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ՍԵՂՄԱԳԻՐ

ԵՐԵՎԱՆ – 2022

NATIONAL ACADEMY OF SCIENCES OF THE REPUBLIC OF ARMENIA
INSTITUTE OF MOLECULAR BIOLOGY

HAIG YOUSEF BABIKIAN

ANTIBACTERIAL, ANTIVIRAL, AND IMMUNOMODULATORY PROPERTIES OF HERBAL VOLATILE ESSENTIAL OILS *IN VIVO* AND *IN VITRO*

SYNOPSIS

of Dissertation Submitted for the Degree
of Candidate of Biological Sciences (Ph.D.) in the Field of
03.00.03. “Molecular and Cellular Biology”

YEREVAN – 2022

Ատենախոսության թեման հաստատվել է ՀՀ ԳԱԱ Մոլեկուլային կենսաբանության ինստիտուտի գիտական խորհրդում:

Գիտական ղեկավար՝	կ.գ.դ., պրոֆ. Տիգրան Կամոյի Դավթյան
Պաշտոնական ընդդիմախոսներ՝	բ.գ.դ., պրոֆ. Գայանե Գուրգենի Մելիք-Անդրեասյան կ.գ.թ. Հովակիմ Սարգսի Զաքարյան
Առաջատար կազմակերպություն՝	Մ. Հերացու անվ. Երևանի պետական բժշկական համալսարան

Ատենախոսության պաշտպանությունը տեղի կունենա 2022 թ. հունիսի 3-ին, ժամը 14:00-ին, ՀՀ ԳԱԱ մոլեկուլային կենսաբանության ինստիտուտում, 042 մասնագիտական խորհրդի նիստում (ՀՀ 0014, ք. Երևան, Հասրաթյան 7):

Ատենախոսությանը կարելի է ծանոթանալ ՀՀ ԳԱԱ մոլեկուլային կենսաբանության ինստիտուտի գրադարանում և http://molbiol.sci.am/ կայքում:

Ատենախոսության սեղմագիրն առաքվել է 2022 թ. ապրիլի 12-ին:

042 մասնագիտական խորհրդի գիտական քարտուղար,
կենս. գիտ. թեկնածու Զ.Ա. Խաչատրյան

Dissertation topic approved at the Scientific Council of the Institute of Molecular Biology NAS RA.

Scientific supervisor:	D.Sc., Prof. Tigran Kamo Davtyan
Official opponents:	D.Sc., Prof. Gayane Gurgen Melik-Andreasyan Ph.D. Hovakim Sargis Zakaryan
Leading organization:	Yerevan State Medical University after M. Heratsi

The defense of the dissertation will be held on 3 June 2022, at 14:00 at the session of the specialized council 042 acting in the Institute of Molecular Biology NAS RA (Hasratyan 7, 0014, Yerevan, RA).

The dissertation is available at the library of the Institute of Molecular Biology NAS RA and at the website http://www.molbiol.sci.am.

Synopsis was sent out on 12 April 2022.

Scientific secretary of the specialized council 042
PhD Z.A. Khachatryan

INTRODUCTION

Problem statement. Modern antimicrobial and antiviral therapies have low efficacy and are limited due to the spread of microorganism strains resistant to antibiotics. The emergence of viable resistant microbes has been proven even against antimicrobial agents, such as vancomycin and cationic antimicrobial proteins, to which resistance has previously been considered impossible. Therefore, the development and application of new, highly effective, antimicrobial, antiviral, and immunomodulatory drugs for applied medicine capable of suppressing the expression of pathogenic factors and co-morbidity and drug-resistance coefficients is necessary. Related to this, the antibacterial, antiviral, and immunomodulatory properties of herbal combined volatile essential oils (EOs) with potential antimicrobial effects on the *in vitro* and *in vitro* antibiotic activity of viruses and polyresistant bacterial strains is of significant interest. The animal livestock industry cannot be separated from antibiotic growth promoters (AGPs), increasing productivity and preventing diseases caused by farm environments. However, AGPs are product residues that are dangerous when consumed excessively; AGPs cause pathogenic microbial resistance. Moreover, the excessive utilization of AGPs in animal farming stimulates bacterial resistance and appears as residue in animal products (meat, milk, etc.), harmful to human health. The EU has banned the use of AGPs in animal farming since 2006, and consumers have become increasingly aware of their food quality and safety issues. Research on the alternative use of AGP 'feed quality for food safety' has garnered much interest, including the alternative the use of growth promoters such organic acids, immunomodulators, probiotics, prebiotics, enzymes, phytogenics, EOs, etc. White spot syndrome (WSS) is a viral infection of penaeid shrimp and one of the most important shrimp diseases worldwide, affecting most commercially-cultured shrimp species (Jha et al., 2006, 2007, 2010; Sudheer et al., 2014). The disease is highly lethal and contagious, killing shrimp quickly; outbreaks have wiped out entire populations of many shrimp farms within a few days throughout the world. The disease is caused by a family of related viruses, subsumed as the WSS baculovirus complex.

Acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS) have been reported in several shrimp-producing countries, such as Vietnam, Malaysia, Thailand, Mexico, and the Philippines (Flegal, 2012; Nunan et al., 2014), with a significant impact on world shrimp production (Leaño, Mohan 2012; Tran et al., 2013); however, the causative agent in AHPND, *Vibrio parahaemolyticus,* has been successfully identified and isolated. The unique symptoms and characteristics of this disease include severe atrophy of the shrimp hepatopancreas, consisting of massive sloughing of hepatopancreas epithelial cells (Lightner et al., 2012). External symptoms in infected shrimp, such as an empty stomach, bluish body color, and shrunken hepatopancreas, can be observed in outbreak ponds, appearing in the culture ponds after 8-45 days of stocking. AHPND can be transmitted experimentally by horizontal transmission, co-hab precipitation, immersion, and reverse gavage. Furthermore, shrimp cultured in earthen bottom ponds were more susceptible to AHPND than the HDPE-lined ponds. Infectious myonecrosis virus (IMNV) is known to cause significant outbreaks and mortality in penaeid shrimp (Lightner, 2004). In Indonesia, the first outbreak occurred in Situbondo in 2006, spreading to East Java, Bali, Lampung, Central Java, West Kalimantan, and West Nusa Tenggara (Sukenda et al., 2011). In Brazil, IMNV causes up to 70% mortality (Melo, 2014). IMNV has been demonstrated to be horizontally transmitted by ingesting infected moribund shrimp and live feed (Taukhid & Nuraini, 2009). Aquaculture experts have continuously attempted to control the spread of the virus by proper husbandry practices, specific pathogen-free (SPF) broodstock, and rapid and sensitive detection kits (Poulos, 2006). IMNV is associated with the characteristics of the WSS virus (WSSV) and causes chronic mortality in ponds; this results in larger losses due to the high feed conversion ratio (FCR), lower survival rate, and higher production costs. IMNV-infected shrimp in stressed conditions show typical white or red muscle in the fifth to sixth

segments, and it is difficult for shrimp with white muscle to recover in the cultured ponds. Herbs have significant potential in clinically useful drugs to effectively prevent and control many viruses of aquaculture origin. Various plant- and plant-derived products have shown significant effects against pathogens, especially in reducing the mortality of viral diseases in shrimp, such as *Phyllanthus niruri,* increasing the survival rate against IMNV from 66.67% to 86.67% (Sivasankar, 2015).

Several types of volatile EOs such as lavender (*Lavandul latifolia*), pine (*Pinus sylvestrisa*), jasmine (*Jasminum officinale*), lemon (*Citrus limon*), cherry (*Prunus avium*), violet (*violet*), gardeniat (*Gardenia jasminoides*), palm (*Cocos nucifera*), rose (*Rosa damascene*), and nettle (*Eucalyptus globules*) are well known and have been studied for their antiseptic, antimicrobial, and anti-inflammatory effects separately, including their antiviral and immunomodulatory properties. Lavender EO consists of monoterpenoids and sesquiterpenoids, whose components are linalol, moderate amounts of lavender-acetate, terpine-4-ol, and lavandulol and minor amounts of 1,8-cineol (eucalyptol) and camphor (Gostner et al., 2014). Pine oil consists of α-terpineol or cyclic terpene spirits and is considered a phenolic disinfectant and mild antiseptic, with antifungal, antibacterial, and antiviral properties (Patra et al., 2015; Koutsaviti et al., 2021). Jasmine oil, known as the "King of Oils" and whose main component is benzylacetate, is used in aromatherapy, phytotherapy, and dermatology as an antibacterial agent (Rath et al., 2008). Lemon contains polyphenols and terpenes, low in lemon juice pH, making it a powerful antibacterial agent (Baser, Buchbauer 2015). The high content of antioxidants in palm oil, such as palmitic acid, stearic acid, oleinic acid, linoleic acid, linolenic acid, and eleostearic acid, is positive for inflammatory bowel function (Gesteiro et al., 2019). The effective antifungal, antibacterial, and antioxidant properties of violet EO are particularly effective in many alternative therapies (Hammami et al., 2011); geniposide and genipine suppress the production of lipid peroxides and oxidative stress in neurons in this EO and can be used as anti-inflammatory antioxidants (Friedman et al., 2002; Kalim et al., 2010). Additionally, coconut oil has powerful antifungal, antibacterial, and antiviral effects (Hilmarsson et al., 2007; Huang et al., 2014).

