole class that acts by inhibiting the gamma-aminobutyric acid (GABA)-gated chloride channel leading to neural hyperexcitability at low doses, paralysis, and subsequently death in insects (Szegedi et al., 2005). Although fipronil has a high specificity towards the invertebrate receptors (Narahashi et al., 2007), it remains a concern to other species due to its high usage, persistence in the environment, and toxicity to non-target organisms (Singh et al., 2021).

Residual fipronil contaminates land surfaces and water bodies by leaching and surface runoff. As such, fipronil is commonly detected not only in agricultural lands but also in the soil and water of metropolitan areas (Cryder et al., 2019; Sprague and Nowell, 2008). For instance, fipronil was detected in surface water within residential landscapes in Florida, USA, at concentrations ranging from 0.5 to 207.3 ng/L (Wu et al., 2015). In Brazil, fipronil is the most frequently detected pesticide in surface water with concentrations ranging from 0.05 to as high as 26.2 µg/L (Albuquerque et al., 2016). In China, fipronil was found in 55.3% out of 789 tap water samples with a median concentration of 0.03 ng/L, ranging up to 5.07 ng/L (Shi et al., 2020). Additionally, fipronil can undergo biotransformation into fipronil sulfone in animals, increasing its toxic effects due to a higher half-life (1.8 days for fipronil sulfone compared to 0.6 days for fipronil) and possible enhanced affinity for the vertebrate $GABA_A$ receptors (Hainzl et al., 1998). Moreover, fipronil shows a high degree of bioaccumulation in animals, with a notable presence in aquatic organisms (Wang et al., 2022; Santillán Deiú et al., 2021; Li et al., 2018).

Zebrafish (*Danio rerio*) is a promising biological platform for toxicity testing of pesticides (Gonçalves et al., 2020). Many studies have already used this model to demonstrate the high acute toxicity of fipronil to aquatic organisms. Indeed, zebrafish larvae exposed to fipronil (2.5 to 15 mg/L) presented disrupted vascular formation with angiogenic failure and inhibited neurogenesis (Park et al., 2020). It also suppressed the expression of genes related to glycogen and glycerate metabolism and affected behavioral responses in larvae following a pulse embryonic exposure in

concentrations ranging from 0.02 μg/L to 4000 μg/L (Eadie et al., 2020). Moreover, fipronil exposure (33 to 5000 μg/L) to zebrafish embryos for 30 hours caused scoliosis, notochord degeneration, and upregulation in the expression of genes involved in detoxification (Stehr et al., 2006). In this context, due to the high toxicity of fipronil and other synthetic pesticides in general, the search for alternative chemical compounds that are environmentally friendly, sustainable, and effective to control arthropod pests has intensified.

Carvacrol is a phenolic monoterpenoid found in essential oils of oregano (*Origanum vulgare*), and thyme (*Thymus vulgaris*). This chemical is well known for possessing a wide range of biological activities as an antimicrobial, antioxidant, and anticancer agent (Sharifi-Rad et al., 2018). Carvacrol has also emerged as a promising alternative compound to control arthropod pests, showing high insecticidal activity (Gong and Ren, 2020; Magierowicz et al., 2019; Youssefi et al., 2019). Additionally, studies have suggested that chemical modification of carvacrol can enhance its biological activity and stability (Nesterkina et al., 2018; Cacciatore et al., 2015). For instance, acetylation of carvacrol increases its stability by conversion of the phenolic hydroxyl group, which is more susceptible to oxidation, into a less oxidation-sensitive ester group (Konig et al., 2021). Moreover, acetylcarvacrol exhibited a greater acaricidal activity (Ramírez et al., 2016), with reduced toxicity to mice when compared to carvacrol (Andre et al., 2016).

A growing number of studies have indicated promising results of natural-based compounds against a wide range of arthropod pests (Lorsbach et al., 2019; Sparks et al., 2019). Despite this, commercially available biopesticides, particularly those based on carvacrol, are scarce. Pavela and Benelli (2016) state that this is mainly due to the lack of comparative toxicological studies between alternative and commercial pesticides. Indeed, few studies have compared the toxicity of carvacrol and acetylcarvacrol (Oliveira et al., 2020; Andre et al., 2016). Moreover, there are no studies, to the best of our knowledge, comparing the toxicity of these chemicals with a commercial pesticide.

The objectives of this study were to compare the effects of fipronil, carvacrol, and acetylcarvacrol on developing zebrafish. As such, we leveraged a series of toxicity assays and measured endpoints related to survivability, hatchability, malformations, morphometrics, mitochondrial bioenergetics, apoptosis, reactive oxygen species as well as locomotor and behavioral activities. Additionally, we analyzed the expression of genes related to swim bladder inflation, oxidative stress, lipid metabolism, and mitochondrial activity. Data from this study are

anticipated to inform on the toxicity of these compounds to early-stage fish and to contribute to the search and validation for environmentally safer alternative pesticides.

2. MATERIALS AND METHODS

2.1 Chemical Preparation

Fipronil (CAS: 120068-37-3, 98% purity) and carvacrol (CAS: 499-75-2, 98% purity) were purchased from Sigma-Aldrich (USA). Acetylcarvacrol (carvacryl acetate) (CAS: 6380-28-5, purity ≥ 95%) was purchased from Extrasynthese Trading Company (France). Stock solutions were prepared in dimethyl sulfoxide (DMSO) (CAS: 67-68-5, purity ≥ 99.9%, Sigma-Aldrich). Oligomycin A (CAS: 579-13-5, 99% purity), carbonyl cyanide-4-phenylhydrazone (FCCP) (CAS: 370-86-5, 98% purity), sodium azide (NaN_3) (CAS: 26628-22-8, 99.5% purity) and rotenone (CAS: 83-79-4, 95% purity) were purchased from Sigma-Aldrich (USA).

Exposure solutions were obtained by adding 10 µL of each stock solution into 10 mL of embryo rearing medium (ERM) yielding final concentrations of 0.01, 0.1, 1, 2.5, 5, 10 and 25 µM for fipronil (4.37 µg/L up to 10.92 mg/L) and 0.01, 0.1, 1, 5, 10, 25, 50, 100 and 200 µM for carvacrol (1.5 µg/L up to 30.04 mg/L) or acetylcarvacrol (1.92 µg/L up to 38.45 mg/L), depending on the endpoint measured. Negative controls were composed of ERM and 0.1% DMSO. The exposure solutions were made fresh each day and vortexed to replace the solution in beakers by 90%. ERM was prepared according to the general methods (https://zfin.org/zf_info/zfbook/chapt1/1.3.html), in 1 L double distilled water (pH = 7.2 ± 0.1), containing 0.8 g NaCl, 0.04 g KCl, 0.00358 g Na_2HPO_4,0.006 g KH_2PO_4, 0.144 g $CaCl_2$, 0.12 g $MgSO_4$, 0.35 g $NaHCO_3$.

2.2 Maintenance and egg production of zebrafish

A breeding colony (AB x Tübingen, *Danio rerio*) is maintained in a flow-through Pentair system at the Animal Care Services at the University of Florida (Cancer Genetics Research Center) and multiple breeding pairs (~10 pairs) from the culture are rotated to generate embryos. Water parameters are kept with the following ranges and conditions: pH value of 7.3 ± 1, conductivity

value of 600 ± 100 μS·cm^{-1}, photoperiod cycle of light/dark was 14 h:10 h, dissolved oxygen concentration > 80% of air saturation, and temperature of 28 ± 1 ˚C. Approximately four breeding pairs of fish were selected before the experiment, and the eggs (~6h post-fertilization, hpf) were collected and rinsed three times with ERM buffer solution. Rearing and staging of zebrafish embryos were conducted according to established protocols (Kimmel et al., 1995). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida, IACUC Study Number: 201708562.

2.3 Exposure regime

Fertilized and normally developing eggs were selected at ~6 hpf using a dissecting microscope. Zebrafish eggs were assigned in a random fashion into experimental groups (ERM, 0.1% DMSO, or one dose of 0.01-25μM for fipronil and 0.01-200 μM for carvacrol or acetylcarvacrol). Six independent experiments were conducted with four to six replicates (beakers containing 20 embryos) to daily assess the survival, deformities, and hatchability of embryos using EVOS™ FL Auto Imaging System (Thermo Fisher Scientific, USA). Deformity assessments included the presence of spinal lordosis and edema (yolk sack/pericardial) as well as kinked tails over the duration of the exposure. Zebrafish were exposed continuously for 7 days, and the exposure solutions were prepared and renewed on daily basis to maintain the quality of the exposure medium.

2.4 Morphometric analysis

For the swim bladder area and body length measurements, 7dpf larvae were euthanized with tricaine (tricaine methanesulfonate, MS-222). Then, they were placed on a glass slide to capture pictures of each fish in profile. The surface area of the swim bladder was measured with the “area measure” feature of ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsbweb.nih.gov/ij/). Body length measurements were conducted using the “straight line” tool of image J. A total of 32 measurements (8 from each replicate beaker) were taken for each treatment consisting of either ERM or 0.1% DMSO or 0.01, 0.1, and 1 μM of fipronil, carvacrol, or acetylcarvacrol.

2.5 Visual motor response and light-dark preference test

The visual motor response (VMR) and light-dark preference test (LDPT) proceeded as previously published (Wang et al., 2018). Two independent exposures were performed for 7 days to assess the dark photokinetic response in zebrafish larvae exposed to either ERM or 0.1% DMSO or 0.01, 0.1, and 1 μM of fipronil, carvacrol, or acetylcarvacrol. Four replicate beakers were prepared for all treatments and each beaker contained 20 zebrafish embryos and 10 mL of ERM. The temperature control unit was acclimated to 26 ◦C. For the VMR, normally developed larvae were selected from each replicate beaker and placed into a 96-well plate (n= 8-12 individuals/treatment), with each well containing 200 μL of ERM. Six independent trials (each trial represents a 96-well plate) were conducted to assess the effects of the three chemicals on larval behavior. The 96-well plate was placed into DanioVision™ Observation Chamber (Noldus Information Technology, Leesburg, VA) with an infrared analog camera (25 frames/ second) to track the activities of zebrafish larvae. All trial data were pooled, and the total distance traveled served as a measure of overall locomotor activity.

For the LDPT, zebrafish larvae at 7 dpf were placed individually into a 12-well plate (Whatman, CAT#: WH7701-1651) containing 1 mL of media. The concentration of 1 μM was not used in the LDPT since it affected the movement of larvae exposed to fipronil in the VMR and we decided to keep it consistent with the other chemicals. Buspirone hydrochloride at 60 μM (CAS: 33386-08-2, Sigma-Aldrich) was used as a positive control in this assay since it is an anxiolytic compound that has been validated in the LDPT with zebrafish larvae (Bai et al., 2016). Twenty trials were conducted with n= 2 fish per treatment per plate (n= 40 larvae per treatment in total). A 12-well plate with a slotted cover over the bottom of the plate created alternating light and dark zones in the plate. Poorly tracked larvae were excluded from the analysis; poor tracking results in the majority of the well lighting up red with a motion that is extremely linear. Data from twenty distinct runs were blended into a single graph to reflect all runs.

2.6 Real-time PCR analysis

Zebrafish larvae (6 hpf) were exposed to either ERM or 0.1% DMSO or 0.1 µM of fipronil, carvacrol, or acetylcarvacrol for gene expression analysis. Two independent exposures were conducted with four beakers per treatment (n = 8 biological replicates per treatment in total). Each beaker contained 20 embryos and exposure conditions were maintained as detailed above. Twelve to fifteen fish from a single beaker were pooled to make one biological replicate. Samples were collected, subjected to liquid nitrogen, and placed into a −80 °C freezer prior to RNA extraction. Nucleic acids were extracted using TRIzol® Reagent as per the manufacturer's instructions and pellets were reconstituted in DNase-RNase-free water. RNA integrity was determined using the RNA 6000 nano kit and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The mean RNA integrity value for the samples was 9.55 ± 0.29.

TURBO DNA free™ Kit was used to remove genomic DNA as per manufacturer's guidelines (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using a T100™ Thermal Cycler (BioRad, USA): 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C for 5 min. The iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA) was used for the synthesis with 500 ng RNA input in accordance with the manufacturer's instructions. The no reverse transcriptase (NRT) controls were created using one sample from each treatment group (n=5 NRTs in total) that had the highest RNA concentration. NRTs were prepared as mentioned above, but the enzyme was not added to the reaction mix. One no-template control (NTC) without RNA template was also included in the plate. Each well of the qPCR plates contained 0.8 µL of forward and reverse primers (approx. 100-200 nM), 3.33 µL of (1/20) diluted cDNA, and 5.025 µL of SsoFast™ EvaGreen® Supermix (BioRad, Hercules, CA, USA). Samples were run in technical duplicate. The CFX Connect™ Real-Time PCR Detection System (BioRad) was used to collect data.

To standardize target expression, four housekeeping genes were used: ribosomal subunit 18 (*rps18*), beta-actin (*β-actin*), tubulin-alpha (*TUB-α*) and ribosomal protein L13a (*RPL13a*) (M-value = 1.2522, CV = 0.5938). CFX Manager TM software (v3.1) was used to obtain normalized expression values for each target gene, and the relative ΔΔCq technique based on BioRad software was applied. The primer sets are given in Supplementary Table 1.

2.7 Reactive oxygen species

Embryos were obtained for ROS assessment as described above in sections 2.2 and 2.3. Using sterile micropipettes, fertilized embryos were equally dispensed into sterile 25 mL glass beakers containing either ERM or 0.1% DMSO or 0.1 µM of fipronil, carvacrol or acetylcarvacrol (n=5 beakers per experimental group), each containing 20 embryos. Two time points were used to assess ROS production (2 and 7 dpf). For the assay, zebrafish embryos/larvae were euthanized in MS-222, transferred from beakers to 1.7 mL microcentrifuge tubes and homogenized in 200 µL of ice-cold phosphate-buffered saline solution (PBS). Samples were then centrifuged at 12,000 g for 20 min at 4 ˚C (LYNX6000, Thermo Scientific). Then, 20 µL of supernatant was transferred to a black fluorescence plate, followed by the addition of 8.3 µL of H_2-DCFDA (CAT #: D399, Thermo Fisher Scientific) (1 mg/mL – diluted in DMSO) and 200 µL of PBS. The mixture was incubated in the absence of light for 30 min at 37 ˚C ± 1.0 ˚C, and then the fluorescence was measured with an excitation at 485 nm and emission at 520 nm using a multi-detection microplate reader (New Synergy™, BioTek). Total protein was determined for each sample using a BCA assay (Thermo Scientific). ROS levels were expressed as normalized signal intensity/(µg/mL) protein.

2.8 Oxygen consumption rates and metabolic profiling

For the oxygen consumption rates (OCR) measurement, a single embryo was selected at random from each of the experimental beakers following a 48-hour exposure to fipronil, carvacrol, or acetylcarvacrol. For this experiment, we exposed 6 hpf embryos (n= 10 per beaker, 4 beakers per treatment) to one of the following conditions: ERM, 0.1% DMSO, and 10 and 25 µM of one of the three chemicals. Due to space limitations, two separate plates were used in the analysis, one containing both controls and fipronil at 10 and 25 µM and the other one containing both controls and carvacrol and acetylcarvacrol at 10 and 25 µM (n=3-4/treatment as biological replicate). OCR was determined using a Seahorse XFe24 Extracellular Flux Analyzer (Seahorse Bioscience, Massachusetts, USA) following previously published guidelines (Souders II et al., 2019). Wave Desktop 2.6 Software (Agilent Technologies, USA) was used to export data to GraphPad PRISM v9.4 (La Jolla, CA, USA). As directed by the Seahorse XF Cell Mito Stress Test Kit User Guide,

the following parameters were calculated: basal respiration, maximal respiration, ATP-linked respiration, proton leak, and non-mitochondrial respiration (User Guide Kit 103015-100, Agilent).

2.9 Acridine Orange staining/Apoptosis assay

The acridine orange staining proceeded as previously described (Tucker and Lardelli, 2007). Briefly, zebrafish larvae were exposed to either ERM, 0.1 % DMSO, or 0.1 µM of fipronil, carvacrol, or acetylcarvacrol for 7 days. Then, they were washed with ERM and stained with 2 µg/mL acridine orange solution (CAS: 65-61-2, Sigma-Aldrich) for 30 min at room temperature in the absence of light. Ten larvae from each biological replicate were used, totaling 50 larvae per treatment. After washing with ERM (5 times for 30 seconds), apoptotic cells were visualized with an EVOS™ FL Auto Imaging System (ThermoFisher Scientific, USA) using a GFP filter at 10x magnification. Fluorescence patches of bright green color denoted apoptotic cells. The fluorescence intensity was quantified using the histogram tool of the ImageJ software.

2.10 Statistical Analysis

GraphPad v9 (La Jolla, CA, USA) was used for statistical evaluation of group differences and graphing. Data were first assessed for normality using a Shapiro-Wilk test prior to ANOVA. Data were $\log_{10}$ transformed in some cases to approximate a normal distribution. Oxygen consumption rates, reactive oxygen species and apoptosis (AO stain) were analyzed with a one-way ANOVA followed by a Dunnett's post-hoc test. For the behavior assay (VMR test and LDPT), a one-way ANOVA was conducted followed by a Holm-Sidak's multiple comparisons test. Data are presented as mean ± S.D. Data that did not pass the normality test even after transformation were subjected to the Kruskal-Wallis test followed by Dunn's multiple comparisons post-hoc test. In this case, the median value is presented for the data. For all endpoints, the significance of the difference was considered at alpha = 0.05.

3. RESULTS

3.1 Survival, deformities, and hatch rate

The survival rate of zebrafish larvae was considerably affected by fipronil exposure. We estimated the concentration-effect values (EC_{50}) for both deformities and mortality following fipronil exposure at 3, 5, and 7 dpf (**Fig. 1A-C**). About half of the population was deformed at 7 dpf in the concentration of 1 μM. Similar concentration caused the death of 50% of the individuals at 7dpf. The most frequent deformities observed included spinal lordosis and yolk sac edema (**Fig. 2**).

Carvacrol and acetylcarvacrol exposure, on the other hand, caused much lower acute toxicity (**Fig. 1 D-I**). The EC_{50} value of carvacrol for both deformities and mortality was higher than 50 μM at 7 dpf, whereas for acetylcarvacrol this value was greater than 70 μM. The most frequent deformities for both chemicals were yolk sac edema and tail malformations. The percent hatch rate was not affected by any of the chemicals at low concentrations (**Fig. 3**). However, fipronil caused a decrease in hatch rate at 2dpf at 5 μM ($H = 13.29$, $p = 0.0386$). The same pattern was observed for carvacrol at concentrations higher than 25 μM ($H = 43.32$, $p < 0.0001$). Acetylcarvacrol only affected hatching at 100 μM ($H = 19.23$, $p = 0.0233$).

3.2 Morphometric analysis

We evaluated the effects of the chemicals on the body length and swim bladder area (**Fig. 4**). Fipronil at 0.1 and 1 μM induced a decrease in body length ($F_{(10, 341)} = 48.62$, $p < 0.0001$) and swim bladder surface area ($F_{(10, 341)} = 46.57$, $p < 0.0001$) of larvae at 7dpf. In fact, larvae exposed to 1 μM fipronil were about 20% shorter than the control. Also, the swim bladder area of these individuals was reduced by approximately 50% compared to the control. No differences were observed for the other chemicals compared to DMSO at the time point evaluated.

3.3 Visual motor response and dark-light preference test

Six independent trials of the VMR test were conducted for a total of 48-72 larvae per treatment. Data for each trial are shown in the supplementary materials (**Fig. S1**). A decrease in locomotor activity was observed for fipronil at 1 μM in the combined data ($F_{(54, 495)} = 11$, $p < 0.0001$) (**Fig. 5**) and this pattern was consistent for most of the individual runs. Acetylcarvacrol induced hyperactivity at 0.1 μM as shown in the combined data. Hyperactivity was also observed for the other chemicals at 0.1 μM in some of the individual runs, but no differences were observed in the combined data compared to the control.

We also measured anxiolytic and anxiety-related behaviors in larval zebrafish using the LDPT. There were no differences in distance moved for any of the chemicals compared to DMSO after a post-hoc test ($F_{(8, 225)} = 2.570$, $p = 0.0106$) (**Fig. 6**). The frequency in the dark zone increased in larvae exposed to buspirone ($F_{(8, 225)} = 2.731$, $p = 0.0068$). Similar results were observed for the cumulative duration in the dark zone ($F_{(8, 225)} = 8.112$, $p < 0.0001$). However, there were no changes in the mean time in the dark zone for larvae exposed to any of the chemicals ($F_{(8, 214)} = 1.126$, $p = 0.3471$).

3.4 Real-time PCR analysis

Select transcripts associated with swim bladder inflation (**Fig. 7**), oxidative stress (**Fig. 8**), and mitochondrial activity (**Fig. 9**) were measured in larval zebrafish exposed to 0.1 μM of either fipronil, carvacrol, and acetylcarvacrol. There were no changes in *acta2* ($H = 3.360$, $p = 0.4995$), *ihha* ($H = 1.249$, $p = 0.8699$) and *pbx1a* ($H = 8.478$, $p = 0.0756$), which are genes related to swim bladder inflation. Additionally, there were no changes in the expression of genes related to toxicity and oxidative stress: *ache* ($H = 3.589$, $p = 0.4645$), *cat* ($H = 3.346$, $p = 0.5017$), *hsp70* ($H = 3.295$, $p = 0.5098$), *hsp90b* ($H = 4.354$, $p = 0.3602$), *sod1* ($H = 3.688$, $p = 0.4498$) and *sod2* ($H = 4.781$, $p = 0.3105$).

Transcript levels of *atp5f1c* ($H = 15.28$, $p = 0.0041$) **(Fig. 9C)** were upregulated in larvae exposed to 0.1 μM fipronil compared to the DMSO control. However, there were no changes in the transcript levels of other genes related to mitochondrial activity at the time point evaluated:

atp06 (H = 10.21, $p = 0.0371$), *atp5f1b* (H = 9.243, $p = 0.0553$), *atp5mea* (H = 9.709, $p = 0.0456$), *cox1* (H = 1.934, $p = 0.7480$) and *cox4i1* (H = 4.831, $p = 0.3051$).

We also assessed the transcript levels of genes related to lipid metabolism, as shown in the supplemental materials (**Fig. S2**). There were no changes in the expression of any of the following genes: *acaca* (H = 3.415, $p = 0.4909$), *acs* (H = 8.635, $p = 0.0709$), *cpt1* (H = 8.786, $p = 0.0667$), *dgat1* (H = 4.977, $p = 0.2897$), *dgat2* (H = 6.301, $p = 0.1778$), *elovl2* (H = 3.557, $p = 0.4692$), *elovl6* (H = 5.373, $p = 0.2511$), *fad2* (H = 2.728, $p = 0.6043$), *mogat3* (H = 7.615, $p = 0.1068$), *pgc1a* (H = 4.158, $p = 0.3850$), *pparg* (H = 3.498, $p = 0.4782$).

3.5 Reactive oxygen species

Zebrafish larvae were exposed to 0.1 µM fipronil, carvacrol, or acetylcarvacrol for either 2 or 7-days. No differences were detected in the ROS levels at 2 dpf compared to DMSO control ($F_{(4, 19)} = 1.912$, $p = 0.1499$) (**Fig. 10A**). However, all chemicals induced an increase in ROS levels at 7 dpf ($F_{(4, 19)} = 4.692$, $p = 0.0084$) (**Fig. 10B**).

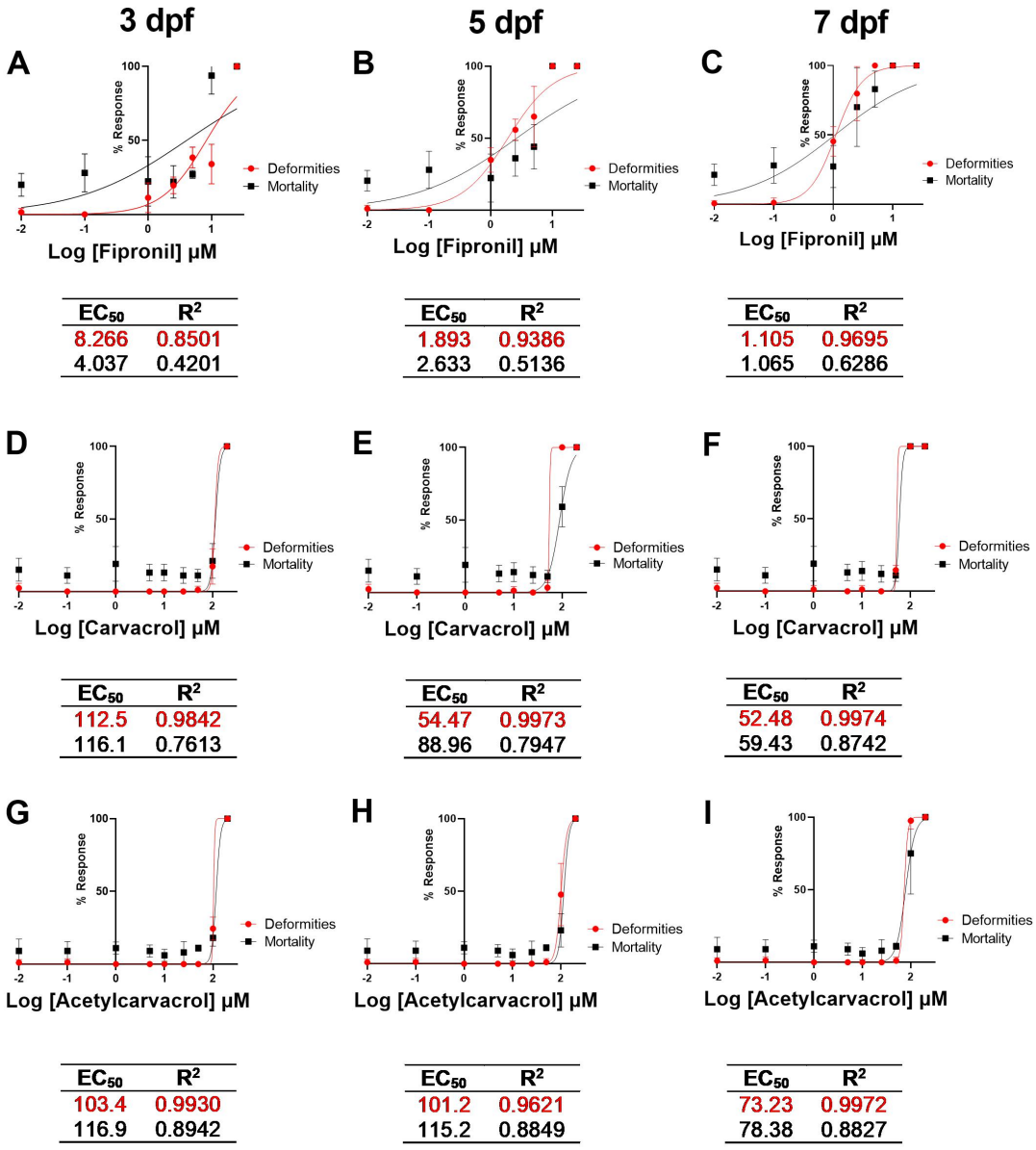
3.6 Oxygen consumption rates and metabolic profiling

The oxygen consumption rate (OCR) of zebrafish embryos (at 54 hpf) was determined following a 48-hour exposure to fipronil (**Fig. 11**) as well as carvacrol and acetylcarvacrol (**Fig. 12**). Exposure to fipronil did not affect basal respiration ($F_{(3, 12)} = 2.150$, $p = 0.1471$), ATP-linked respiration ($F_{(3, 12)} = 2.148$, $p = 0.1473$), maximal respiration ($F_{(3, 12)} = 2.830$, $p = 0.0833$), proton leak ($F_{(3, 12)} = 0.4741$, $p = 0.7061$), and non-mitochondrial respiration ($F_{(3, 12)} = 0.4951$, $p = 0.6924$).