Aim and objectives. This study aimed to investigate the antibacterial, antiviral, and immunomodulatory properties of blended volatile EO (consisting of *Lavandula latifolia*, *Pinus sylvestris*, *Jasminum officinale*, *Citrus limon*, *Prunus avium*, *Viola odorata*, *Gardenia jasminoides*, *Cocos nucifera*, *Rosa damascene*, and *Eucalyptus globules*) *in vivo* and *in vitro*.

Research objectives are:

- To study the antifungal and antimicrobial effects of natural oil formulations (NOFs) of EO blends on the pro- and anti-inflammatory activation of human peripheral blood mononuclear cells (PBMCs) and production of cytokines *in vitro*.
- To evaluate the efficacy of NOFs in improving the clinical performance of white leg shrimp (*Penaeus vannamei*) in AHPND or EMS bacterial challenge models.
- To determine the effectiveness of NOFs against IMNV in white leg shrimp, *Penaeus vannamei*.
- To investigate the efficacy of NOF in improving the clinical performance of white leg shrimp (*Penaeus vannamei*) in a WSSV challenge model.
- To evaluate the impact of NOF on the productivity and blood parameters of calves during growth and transportation periods.
- To study the efficacy of NOFs and NOF liquids on the productivity of broiler chickens as an antibiotic replacement strategy.

Scientific and practical significance of the results. In this study, we demonstrated that a NOF, prepared from a mixture of *Lavandula latifolia*, *Pinus sylvestris*, *Jasminum officinale*, *Citrus limon*, *Prunus avium*, *Viola odorata*, *Gardenia jasminoides*, *Cocos nucifera*, *Rosa damascene*, and *Eucalyptus globules* showed antiviral, anti-inflammatory, and antimicrobial activities against

bacteria and fungi. The NOF indicated moderate antimicrobial effects against six bacterial strains (*Staphylococcus aureus*, *Staphylococcus epidermidis, Escherichia coli, Salmonella enterica serovar Typhimurium, Pseudomonas aeruginosa,* and *Micrococcus luteus*) and two fungal *Candida albicans* strains *in vitro*. We observed a prominent suppression of proinflammatory cytokine production by LPS-primed PBMCs in the presence of the NOF. Therefore, our results suggest that NOFs may serve as a promising pharmaceutical agent with combined anti-inflammatory, antimicrobial, and fungal action.

A bioassay trial was conducted to determine the efficacy of the developed NOF product as an anti-AHPND, with results demonstrating that the cumulative mortality reached 56.7% in the positive control, 23.3% in the 80-ppm NOF group, and 13.3% in the 40-ppm NOF group at day post infection (dpi) 8, whereas no mortality was recorded in the negative control. The relative percent survivals of the 80-ppm and 40-ppm groups were 64.7% and 76.5%, respectively. The trial results showed that the developed NOF had a significant effect against AHPND-*Vibrio parahaemolyticus* under controlled conditions. The IMNV trial results of the *Penaeus vannamei* shrimp showed that the developed blended oil extract formulation had a significant effect against IMNV under controlled conditions. The developed product is user-friendly as it is mixed and incorporated into the shrimp feed in specific amounts to have antiviral properties. The NOF efficacy study results to improve the clinical performance of *Penaeus vannamei* white leg shrimp in the WSSV challenge model showed that the developed blended oil extract formulation has a significant effect against WSSV in controlled conditions. The developed product was incorporated into shrimp feed in a specific amount to have antiviral properties.

The study results evaluating the impact of NOF in productivity and blood parameters on calves during their growth and transportation period demonstrated that the addition of NOF (2 kg/ton feed) increased their health status and tended to reduce bodyweight loss during transportation. The NOF improved the economic value after the 122-day feedlot program. Providing 2-kg/ton NOF feed and NOF liquid (0.5 mL) drinking water to broiler chickens was effective in increasing the productivity and health of broiler chickens, characterized by a higher increase in final weight and lower mortality compared to control group chickens. The 2-kg/ton NOF feed and 0.5-ml/L NOF drinking water were proven safe (not toxic to chickens) based on liver function, kidney function, and description of the pathology (PA) and histopathology (HP) of the liver and kidney.

Approbation. Proceedings of the dissertation have been presented at "Third International Conference on Aquaculture & Fisheries," Sep 29 - Oct 1, 2016, London, UK; "BIT's 5th Annual World Congress of Aquaculture and Fisheries-2016," Nov 4-6, 2016, Qingdao, China; "2nd International Conference on Aquaculture and Fisheries," Aug 28-29, 2020, Kuala Lumpur; "ClimFishCon," Feb 11-14, 2020, Le Meridien Kochi, Kochi, India; speaker at "10th International Conference of Aquaculture Indonesia," Oct 28-30, 2021, Semarang, Indonesia; "15th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases," Nov 2-5, 2021, New Orleans, USA, Live and On-demand for and by Elsevier; "3rd online International Conference in Aquaculture and Fisheries", July 5-6, 2021; at the meetings of the scientific councils of the Research Institute of Epidemiology, Virology and Medical Parasitology named after A.B. Alexanian Ministry of Health of RA (2020) and the Institute of Molecular Biology of NAS RA (2021-2022).