There were also no changes in OCR for carvacrol and acetylcarvacrol compared to DMSO: basal respiration: ($F_{(5, 14)} = 1.029$, $p = 0.4380$), ATP linked respiration: ($F_{(5, 14)} = 1.748$, $p = 0.1886$), maximal respiration: ($F_{(5, 14)} = 0.7837$, $p = 0.5781$) and non-mitochondrial respiration ($F_{(5, 14)} = 0.9277$, $p = 0.4921$). An outlier was detected for the proton leak in the DMSO group (Grubbs' Test; $p < 0.05$, z = 1.48) (**Fig. 12E**), showing a highly negative result (-38.07). We represented the data without the outlier, but here point out that no changes were observed among treatment groups compared to DMSO in the presence ($F_{(5, 14)} = 2.102$, $p = 0.1256$) or absence ($F_{(5, 13)} = 3.235$, $p = 0.0408$) of the outlier.

3.7 Acridine Orange staining/Apoptosis assay

The effects of the chemicals on zebrafish larvae apoptosis were assessed at 7 dpf. No significant differences were observed between the treatment and control groups ($F_{(4, 245)} = 1.695$, $p = 0.1517$) (**Fig. 13**).



EC_{50}	R^2
8.266	0.8501
4.037	0.4201

EC_{50}	R^2
1.893	0.9386
2.633	0.5136

EC_{50}	R^2
1.105	0.9695
1.065	0.6286

EC_{50}	R^2
112.5	0.9842
116.1	0.7613

EC_{50}	R^2
54.47	0.9973
88.96	0.7947

EC_{50}	R^2
52.48	0.9974
59.43	0.8742

EC_{50}	R^2
103.4	0.9930
116.9	0.8942

EC_{50}	R^2
101.2	0.9621
115.2	0.8849

EC_{50}	R^2
73.23	0.9972
78.38	0.8827

Figure 1. Concentration-effect-curves (EC) of lethality and deformities of early staged zebrafish exposed to fipronil (A-C), carvacrol (D-F), or acetylcarvacrol (G-I) for 3, 5, and 7 days.

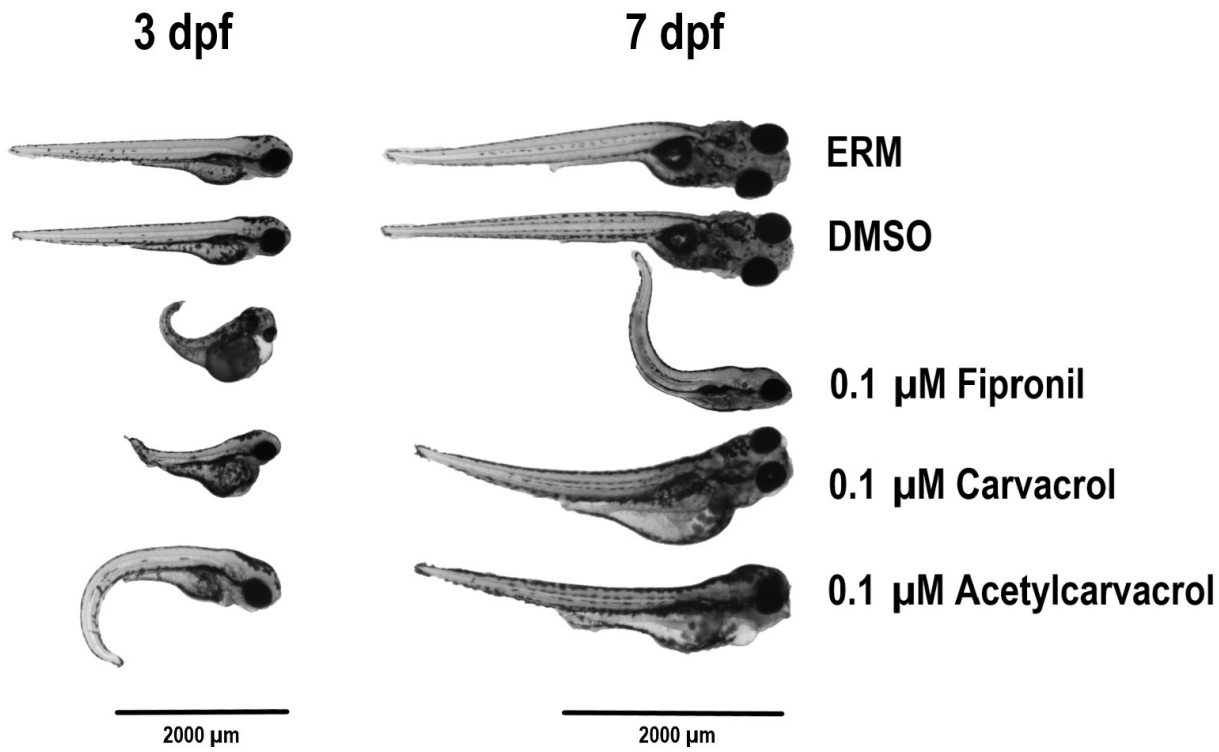


Figure 2. Selected photomicrographs of morphological deformities observed in zebrafish larvae at 3 and 7 days after being exposed to ERM (control), 0.1% DMSO (solvent control), 0.1 µM fipronil, carvacrol or acetylcarvacrol. The scale bar in each picture is 2000 µm. Deformities observed included pericardial and yolk sac edema, bent tail, and spinal curvature.

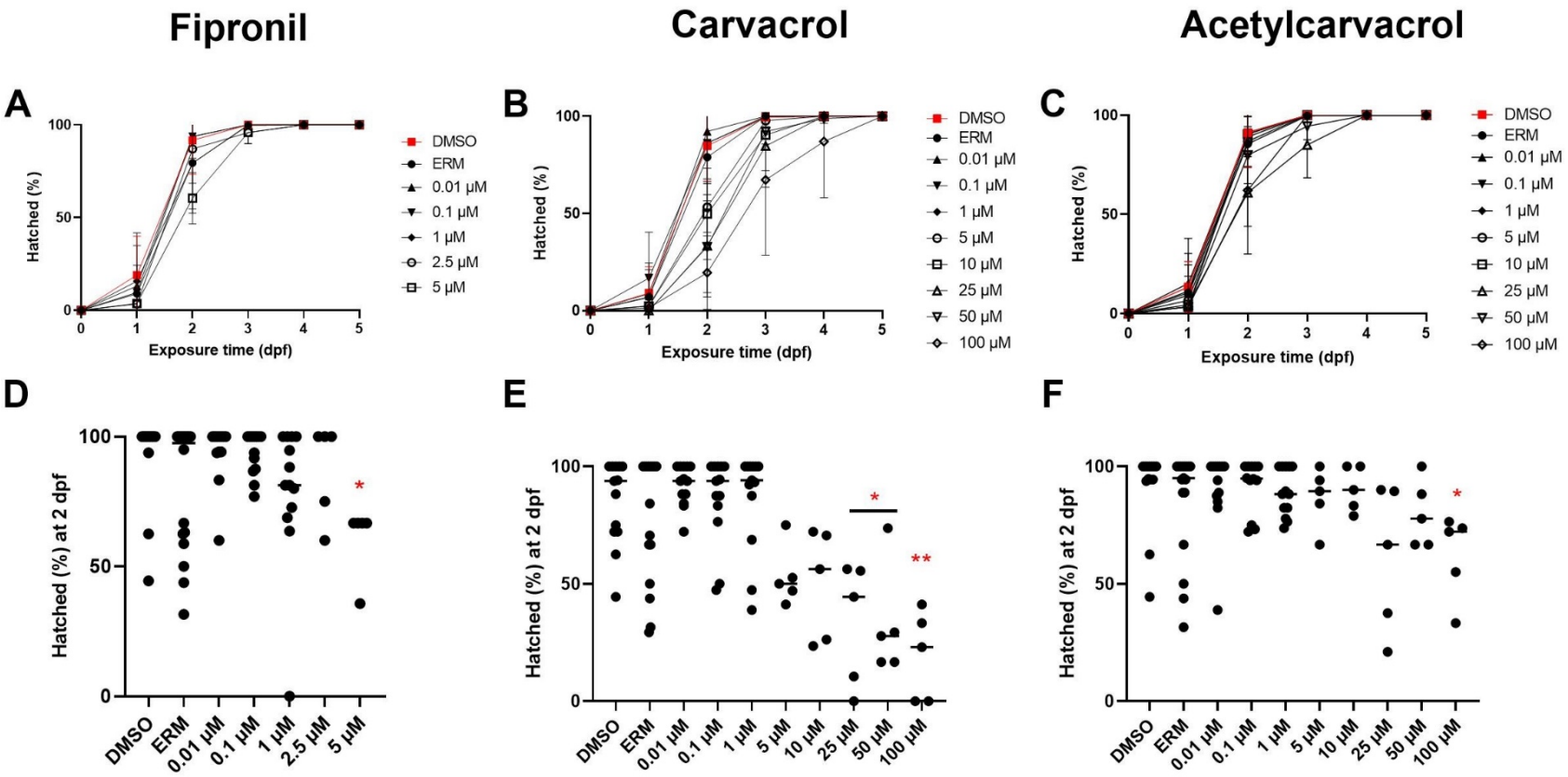


Figure 3. Percent of hatched zebrafish exposed to fipronil, carvacrol, or acetylcarvacrol. (A-C) Percent hatch over five days. (D-F) Percent of hatched zebrafish at 2 dpf (one-way ANOVA followed by Dunnett's post-hoc test, n = 5-17 beakers, 20 fish per beaker, *$p \leq 0.05$, **$p \leq 0.01$).

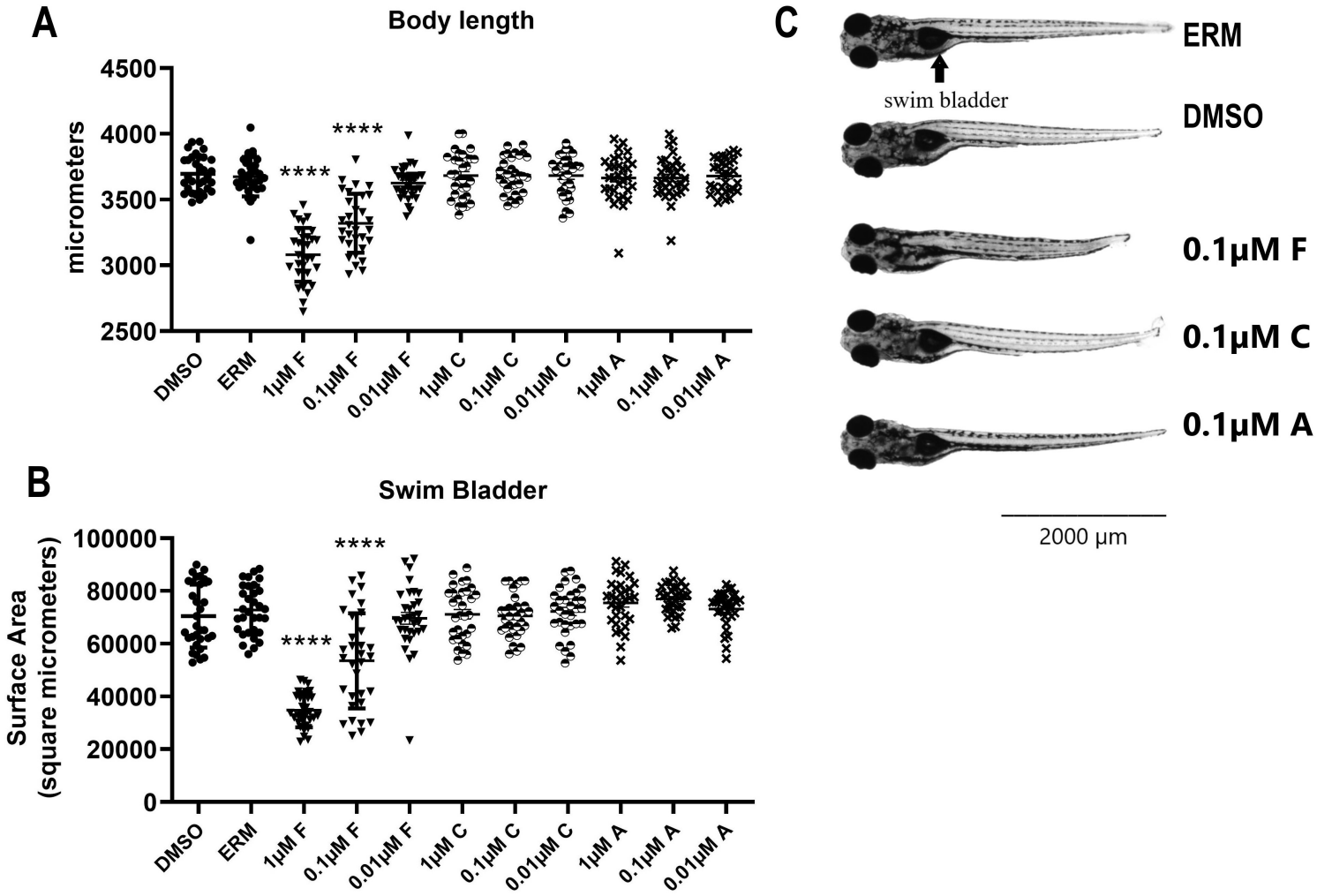


Figure 4. Morphometric measurements of zebrafish larvae exposed continuously to fipronil (F), carvacrol (C), or acetylcarvacrol (A) for 7 days. (A) Body length measurements (µm). (B) Swim bladder surface area (µm^2). (C) Representative micrographs of zebrafish larvae. Scale bar = 2000 µm. Note smaller body length and surface area of the swim bladder in larvae exposed to 1 µM and 0.1 µM fipronil (one-way ANOVA followed by Dunnett's post-hoc test, n = 50/group, **** $p \leq 0.0001$).

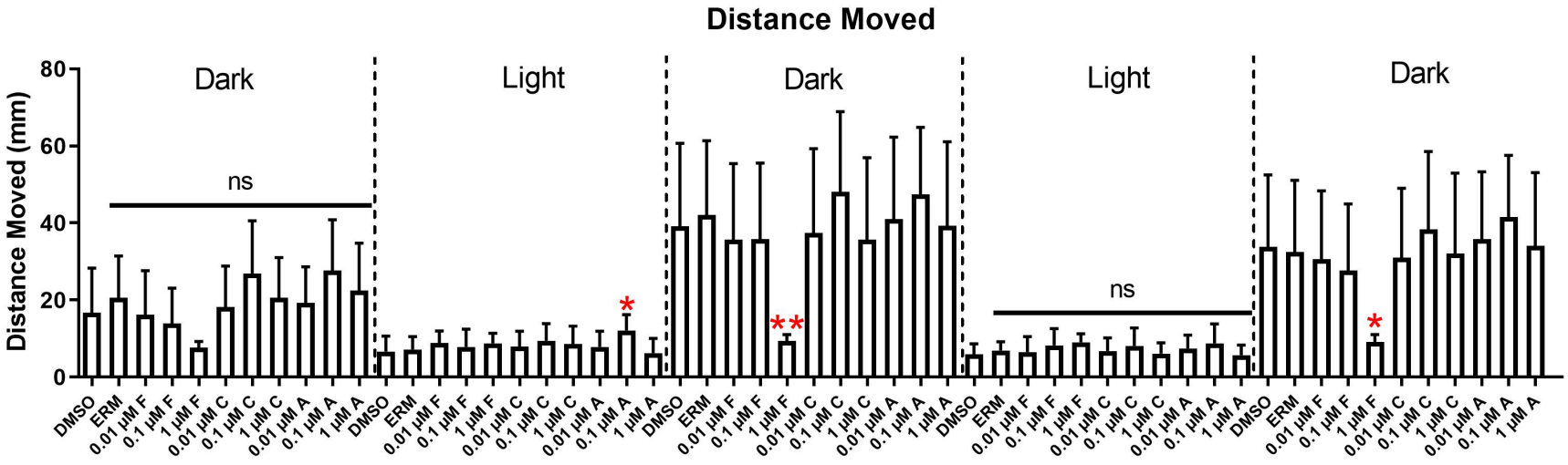


Figure 5. Visual Motor Response Test for zebrafish larvae exposed continuously to fipronil (F), carvacrol (C), or acetylcarvacrol (A) for 7 days. Light-dark cycles are organized in 10-minute intervals. Data from six independent trials are merged as a single graph. Mean (±SD) distance moved (mm) per minute intervals per experimental group. One-Way ANOVA followed by Holm-Šídák's multiple comparisons test, ns = non-significant, *p ≤ 0.05, **p ≤ 0.01, n = 48- 72 larvae per treatment.

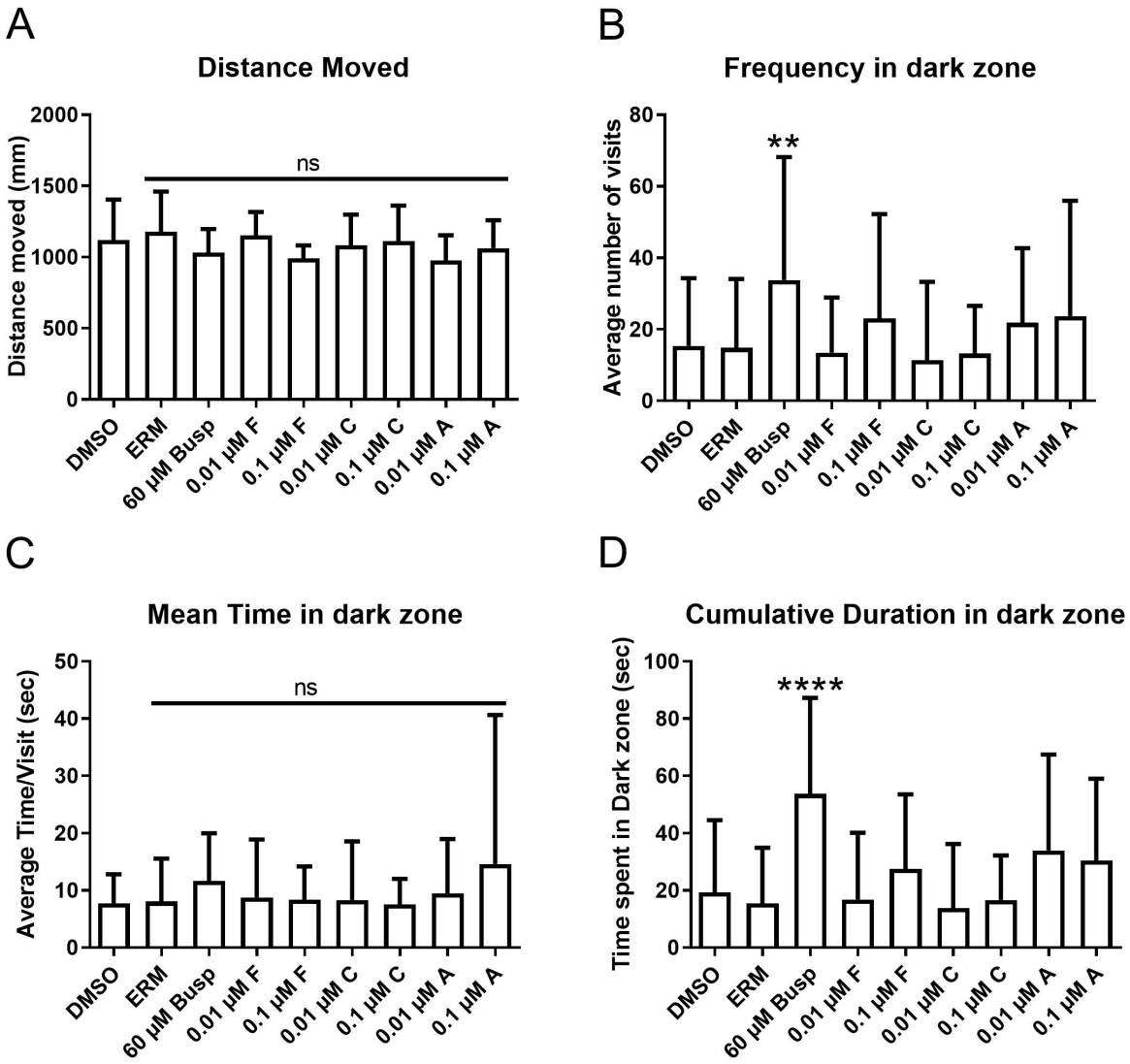


Figure 6. Light-dark preference test (LDPT) showing anxiolytic behavior of zebrafish larvae exposed continuously to fipronil (F), carvacrol (C), or acetylcarvacrol (A) for 7 days. (A) Total distance moved (mm); (B) Mean time in dark zone; (C) Frequency in dark zone; (D) Cumulative duration in dark zone. Data are presented as mean ± SD (One-Way ANOVA followed by Holm-Šídák's multiple comparisons test, ns = non-significant, **$p \leq 0.01$, **** $p \leq 0.0001$., n = 40 larvae per treatment).

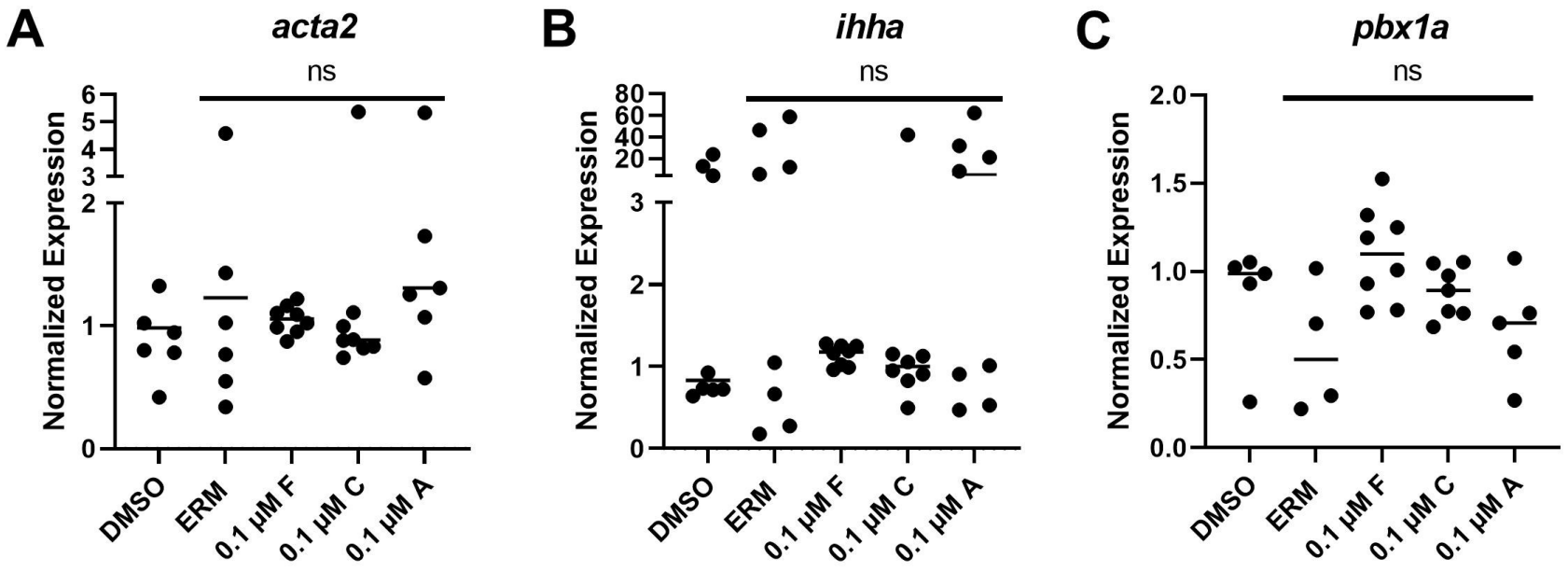


Figure 7. Expression levels of transcripts related to swim bladder inflation of zebrafish larvae exposed continuously to either 0.1 µM fipronil (F), carvacrol (C), or acetylcarvacrol (A) for 7 days. (A) *acta2*, Actin Alpha 2, Smooth Muscle; (B) *ihha*, Indian hedgehog; (C) *pbx1a*, PBX Homeobox 1. The horizontal line represents the group's median value (Kruskal Wallis followed by Dunn's multiple comparison test, ns = non-significant, $p > 0.05$, n = 8 biological replicates/treatment).