Publications. The main results of the dissertation are published in 12 papers and 9 presentation abstracts at international scientific conferences.

Structure. This dissertation comprises 116 pages of computer-formatted English text, including 29 tables and 24 figures, consisting of the following sections: Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusion, References (including 198 sources), and Appendix (pp. 117-161).

MATERIALS AND METHODS

In Silico Modeling. Multiple sequence alignment and the protein sequence similarity between *V. parahaemolyticus* $PirA^{vp}/PirB^{vp}$ and bacterial toxins, protein homology modeling and protein-protein docking, structural analysis of the cytotoxic mechanism of *V. parahaemolyticus* $PirA^{vp}$ toxin, structure-based ligand-binding site and druggability prediction for *V. parahaemolyticus* $PirA^{vp}/PirB^{vp}$ and B. *thuringiensis* Cry toxins as well as identification of *V. parahaemolyticus* $PirA^{vp}/PirB^{vp}$ and *B. thuringiensis* Cry toxin potential inhibitors by structure-based virtual screening and molecular docking were used**.**

NOF preparation and composition. Individual oils in EO mixture were obtained from Demeter Agro Research and Improvements Pty Ltd, New Directions Australia Pty Ltd, and Australian Botanical Products Pty Ltd. Each EO is obtained through the steam distillation process and was thoroughly checked for quality and chemical compositions based on European Pharmacopeia. EOs were declared to pass the quality check, the EO mixture was conducted at a specific concentration and sequence. The mixing process takes 45-60 min at a temperature of 40 °C.

Oil composition analysis by GC-MS. GC-MS oil analysis was performed on a DANI Master GC gas chromatograph, coupled with a time-of-flight mass spectrometer (TOF MS). Compound identification was based on the comparison of retention indices and mass spectra of most of the compounds with data generated under identical experimental conditions.

Antibacterial assay. The following microorganisms were purchased from the American Type Culture Collection and used as test strains: *Staphylococcus aureus* ATCC-6538, *Staphylococcus epidermidis* ATCC-12228, *Escherichia coli* ATCC-8739, *Salmonella enterica serovar Typhimurium* ATCC-14028, *Pseudomonas aeruginosa* ATCC-9027, *Micrococcus luteus* ATCC-10240, yeast *Candida albicans* ATCC-10231, and *Candida albicans* NCTC-885-653. NOF screening for antibacterial activity was performed using the disk diffusion method. Broth microdilution susceptibility assay was performed to determine the MIC and MBC in 96 well micro-titer plate. The inhibition of bacterial growth was revealed by the addition of a resazurin and reincubation for 3 h. MIC was determined by the permanence of blue coloration in the wells.

In vitro stimulation of PBMCs with plant extracts, and LPS. PBMCs were cultured in a 24-well flat-bottom plate at $1x10^6$cells/ml in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 2-mM L-glutamine in duplicates for each sample in a humidified 5% CO_2 incubator for 24 h at 37 °C. The culture supernatants were harvested after the incubation period, centrifuged to remove cell debris, and stored at −80 °C for cytokine assays. Cytokine content was determined using human IL-1β, IL-6, IL-10, IL-2, TNF-α, and IFNg ELISA MAX Deluxe kits.

White Leg Shrimp (Penaeus vannamei) in an AHPND Bacterial Challenge Model. White leg shrimp larvae (PL10) were spawned and nursed to 0.5 to 1 g/individual (the size most affected by AHPND) in a recirculation system at the shrimp hatchery and nursery of the College of Aquaculture and Fisheries, Cantho University. The weight and length of 30 shrimp were measured prior to applying the NOF and challenge. Each aquarium contained 25 L of seawater (15‰) with a continuous aeration system, and the temperature was maintained at 26-30°C. Experimental shrimp were stocked with a density of 40 individuals/aquaria (before challenge) and 15-20 individuals/aquaria (during challenge). Different concentrations of NOF (40 ppm and 80 ppm) were applied to obtain the best dose to act against AHPND. V36 bacterial strain cultures were grown for 24 h in TSB supplemented with 1.5% NaCl at 28°C. The OD corresponding to 10^8 cells ml^{-1} was diluted in seawater. Shrimp were immersed for 15 min in this bacterial culture with continuous aeration. The bacterial solution and shrimp were transferred to aquaria containing seawater to reduce the bacterial concentration to 10^6 cells ml^{-1}. No water was exchanged for the 48-h after the challenge. Then, 30% of the water was renewed every 2 days. Cumulative mortality, relative percent survival, severity and time of clinical sign onset, PCR

(IHHNV, WSSV, MBV, TSV, *Vibrio parahaemolyticus* causing AHPND) after and before the challenge were determined.