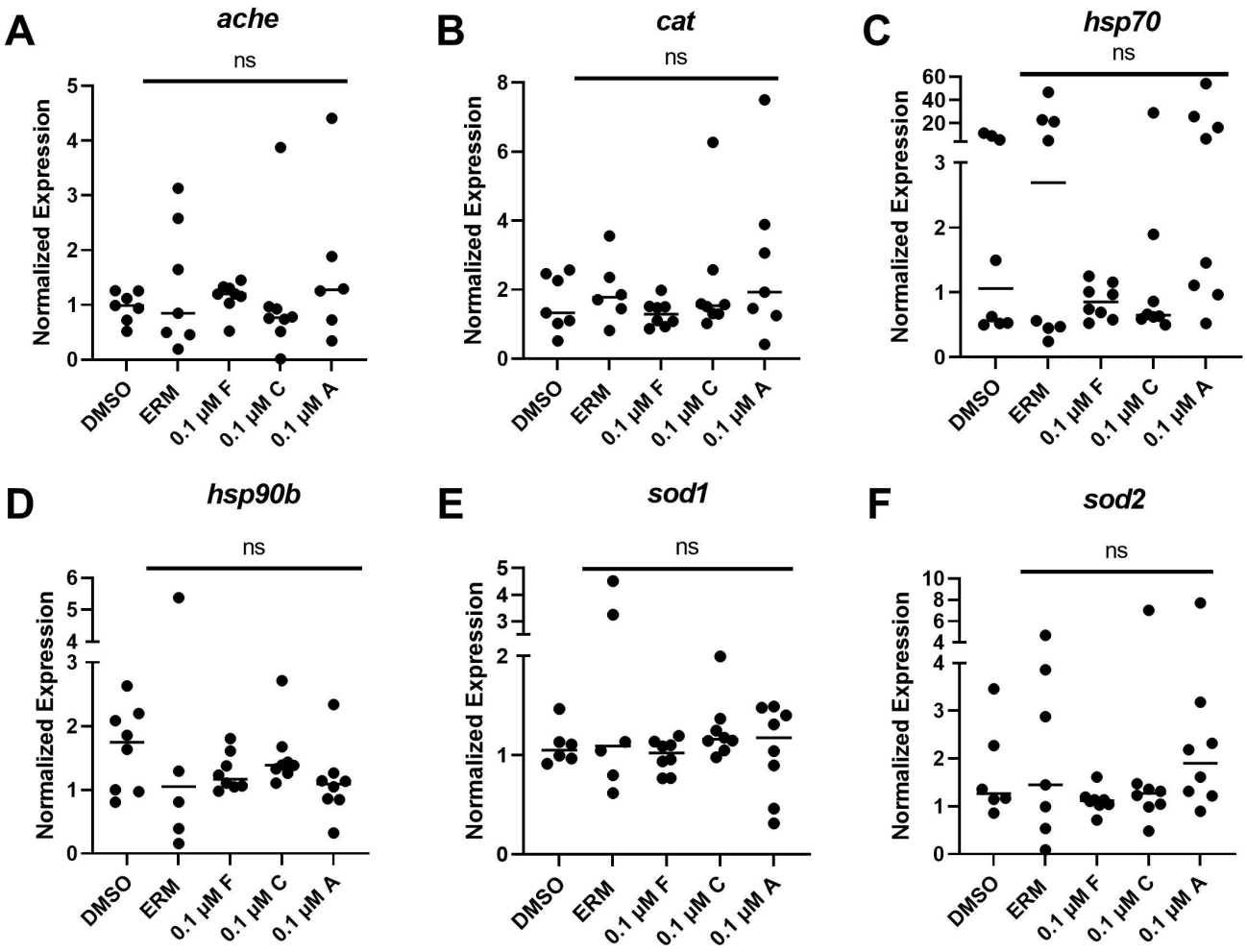


Figure 8. Expression levels of transcripts related to toxicity and oxidative stress of zebrafish larvae exposed continuously to either 0.1 µM fipronil (F), carvacrol (C), or acetylcarvacrol (A) for 7 days. (A) *ache*, Acetylcholinesterase; (B) *cat*, Catalase; (C) *hsp70*, Heat shock protein 70; (D) *hsp90b*, Heat shock protein 90b; (E) *sod1*, superoxide dismutase 1; (F) *sod2*, superoxide dismutase 2. The horizontal line represents the group's median value (Kruskal Wallis followed by Dunn's multiple comparison test, ns = non-significant, $p > 0.05$, n = 8 biological replicates/treatment).

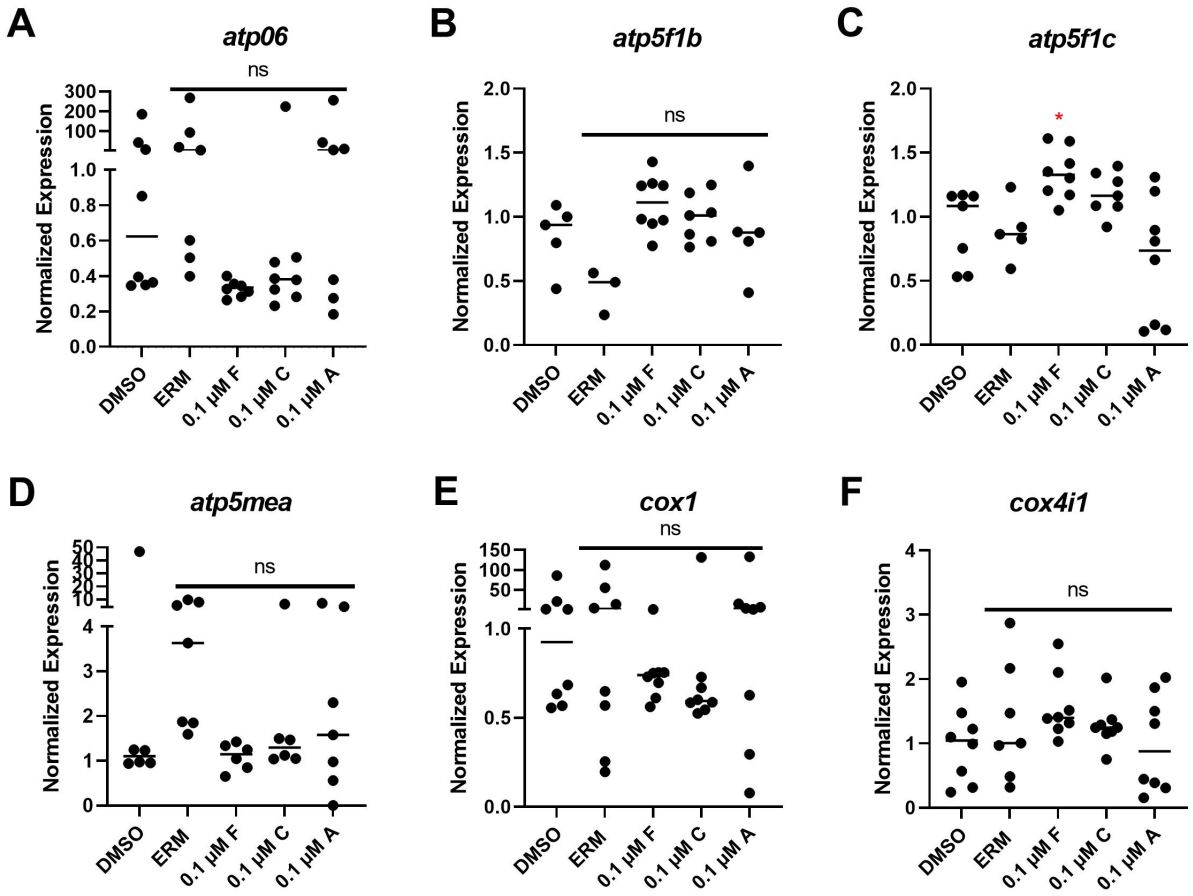


Figure 9. Expression levels of transcripts related to the mitochondrial activity of zebrafish larvae were exposed continuously to either 0.1 µM fipronil (F), carvacrol (C), or acetylcarvacrol (A) for 7 days. (A) *atp06*, ATP synthase F_0 subunit 6; (B) *atp5f1b*, ATP Synthase F_1 Subunit Beta; (C) *atp5f1c*, ATP Synthase F_1 Subunit Gamma; (D) *atp5mea*, ATP synthase membrane subunit ea; (E) *cox1*, mitochondrially encoded cytochrome c oxidase I; (F) *cox4i1*, Cytochrome C Oxidase Subunit 4I. The horizontal line represents the group's median value (Kruskal Wallis followed by Dunn's multiple comparison test, ns = non-significant, * $p \leq 0.05$, n = 8 biological replicates/treatment).

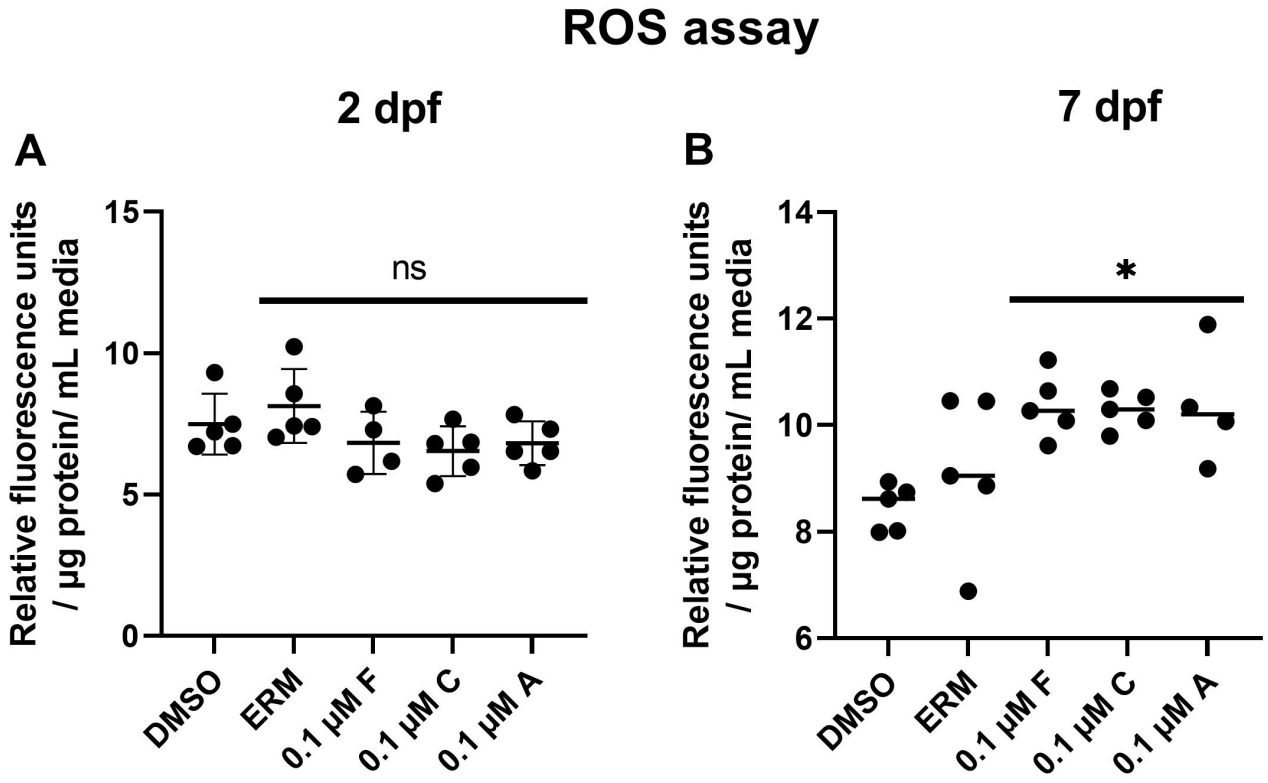


Figure 10. Reactive oxygen species present in 2 dpf (A) and 7 dpf (B) zebrafish exposed continuously to either 0.1 µM fipronil (F), carvacrol (C), or acetylcarvacrol (A). Results are expressed as relative fluorescence/(µg/mL protein) (one way ANOVA followed by Dunnett's post-hoc test, n = 5 biological replicates/treatment , ns = non-significant, * $p \leq 0.05$).

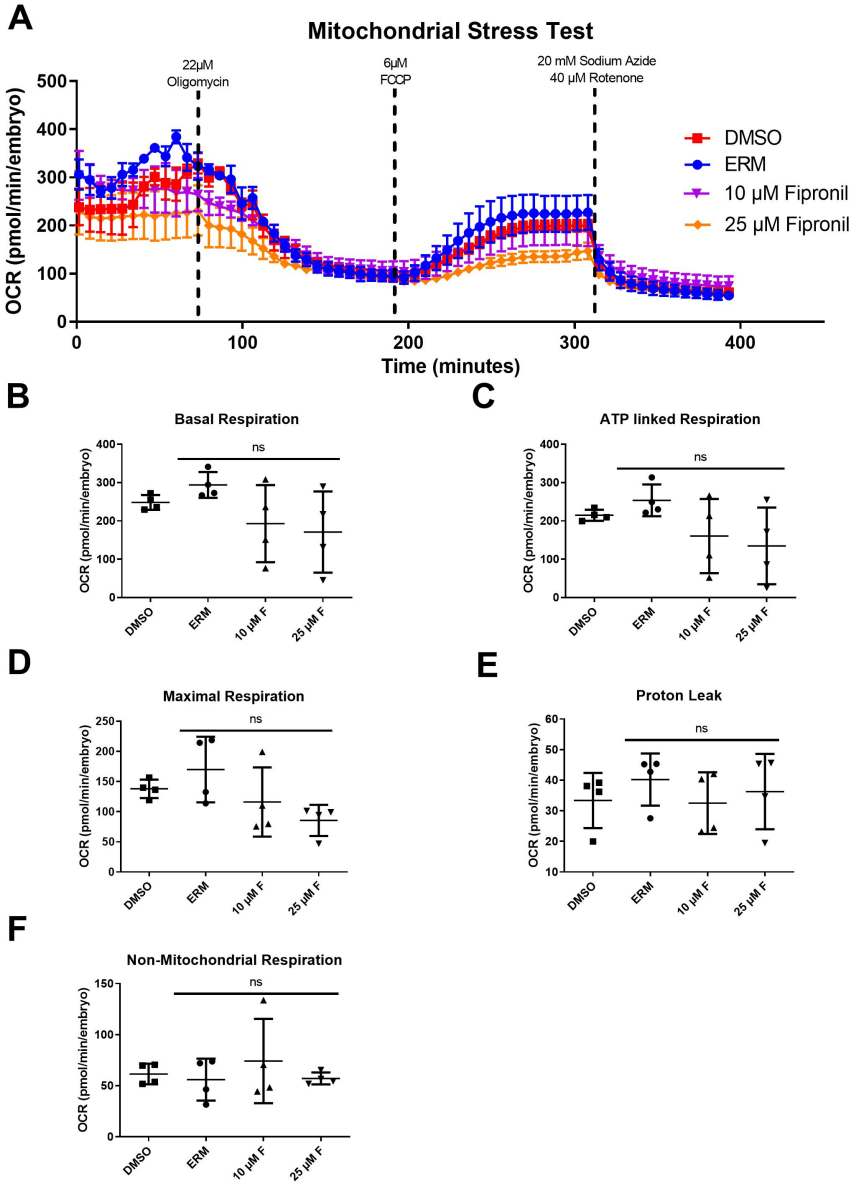


Figure 11. Oxygen consumption rate (OCR, *p*mol/min/embryo) in 54 hpf zebrafish embryos treated with 10 and 25 µM fipronil (F). (A) OCR over time; (B) Basal respiration; (C) ATP-linked respiration; (D) Maximal respiration; (E) Proton leak; (F) Non-mitochondrial respiration. Data are presented as mean ± SD (one-way ANOVA followed by Dunnett's post-hoc test, n = 3-4/group, ns = non-significant, $p > 0.05$).