Infectious Myonecrosis Virus (IMNV) in White leg Shrimp *Penaeus vannamei.* A bioassay trial was set up using 10 aquaria for each group. The seawater utilized during the experiment was prepared by sedimentation, filtration, and disinfection using 30-ppm active chlorine; finally, it was treated with ultraviolet radiation. The trial duration was 30 days that is, 5 days of acclimatization, 14 days of anti-IMNV feed feeding and NOF application and observation, viral challenge on day 15, and then feeding and observation for 10 days. The shrimp in the treatment group were fed experimental feed, whereas regular feed was fed to the control. There was no change in the feed type, quality, or feeding rate throughout the experiment. The liquid supplement, NOF, was applied daily in experimental tanks throughout the experiment. Shrimp were challenged with a lethal IMNV dose (log 5) by injection. The collected tissue containing viable IMNV was stored at -80 C by adding sterile glycerol until further use. IMNV was confirmed by nested PCR analysis and HP.

White Leg Shrimp (Penaeus vannamei) in White Spot syndrome Virus (WSSV) Challenge Model. The WSSV was extracted using ultracentrifugation following the standard method. The pure virus was injected into the SPF shrimp. The virus quantification in shrimp tissue was performed using the qPCR method. Treatment group: 1.6 g (12 slices of muscle for 12% of shrimp per tank (5% of biomass per tank) of WSSV infected tissue (log 9) are dipped into a 20-ml of stock solution of NOF for 2 h in the refrigerator. Positive Control: 1.6 g of sub-lethal WSSV infected tissue are dipped into 20 ml of TS-Buffer for 2 h in the refrigerator. Negative Control: 1.6 g of normal tissue and dipped into 20 ml of TS-Buffer for 2 h in the refrigerator. The remaining shrimp from all the groups were tested by PCR and HP for the presence of WSSV.

Impact of NOF in Productivity and Blood Parameter on Calves during the Growth and Transportation Period. The experiment was conducted at the Faculty of Animal Science for 10 weeks; 2 weeks of calf adaptation, followed by 8 weeks of the growth period. A total of 24 Brahman Cross calves were assigned to the treatment groups; each treatment consisted of two replicates with six calves each. The treatments were as follows: control with no dietary NOF treatment and a group with dietary NOF (2 kg/ton). Feed intake was recorded daily throughout the experimental period, and the average daily feed was calculated. Calves were weighed individually before treatment and at weeks 4 and 8. Body weight gains calculated as weight gain (final bodyweight-initial bodyweight) and the average daily gain (ADG) were calculated for the growth period, and the FCR was calculated by dividing the total feed intake per group by the total body weight gain per animal for the growth period. Plasma concentrations of glucose, urea-N, total protein, albumin, globulin, and hematology parameters were determined by autoanalyzer.

Efficacy of NOF and NOF Liquid on Productivity of Broiler Chicken as an Antibiotic Replacement Strategy. A total of 480 experimental animals used in this study were day-old chicks (DOC). The experimental design was a completely randomized design with control and 2 treatment groups and 160 replications. Parameter measurements were performed on broiler productivity, including feed consumption, drinking consumption, body weight gain, final bodyweight, mortality, and FCR. Meanwhile, parameter measurements were also conducted on the health of broiler chickens, including red blood count, hematocrit, and hemoglobin, white blood count, blood biochemistry (triglycerides, total cholesterol, HDL, LDL, total protein, and albumin), and testing for antibody titers. Antibody titer testing was conducted by taking blood serum twice, 7 days after the application of the ND vaccine I and 7 days after the application of the ND II vaccine (booster). In addition, parameters were measured to assess the safety of NOF and NOF liquid preparations, including liver function and kidney function. The safety data of the

preparations were collected by observing the anatomical PA and HP of the liver and kidneys on the 35th day after treatment.

Statistical and data analyses were performed using the statistical software Graph Pad Prism 5.01 (Graph Pad Software, USA). Data were compared using one-way repeated measures ANOVA. Normal distribution was checked visually from distributions using the Shapiro-Wilk's W test. Statistical significance was set at $P \leq 0.05$. The results were expressed as the mean and standard error of the mean. Data were also analyzed using SPSS software, version 16 (IBM, 2011). The Kaplan-Meier estimator, also known as the product limit estimator, is a non-parametric statistic used to estimate the survival function from lifetime data.

RESULTS AND DISCUSSION

Designing Natural Antimicrobials to Block the Cytotoxic Effects of Vibrio parahaemolyticus PirAvp/PirBvp Toxins. Natural antimicrobials could be effective in reducing bacterial pathogenicity via direct or indirect anti-virulence activities. Compounds exerted direct anti-virulence activity by suppressing four major potential pathogenic mechanisms of V. parahaemolyticus-induced AHPND in *Penaeus vannamei* shrimp, including PirBvp/PirAvp toxin interaction with *P. vannamei* shrimp APN- and ALP-receptors, PirBvp/PirAvp toxin homodimer, and PirAvp/PirBvp heterotetramer formation. Other mechanisms by which natural antimicrobials can reduce virulence based on their involvement in bacterial physiology may include disruption of membrane integrity and composition, increased membrane fluidity, alteration of efflux pumps, ion channels, adherence to epithelial cells, and increases in host defense mechanisms, such as antioxidant, anti-inflammatory, and innate immune responses against bacterial cells. The phytochemical constituents of NOF were analyzed using GC-MS with stationary phase nonpolar columns, leading to the identification of a total of 97 different compounds from n-hexane extracted oil samples (Figure 1). Eighty volatile constituents were identified, and their corresponding biological activities, targets, and molecular functions are described. Alfa-terpineol (6,98%), anozol (6,89%), polypropylene glycol (4,91%), anisylacetone (4.55%), 1,1'-oxybis-2-propanol (4,19%), eucalyptol (3,91%), and linalool (2.45%) were the main components. The minor constituents included phenylethyl alcohol, oleic acid, benzyl acetate, anethole, 2-bornanone, camphor, isoamyl acetate, camphene, borneol acetate, methyl anthranilate, limonene, linalyl acetate (bergamiol), eugenol, borneol, citronellol, coumarin α-phellandrene, and other compounds. Most constituents with highly biologically active compounds and well-known antibacterial, antifungal, and antimicrobial agents, with potent anti-inflammatory molecules, including NF-κB and MAPK pathway inhibitors, LPS-induced COX-2 expression inhibitors, and STAT3/TLR4 signaling pathway inhibition.

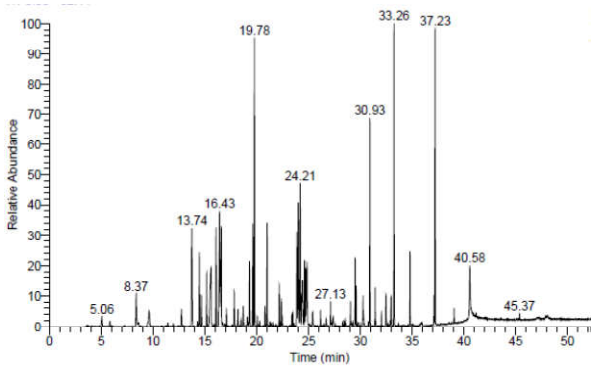


Figure 1. GC-MS analysis of NOF was performed on a GC gas chromatograph coupled with time-of-flight mass spectrometer. The oven program started with an initial temperature of 40 °C, was held for 4 min, then the oven temperature was heated at 5 °C/min to 80°C, held for 5 min, and the oven temperature was heated at 5 °C/min to 260 °C, and finally held isothermally for 25 min. For Master TOF MS detection, an election ionization system, with ionization energy of 70 eV was used.

NOF Compounds, including anethole, anisylacetone, borneol, phenylethyl alcohol, polypropylene glycol, and tripropylene glycol could be potent suppressors of four major potential pathogenic mechanisms of V. parahaemolyticus-induced AHPND in *P. vannamei* shrimp, including PirAvp toxin interaction with *P. vannamei* shrimp APN- and ALP-receptors, PirAvp toxin homodimer, and PirAvp/PirBvp heterotetramer formation.
Selected NOF molecules listed in Table 1 form polar interactions with hotspot Lys67, Val68, Gln54, and Gln56; Arg39 and His111; and common Ser28 and adjacent amino acid residues, responsible for the $PirA^{vp}$ toxin-APN/ALP-receptor binding (in Table 1, these residues are highlighted in blue and yellow, respectively).

Table 1. Docking of the 15 selected NOF molecules at the binding sites of the *V. parahaemolyticus* $PirA^{vp}$ toxin.