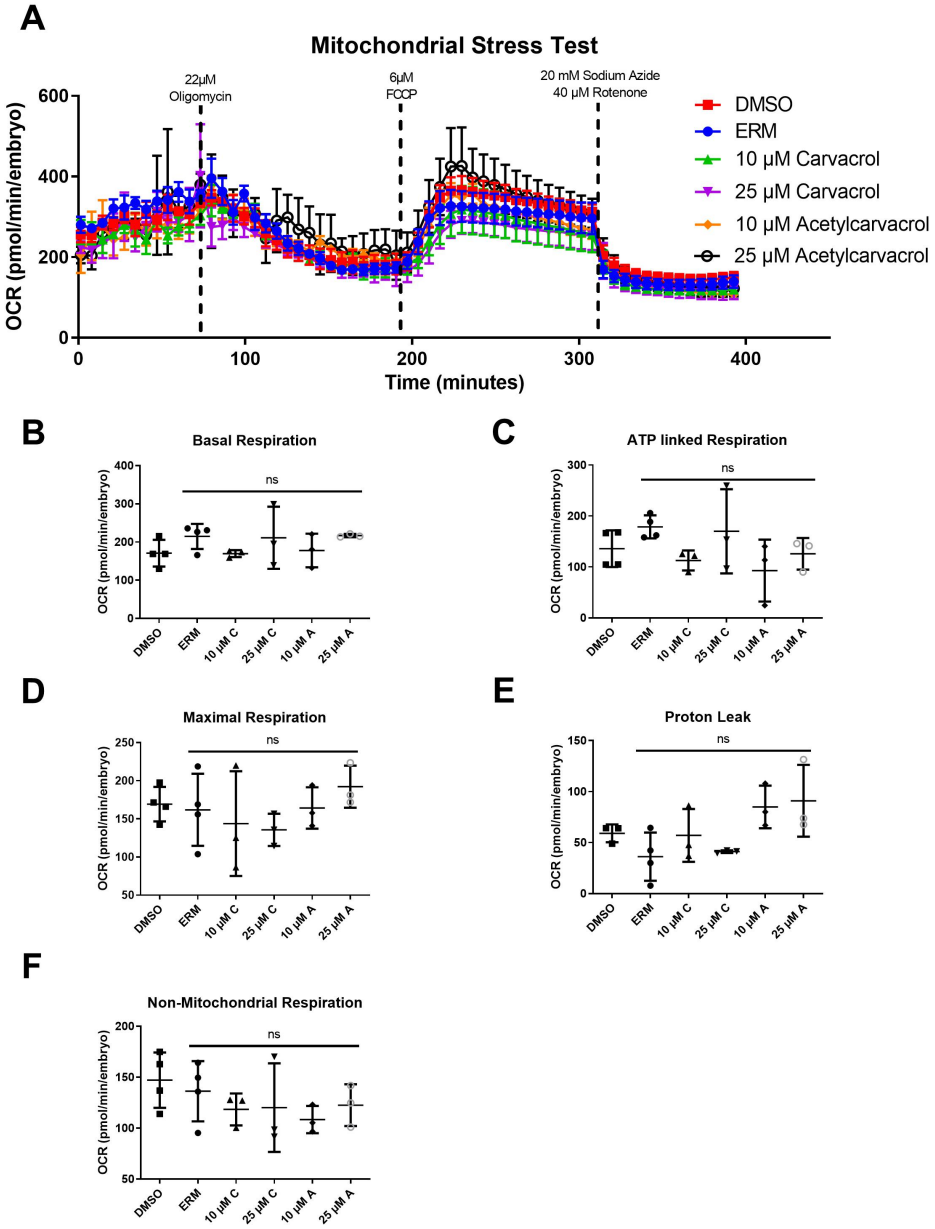


Figure 12. Oxygen consumption rate (OCR, pmol/min/embryo) in 54 hpf zebrafish embryos treated with 10 and 25 μM carvacrol (C) and acetylcarvacrol (A). (A) OCR over time; (B) Basal respiration; (C) ATP-linked respiration; (D) Maximal respiration; (E) Proton leak; (F) Non-mitochondrial respiration. Data are presented as mean ± SD (one-way ANOVA followed by Dunnett's post-hoc test, n = 3-4/group, ns = non-significant, $p > 0.05$).

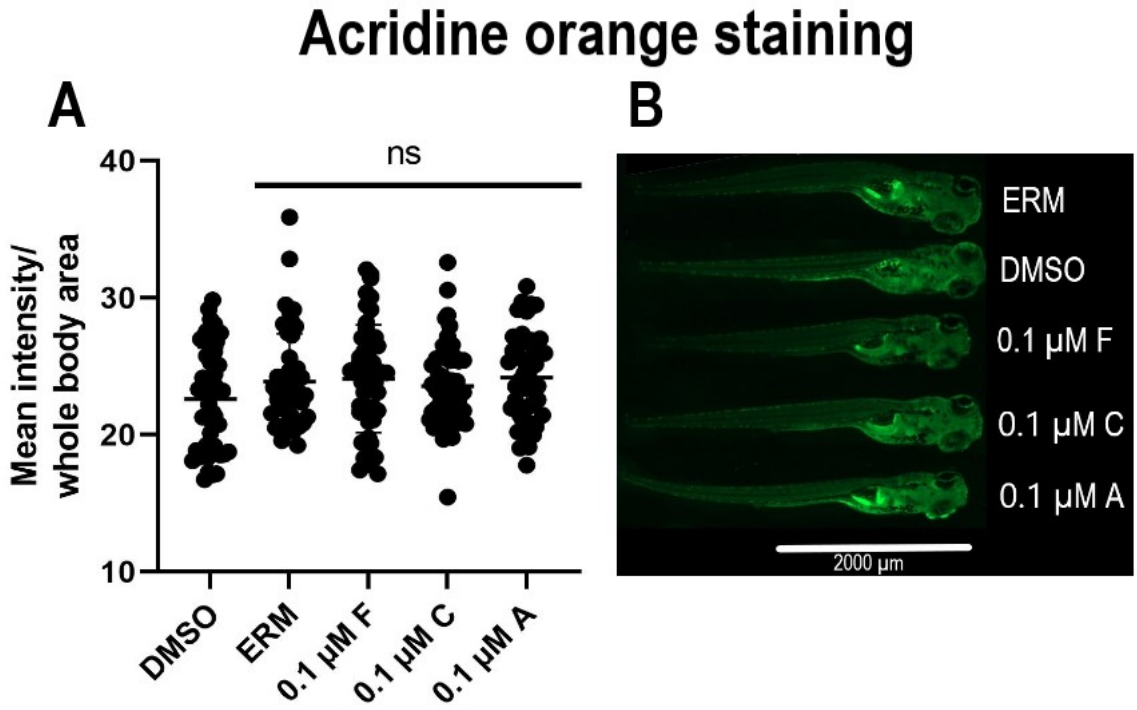


Figure 13. (A) Fluorescence intensity in zebrafish larvae exposed to 0.1 µM fipronil (F), carvacrol (C), and acetylcarvacrol (A) at 7dpf. Data represented as the mean value of the group (±SD) (one-way ANOVA followed by Dunnett's post-hoc test, n = 50/group, ns = non-significant, $p > 0.05$). (B) Representative photomicrographs of zebrafish larvae stained with acridine orange (AO) dye after being exposed to 0.1 µM fipronil (F), carvacrol (C), and acetylcarvacrol (A) at 7dpf. The scale bar in each picture is 2000 µm.

4. DISCUSSION

The search for alternative pesticides is needed due to pest resistance and to reduce contamination of important planet sources, such as water, air, and soil. In this sense, plant-derived compounds have emerged as promising alternatives to the use of synthetic pesticides because of their relatively low cost, abundance, and action by several mechanisms (Souto et al., 2021). However, the lack of comparative toxicological studies is a limiting factor for their commercialization (Pavela and Benelli, 2016). Herein, we compared the toxicity of fipronil, a widely used synthetic pesticide that is known for its high toxicity to non-target organisms, with two natural-based chemicals that are candidates to replace it.

Survivability is evaluated in almost all toxicity studies with developing zebrafish. It enables comparison of the potency of chemicals, and it is used to determine sub-lethal concentrations for further assessment of molecular endpoints. In our study, fipronil presented the lowest EC_{50} value for mortality at 3, 5, and 7 dpf, suggesting high acute toxicity. Carvacrol and acetylcarvacrol, on the other hand, were approximately 60 and 80 times less lethal than fipronil at 7 dpf, respectively. Interestingly, EC_{50} for fipronil at 3 dpf was ~8 μM, dropping to ~1 μM at 7 dpf. This same effect was not observed for the other chemicals, which decreased the EC_{50} by about 50% at 7 dpf. We hypothesize that it is due to the bioaccumulation capacity of fipronil (Xu et al., 2019; Konwick et al., 2006), enhancing its toxic effects over time. Oppositely, carvacrol and acetylcarvacrol would seem to be less likely to bioaccumulate because of their higher volatility (Konig et al., 2021).

Early-staged zebrafish develop deformities that can also be used to evaluate the potency and toxicity of chemicals. Our results show that EC_{50} curves for deformities and mortality followed a similar pattern for all chemicals. For fipronil, spinal lordosis, reduced body length, and yolk sac edema were the most frequently recorded deformities. Previous studies have also found similar results (Eadie et al., 2020; Xu et al., 2018). A transcriptomic analysis of zebrafish larvae revealed suppression of gene networks related to bone, skeletal structure, muscle development, and metabolism following exposure to 200 μg/L fipronil (Eadie et al., 2020). Thus, our data are consistent with the effects of this chemical on morphological development.

Deformities in zebrafish exposed to carvacrol and its derivative were found in low frequencies and mostly at high concentrations (> 50 μM), suggesting that these chemicals present reduced acute toxicity. This is in agreement with a study that investigated the effects of oral and

intraperitoneal administration of acetylcarvacrol to male and female Swiss mice and found no differences in behavior, body weight, water intake, food consumption, waste production, or biochemical and hematological parameters in treated animals up to 1000 mg/kg (Oliveira et al., 2020).

The timing of hatch can also be important as a toxicological endpoint. The secretion of the hatching enzyme into the perivitelline space of the egg allows it to degrade the eggshell and cause hatching (Skauli et al., 2000). A delay in hatchability was observed for fipronil at 5 µM, carvacrol in concentrations greater than 25 µM, and acetylcarvacrol at 100 µM. Thus, there was a lower effect of acetylcarvacrol on hatchability. We believe that this delay is an attempt of the embryos to reduce the contact surface with the chemicals, as the chorion exerts a protective effect (Wang et al., 2021).

Inflation of the gas bladder, commonly referred to as the swim bladder, begins at 3 dpf. It is a vascularized internal organ that plays an important role in buoyancy and locomotor activity (Robertson, 2007). In our study, 7 dpf zebrafish larvae exposed to 0.1 and 1 µM fipronil presented with a decrease in swim bladder surface area, but no differences were found for carvacrol or its derivative. An uninflated swim bladder was also observed in 5 dpf zebrafish larvae exposed to 400 µg/L fipronil (Yan et al., 2016). Interestingly, this alteration would seem to be frequently found following exposure to other pesticides as well (Huang et al., 2021; Yang et al., 2021; Ma et al., 2019). Thus, it is an essential endpoint to be measured during the toxicity assessment of chemicals with biocidal activity. Indeed, alterations in the swim bladder can affect the ability of fish to search for food and escape from predators, most likely leading to the death of the organisms (Ma et al., 2019).

Zebrafish larvae exposed to 1 µM fipronil showed hypoactivity in the VMR test conducted in our study. We believe it was due to the uninflated swim bladder in larvae exposed to fipronil since deformed larvae (e.g. spinal lordosis) were excluded from the analysis. Yang et al. (2021) state that the deflation of the swim bladder has been attributed to a decline in thyroid hormones (THs) as well as alterations in the expression of genes related to swim bladder inflation, such as *acta2*, *ihha,* and *pbx1a*. We measured relative transcript abundance for these genes but found no differences in larvae exposed to fipronil. We suggest that it may be due to the time point evaluated (7 dpf) or possible alterations in THs, as fipronil exposure has already been shown to decrease their levels in zebrafish (Xu et al., 2019).