Ligand name	**docking score (kcal/mol)**	**protein-ligand affinity (pKd)**	**Amino acid residues interaction**	**Toxin biological activity modulation**	
alpha-terpineol	-6.5	4.94	*Top 1 scoring* **vdW**:Tyr90, Asn87; **Pi-sigma**:Trp57; **Pi-Alkyl:**Trp57, Phe89; **UAA:**Asn87, Ala88 *Top 9 scoring* ***H-bond***: Asn87, Ser28	**APN-receptor binding**	Yes
				ALP-receptor binding	Yes
				$PirA^{vp}$ homodimer formation	Yes
				$PirA^{vp}$/$PirB^{vp}$ heterotetramer formation	**Yes** 15-42 52-79
anisaldehyde	-6.7	6.19	*Top 1 scoring* **H-bond**:Asn87; **vdW**: Phe89, Asn87, Ala88, Lys67, Asp86: **Pi-Alkyl:** Phe89; **Alkyl**:Ala69; **Pi-Pi:** Trp57 *Top 9 scoring* **H-bond**: Asn87, Asp27, Glu25, Lys67, Asp86	**APN-receptor binding**	Yes
				ALP-receptor binding	Yes
				$PirA^{vp}$ homodimer formation	Yes
				$PirA^{vp}$/$PirB^{vp}$ heterotetramer formation	**Yes** 15-42 52-79
anisylacetone	-6.1	4.97	*Top 1 scoring* **H-bond**:Asn87, Trp57; **vdW**:Tyr90, Phe89, Asn87, Ala88, Ala69, Trp57 *Top 9 scoring* **H-bond**:Trp57, Asn87, Ser28	**APN-receptor binding**	Yes
				ALP-receptor binding	Yes
				$PirA^{vp}$ homodimer formation	Yes
				$PirA^{vp}$/$PirB^{vp}$ heterotetramer formation	**Yes** 15-42 52-79
anozol	-7.0	5.35	*Top 1 scoring* **H-bond**:Asn87; **vdW**: Val68, Ala69, Ala88, Pro60, Tyr90, Gly58; **Pi-Alkyl**:Phe89, Ala59; **Pi-Sigma**:Trp57 *Top 9 scoring* **H-bond**:Asn87	**APN-receptor binding**	Yes
				ALP-receptor binding	No
				$PirA^{vp}$ homodimer formation	Yes
				$PirA^{vp}$/$PirB^{vp}$ heterotetramer formation	**Yes** 52-79

Table 1 continued

benzyl acetate	-5.5	4.47	*Top 1 scoring* **H-bond**: Asn87; **vdW**: Phe89, Ala88, Asn87; **Pi-Pi**: Trp57 *Top 9 scoring* **H-bond**:Asn87, Lys70, Asp86, Gln56, Gln54	**APN-receptor binding**	Yes
				ALP-receptor binding	No
				PirA^{vp} homodimer formation	Yes
				PirA^{vp}/PirB^{vp} heterotetramer formation	**Yes** 52-79
phenylethyl alcohol	-5.4	4.12	*Top 1 scoring* **H-bond**: Trp57, Val68; **vdW**: Ala88, Ala59, Pro60, Tyr90, Lys67, Ala69, Asn87; **Pi-Pi**: Trp57 *Top 9 scoring* **H-bond**: Asn87, Trp57	**APN-receptor binding**	Yes
				ALP-receptor binding	No
				PirA^{vp} homodimer formation	Yes
				PirA^{vp}/PirB^{vp} heterotetramer formation	**Yes** 52-79
tripropylene glycol	-4.7	4.97	*Top 1 scoring* **vdW**: Ala88, Tyr90, His111,Arg39, Asn87; **Pi-Alkyl**: Trp57, Tyr90, Phe89 *Top 9 scoring* **H-bond**: Asn87, Phe89, Glu113, Arg84, Gln83, Asp86, Ser40	**APN-receptor binding**	No
				ALP-receptor binding	Yes
				PirA^{vp} homodimer formation	Yes
				PirA^{vp}/PirB^{vp} heterotetramer formation	**Yes** 15-42 52-79

These results suggested that seven NOF compounds could bind Po-1 PirA^{vp} ligand-binding pocket amino acid residues and subsequently prevent the interaction of *V. parahaemolyticus* PirA^{vp} toxin with the APN and ALP receptor active sites and attenuate the loss of enzymatic activity and cytotoxicity of target cells. In addition, all of these NOF molecules interacted with different key amino acid residues responsible for PirA^{vp}/PirA^{vp} homodimer formation (in Table 1, these residues are red underlighted), possibly resulting in homodimer formation or disturbed existing PirB^{vp}/PirB^{vp} homodimers in infected cells. Moreover, all the NOF compounds were able to disturb and prevent the PirB^{vp}/PirA^{vp} heterotetramer structure, interacting with 15-WTVEPNGGVTEVDSKHTPIIPEVGRSVD-42 and 52-TIQYQWGAPFMAGGWKVAKSHVVQRDET-79 sequences of PirA^{vp}.