Hyperactivity was observed for larvae exposed to 0.1 μM acetylcarvacrol in the combined VMR data. The same concentration of carvacrol and fipronil was responsible for increased locomotor activity, as revealed in several independent experiments. Conversely, anxiolytic-like behavior was not affected by exposure to any of the chemicals. Hyperactivity is likely due to the irritant effects of chemical exposure on zebrafish larvae, likely leading to an innate immune response (Martin et al., 2021).

Increased ROS production may be caused by several factors, such as the activation of the innate immune system and disrupted mitochondrial function (Xu et al., 2013). Our results demonstrate that all chemicals induced ROS at 7 dpf, but not at 2 dpf. Physiological concentrations of ROS are necessary to support redox signaling events, but an unbalanced ROS production causes damage in macromolecules, leading to injury, cell death, and disease (Gammella et al., 2016). As a response, detoxifying enzymes (e.g., sod, cat) increase their activity to deal with harmful oxygen species. We measured the relative transcript abundance of genes related to detoxification but found no differences following exposure to any of the chemicals. We believe there was no sufficient antioxidant response in terms of enzymatic activity to prevent the harmful effects of increased ROS production (Woolley et al., 2013).

Mitochondria is a major source of ROS and disrupted function in this organelle will lead to augmentation in their production (Murphy, 2009). In this sense, we investigated the mitochondrial function of developing zebrafish exposed to fipronil, carvacrol, and acetylcarvacrol. Fipronil exposure upregulated the transcript levels of *atp5f1c*. This gene codes for the gamma subunit of the F_1 central stalk of the ATP synthase (complex V of the electron transport chain). Complex V plays a critical role in the synthesis of ATP, the primary currency for energy used in the life (Patel et al., 2020). We hypothesize that fipronil may impair mitochondrial function. As a response, the organism upregulated the expression of *atp5f1c* to increase the production of ATP. Conversely, the opposite effect was observed in a study conducted with primary immortalized mesencephalic dopaminergic rat cells (N27) that found a ~40–60 % reduction in basal respiration, as well as reduced oligomycin-induced ATP production at 50 μM fipronil (Souders II et al., 2021). We assessed mitochondrial activity in zebrafish embryos (2 dpf) exposed to fipronil and the other chemicals, but no differences in these parameters were detected. We argue that the protective effect exerted by the chorion at the beginning of the exposure may have prevented detectable altered mitochondrial function at this time point (Wang et al., 2021). However, fipronil may alter the

function of this organelle over time, as indicated via gene expression analysis thus, affecting metabolic activity on the level of the whole organism.

A metabolomic study in zebrafish larvae exposed to 40 µg/L fipronil revealed changes in lipid profiles. For instance, there was a decrease in the concentration of lipids such as cholesterol, *n*-pentadecanoic acid, and propanoic acid (Wang et al., 2016). Considering this evidence, as well as our results showing that fipronil exposure caused a decrease in body length and upregulation of *atp5f1c*, we measured the expression of genes related to lipid metabolism. The transcript levels of eleven lipid genes were assessed following exposure to fipronil, carvacrol, and acetylcarvacrol. However, we observed no changes in the expression of these genes suggesting that lipid metabolism may not be a significant target of chemical exposure.

Elevated levels of apoptotic cells are often seen as an outcome of disrupted metabolism and increased ROS production (Yamashita, 2003). A study that exposed zebrafish embryos for 72h at 15 mg/L (~12 µM) fipronil found a high number of apoptotic cells in the eye and brain (Park et al., 2020). We exposed zebrafish larvae to an environmentally relevant concentration of fipronil (0.1 µM) and found no differences compared to the negative control. Moreover, neither carvacrol nor its derivative induced apoptosis at this concentration. A study that exposed male albino rats for 8 weeks to fipronil showed that this pesticide activated oxidative, inflammatory, and apoptotic pathways in rats' brains (Khafaga et al., 2021). Therefore, we believe chronic exposure to low concentrations of fipronil could indeed affect cell death, but this hypothesis requires further investigation.

In conclusion, fipronil exerted acute toxicity to developing zebrafish even at low concentrations (0.1 µM). This pesticide caused hatching delay, affected survivability, and induced deformities such as spinal lordosis and yolk sac edema. Zebrafish larvae exposed to fipronil exhibited uninflated swim bladder, which led to reduced locomotor activity. Carvacrol and acetylcarvacrol, on the other hand, exerted low acute toxicity to early staged zebrafish, affecting survival and deformities only at high concentrations (> 50 µM). Acetylcarvacrol presented a lower lethality compared to carvacrol, suggesting that acetylation of this molecule may indeed reduce its toxicity. All chemicals induced an increase in ROS production at 7 dpf, but an alteration in the expression of a mitochondrial gene was only found in larvae exposed to fipronil. There were no changes in mitochondrial bioenergetics and apoptosis following exposure to any of the chemicals.

Thus, our results show that carvacrol and its derivative are much less toxic to aquatic organisms compared to fipronil.

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Statements and Declarations

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Declaration of competing interest

The authors declare that there are no competing interests that could influence the work reported in this manuscript.

Author Contributions

IK, NI, EH, CE, EI, and CLS conducted experiments. IK and CJM wrote the manuscript. SM and CJM conceptualized and supervised the project. CJM funded the study. All authors read and approved the final version of the manuscript.

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SUPPLEMENTAL DATA

Toxicity assessment of carvacrol and its acetylated derivative in early staged zebrafish (*Danio rerio*): safe alternatives to fipronil-based pesticides?

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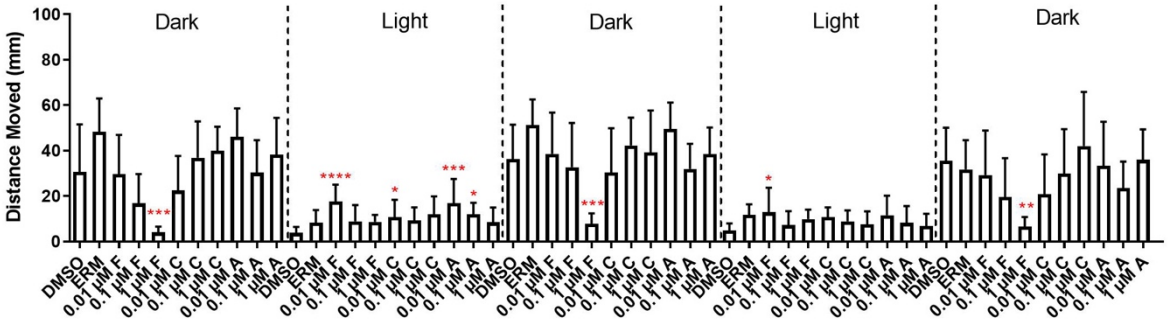
First trial



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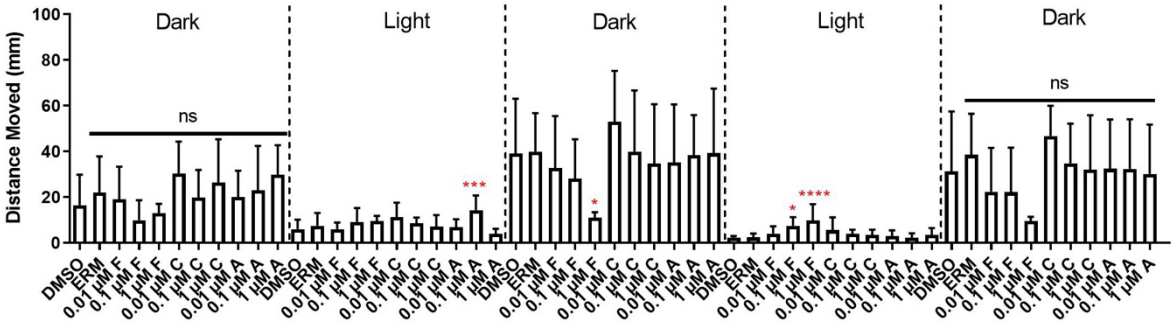
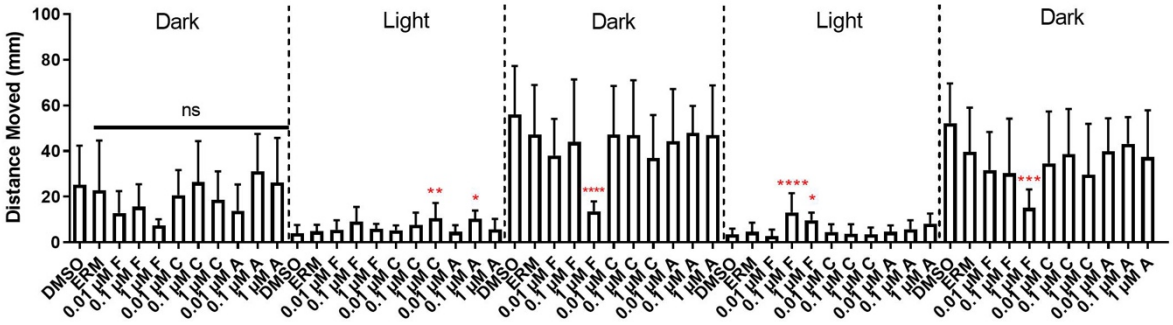
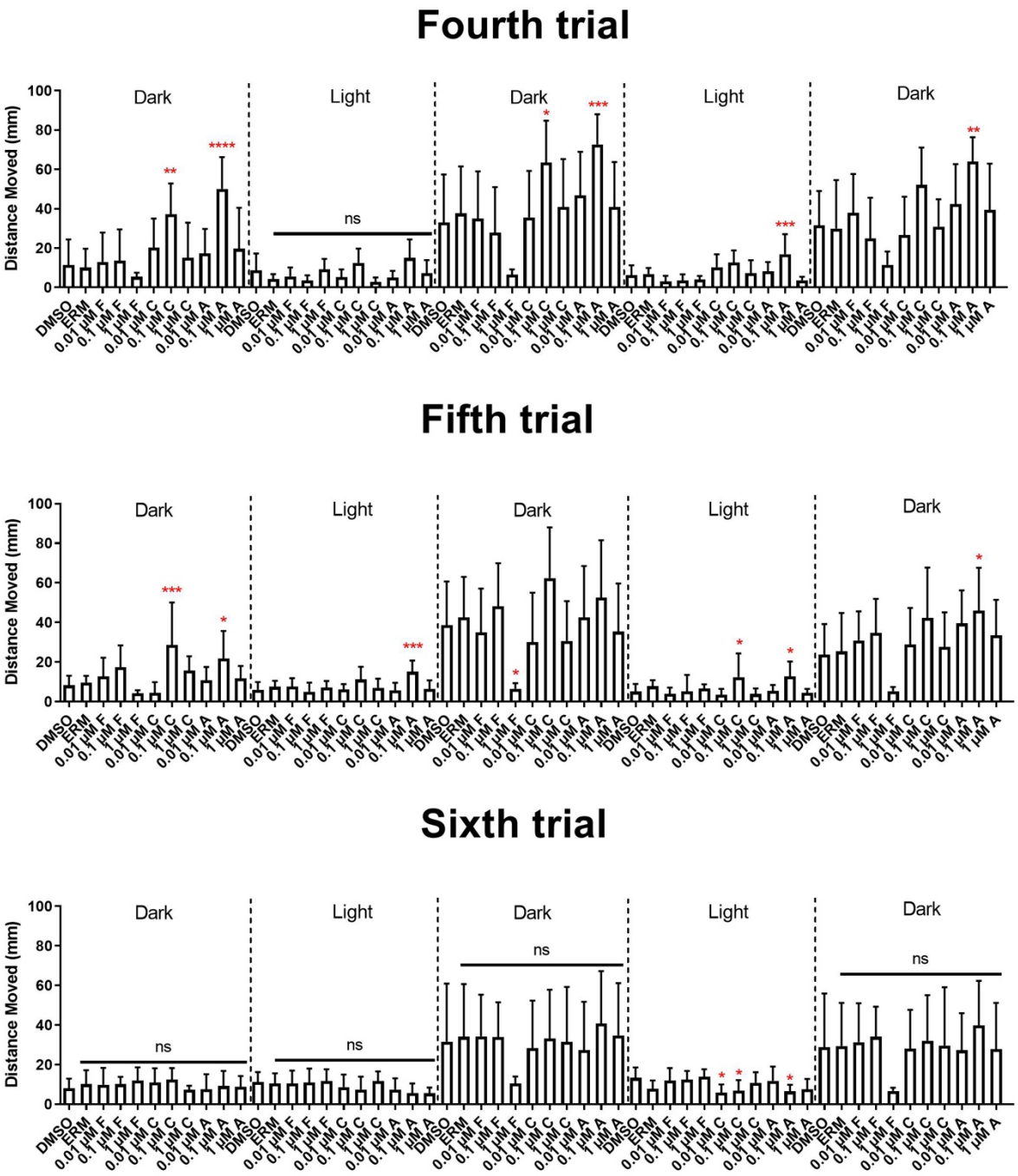
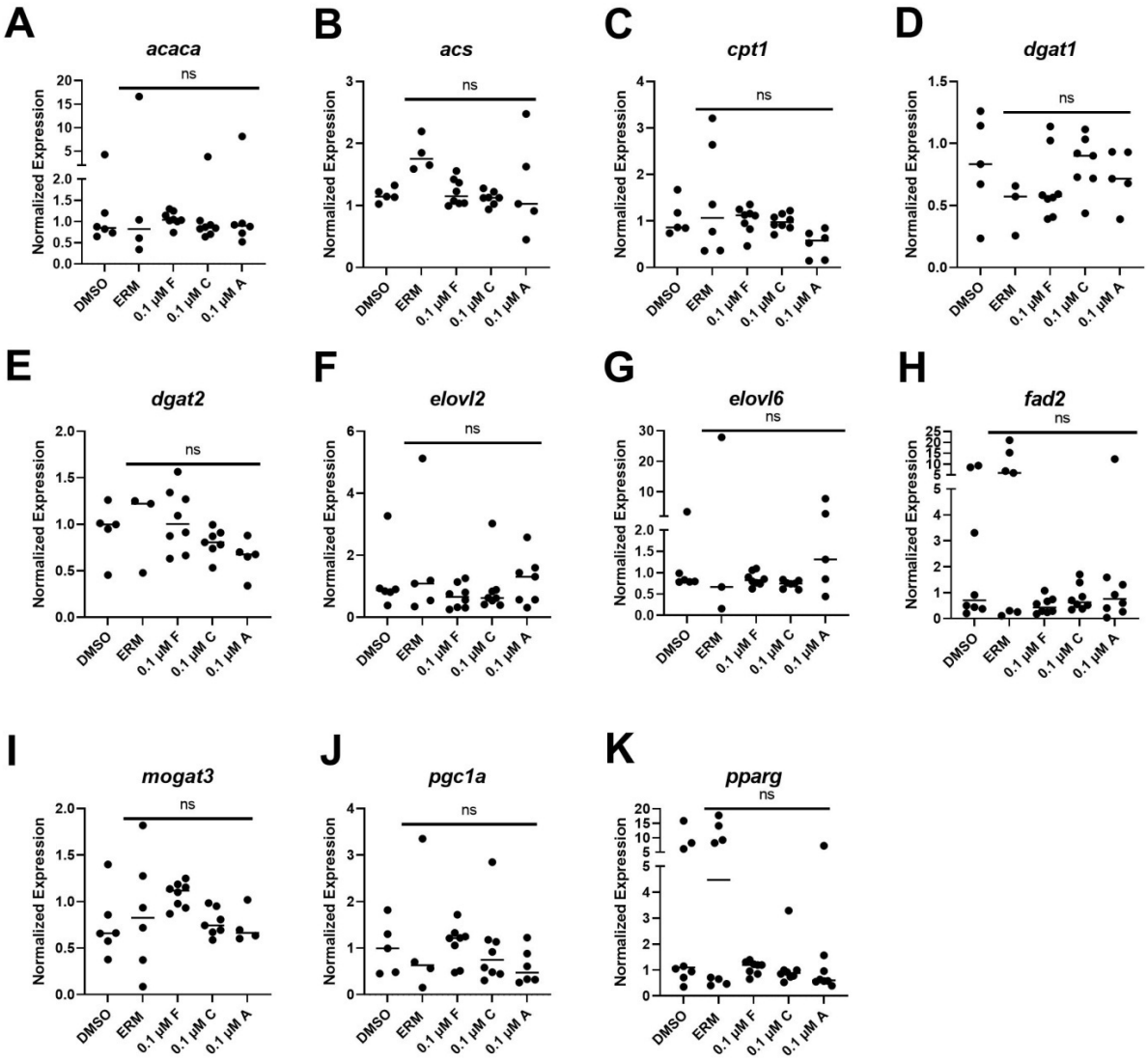


Third trial





Supplemental Figure 1. The activity of 7-dpf zebrafish larvae exposed to fipronil, carvacrol, and acetylcarvacrol. Distance moved in each light and dark zone (10-minute bins) for six independent experiments. Mean values are depicted by the columns in each dark-light phase (mean ± S.D.) (One-Way ANOVA with a Holm-Šídák's multiple comparisons test, n=8-12 per treatment). Asterisk denotes a significant difference at *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, **** $p \leq 0.0001$.



Supplemental Figure 2. Expression levels of transcripts related to lipid metabolism of zebrafish larvae exposed continuously to either 0.1 µM fipronil (F), carvacrol (C) or acetylcarvacrol (A) for 7 days. (A) *acaca*, Acetyl-CoA Carboxylase Alpha; (B) *acs*, Acetyl-coenzyme A synthetase; (C) *cpt1*, carnitine palmitoyltransferase 1A; (D) *dgat1*, Diacylglycerol O-Acyltransferase 1; (E) *dgat2*, Diacylglycerol O-Acyltransferase 2; (F) *elovl2*, Fatty Acid Elongase 2; (G) *elovl6*, Fatty Acid Elongase 6; (H) *fad2*, Delta(12)-fatty-acid desaturase; (I) *mogat3*, Monoacylglycerol O-Acyltransferase 3; (J) *pgc1a*, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; (K) *pparg*, Peroxisome Proliferator-Activated Receptor Gamma. Horizontal line represents the group's median value (Kruskal Wallis followed by Dunn's multiple comparison test, ns = nonsignificant, $p > 0.05$, n = 4-8 biological replicates/treatment).

Supplemental Table 1. Primers used for real-time PCR analysis.

Gene Symbol	Gene Name	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Reference
Housekeeping				
β-actin	Beta-actin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	McCurley and Callard, 2008
rps18	Ribosomal subunit 18	TCGCTAGTTGGCATCGTTTATG	CGGAGGTTCGAAGACGATCA	McCurley and Callard, 2008
rpl13a	Ribosomal protein L13a	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC	Zucchi et al., 2011
TUB-α	Tubulin-alpha	TTTGTGCACTGGTACGTGGG	CCACACTCTCAGCTCCAACCTC	Kataba et al., 2020
Swim bladder inflation				
acta2	Actin Alpha 2, Smooth Muscle	GTGCTACGTGGCTCTGGACT	TGACCTGTCCATCGGGAAGC	Yang et al. 2021
ihha	Indian hedgehog	TGA GTC CAA AGC TCA CAT CCA	AGG CTG GAA AACAACCACCG	Jantzen et al., 2016
pbx1a	PBX Homeobox 1	GAAGCCGGCTCTGTTCAACG	CGCATGAGCTGTGCATCTGG	Yang et al. 2021
Oxidative stress				
ache	Acetylcholinesterase	GCTAATGAGCAAAAGCATGTGGGC	TATCTGTGATGTTAAGCAGACGAGGCA	NM_131846.2
cat	Catalase	CTCCTGATGTGGCCCGATAC	TCAGATGCCCGGCCATATTC	Sarkar et al., 2014
hsp70	Heat shock protein 70	GAAGACGGCATCTTTGAGGTGA	GGGCCCTCTTGTTCTGACTGAT	Hahn et al., 2014
hsp90b	Heat shock protein 90b	TCAGTTCATCGGTTACCCAAT	TTTGTCTTTGTCTTCGCCTG	Zhang et al., 2017
sod1	Superoxide dismutase 1	CGTCTATTTCAATCAAGAGGGTG	GATGCAGCCGTTTGTGTTGTC	Lin et al., 2009
sod2	Superoxide dismutase 2	CTTGGGATAGATGTCTGGG	GTGGTCTGATTAATTGTGCG	Lin et al., 2009
Mitochondrial activity				
atp06	ATP synthase F0 subunit 6	TTATCCTCGTTGCCATACTTC	AGTTGGTTTGTGAATCGTCC	Jin et al., 2010
atp5f1b	ATP Synthase F1 Subunit Beta	CTCAATGCCCTGGAAGTAGC	GAACCT TCTGACCACGAACC	Park et al., 2021
atp5f1c	ATP Synthase F1 Subunit Gamma	TTGAAGGACATCACCATTCG	GCT TCAGGGACCTCTCAGC	Park et al., 2021
atp5mea	ATP synthase membrane subunit ea	GATTACCTGAAGCCCATTGC	AAATGCGCTCTTGTTCTTCC	Lee et al., 2021
cox1	MT-CO1 (mitochondrially encoded Cytochrome c oxidase I)	ACTTAGCCAACCAGGAGCAC	GGGTGGAAGAAGTCAGAAGC	Northam and LeMoine, 2019
cox4i1	Cytochrome C Oxidase Subunit 4I	GGCAACTACGGCATTTCGTC	CGACCTTCGCAACTCCATGT	NM_214701.1
Lipid metabolism				
acaca	Acetyl-CoA Carboxylase Alpha	ACGCTTTGTTGTCATGGTGA	TTCTTGTGCAGCAATTCAGG	Pan et al., 2019
acs	Acetyl-coenzyme A synthetase	TGACAGCACAAGCACAAAAC	TTCCACTCGTGAAGCAAATC	Pan et al., 2019
cpt1	Carnitine palmitoyltransferase 1A	ATGAGGAGCACCAAAGAATG	TGGGAAAAGCGTAAAGAAAG	Pan et al., 2019
dgat1	Diacylglycerol O-Acyltransferase 1	GCTCAGATCGCCGTCTTCTT	CGTTGCCATAGTTACCCCTC	Pan et al., 2019
dgat2	Diacylglycerol O-Acyltransferase 2	ATAGAGGAGCCGACTCAGGA	GAGTGTTCGTTTGGACAGTTAT	Pan et al., 2019
elovl2	Fatty Acid Elongase 2	GTTCCATGTGGTTTCCCACT	TTGGCTCCGTTTAGAGAGGA	Pan et al., 2019
elovl6	Fatty Acid Elongase 6	TCGTGTTACGCAAGCAGAAG	CACCATCTGGGTGATCTGTG	Pan et al., 2019
fad2	Delta(12)-fatty-acid desaturase	TACCTGAAGCCGCTGCTAAT	ACGGACAGATGACCGAAGTC	Pan et al., 2019
mogat3	Monoacylglycerol O-Acyltransferase 3	GGAGGTCACAGTGGGTCAGA	CGTGCAGAAGTTCCCGAATC	Pan et al., 2019
pgc1a	Peroxisome Proliferator Activated Receptor Gamma, coactivator 1 alpha	AGATGGGGACGTGACCAATG	GGGGTTTTCTGTCTTGGCAAC	Guo et al., 2021
pparg	Peroxisome Proliferator Activated Receptor Gamma	CTGCCGCATACACAAGAAGA	TCACGTCACTGGAGAACTCG	Guo et al., 2021

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PART IV – Final discussion

Hundreds of papers reporting the use of natural products to control arthropod pests are published each year. Despite the description of the impressive results of these chemicals, pesticide formulations based on natural compounds are not commonly seen on the market. The main reasons for this are well discussed in a review conducted by Pavela and Benelli (2016) and I point them out here:

1. Relatively low stability of natural compounds to oxidation;
2. The development of a biopesticide would require a homogeneous chemical composition. Thus, being important to evaluate the biological activity of isolated chemicals and not only the mixture present in essential oils;
3. The lack of comparative toxicological studies with commercial pesticides.

We tried to overcome these issues in this study. Firstly, we used the acetylated derivative of carvacrol, which is more stable to oxidation (Konig et al., 2021), presents increased biological activity (Ramírez et al., 2016) and reduced toxicity to mice (Andre et al., 2016). Secondly, we assessed the toxicity of carvacrol and its derivative in their isolated form. Finally, we compared our results with a commercially available pesticide vastly used worldwide.

Our results showed that fipronil is indeed highly toxic to both models evaluated: human blood and zebrafish. There is an urgent need to replace this chemical by environmentally safer pesticides. Sadly, fipronil one of the most frequently used pesticides in Brazil and it is found in high concentrations in surface water (Albuquerque et al., 2016). Our study conducted with zebrafish revealed that exposure to environmental relevant concentrations of fipronil impairs the inflation of the swim bladder of larvae, decreases their body length, induces oxidative stress, reduces their locomotor activity and alters the expression of a gene critical for the mitochondrial activity. In the human blood model, fipronil exposure reduced leukocyte viability, altered the morphology of erythrocytes, and induced fragmentation of the DNA.

Carvacrol and its derivative presented reduced toxicity to zebrafish and human blood cells when compared to fipronil. Additionally, acetylation of carvacrol would seem to be a promising alternative to reduce the toxicity of carvacrol to these models. Indeed, acetylcarvacrol was considerably less lethal than carvacrol to zebrafish larvae and was less toxic to human blood cells and DNA molecules even at higher concentrations. Taken together, our data show that carvacrol and acetylcarvacrol can be used as a replacement of fipronil in terms of toxicity. However, it is important to compare the biological activity these chemicals and

other natural compounds in general to commercial pesticides. Assessing the pesticide activity of a chemical without being possible to compare the results with commercially available ones may bias the conclusion of the study and interpretation by authorities.

Conclusion

Fipronil was highly toxic to human blood cells and developing zebrafish. Exposure to this chemical decreased leucocyte viability, induced hemolysis and morphological alterations in erythrocytes, and caused fragmentation of DNA. In the zebrafish model, this chemical impaired the inflation of the swim bladder of larvae leading to hypoactivity in the behavior assay. Additionally, it upregulated the expression of a gene related to mitochondrial activity and was considerably more lethal than carvacrol and its derivative. Acetylcarvacrol was less toxic than carvacrol and fipronil in both models evaluated. Indeed, it was 70 times less lethal than fipronil to developing zebrafish. However, carvacrol presented low toxicity as well, particularly at decreased concentrations. Finally, comparative toxicological studies should be carried out more often, particularly those comparing natural-based compounds to commercial pesticides. Thus, we can advance in the search for environmentally friendly alternative pesticides.

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