Alteration of cytokine production by NOF in PBMC culture. We examined the dose-dependent effect of an essential volatile oil blend on the activation state of PBMCs, particularly cytokine production. The production of IFN ($p < 0.05$) significantly increased in cells treated with NOF at 6×10^{-3} µg/µL. In contrast, the secretion of IL-6 ($p < 0.01$) by PBMCs decreased following induction with NOF (6×10^{-2} µg/µl). We observed a prominent anti-inflammatory effect of NOF on PBMCs primed with LPS. In particular, the ability of NOF to inhibit the secretion of several pro-inflammatory cytokines was demonstrated (Figure 2). Namely, IFNg ($p < 0.01$) was downregulated by the highest concentration of NOF (6×10^{-2} µg/µl). Similarly, production of IL-1β was significantly decreased by all concentrations of NOF (6×10^{-2} µg/µl, 6×10^{-3} µg/µl, and 6×10^{-4}µg/µl). Supplementing the medium with the two highest concentrations of NOF (6×10^{-2} µg/µl, 6×10^{-3} µg/µl) reduced the levels of IL-6 ($p < 0.05$) and TNFα ($p<0.01$) compared with the

cells incubated only with LPS. The production of anti-inflammatory IL-10 ($p < 0.01$) was also significantly decreased in this group by NOF (6×10^{-2} µg/µl, 6×10^{-3} µg/µl, and 6×10^{-4} µg/µl).

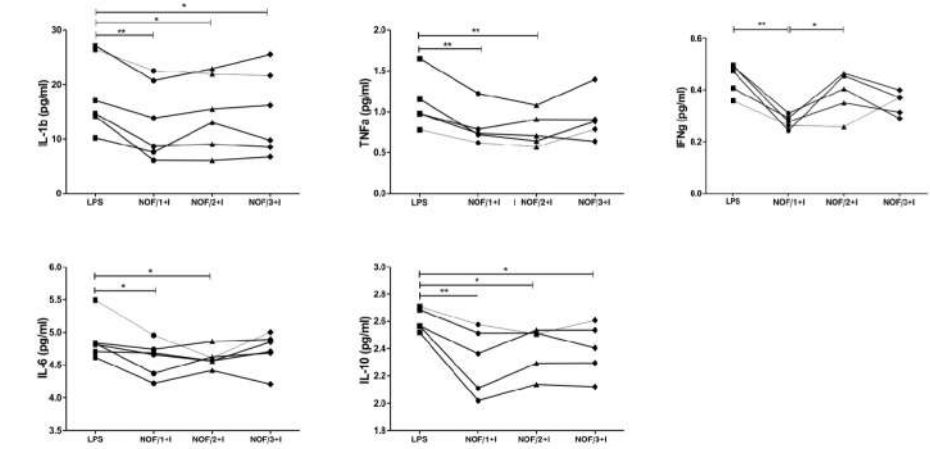


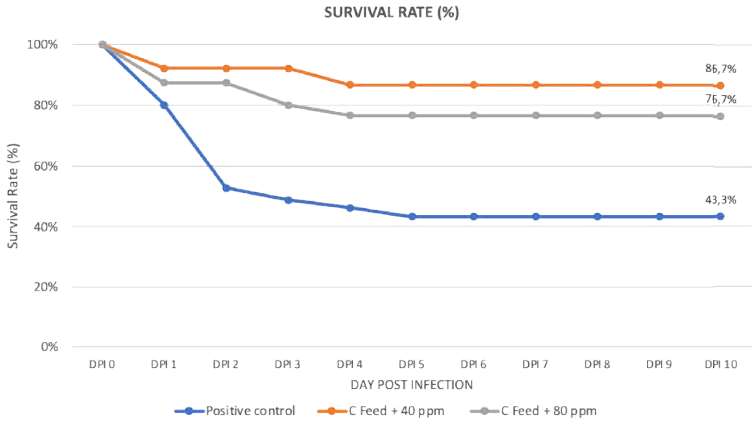
Figure 2. Cytokines production by PBMCs, isolated from 6 healthy donors, stimulated with LPS (100 ng/ml) and LPS + NOF (concentrations: NOF/1 (6×10^{-2} µg/µl), NOF/2 (6×10^{-3} µg/µl), and NOF/3 (6×10^{-4} µg/µl) for 24 h. *p<0.05, **p<0.01, ***p<0.001.

Screening for antimicrobial activity. The degree of inhibition was determined by the values of the NOF IZ diameter). The NOF showed moderate (+) inhibitory activity against four of the six tested bacterial strains. The inhibition has been recorded against gram-positive *Micrococcus luteus* (IZ, 10.13 ± 0.8762 mm), *Staphylococcus aureus* (IZ, 10.50 ± 1.756 mm), and *Staphylococcus epidermidis* (IZ, 8.3 ± 0.7 mm) and gram-negative *Pseudomonas aeruginosa* (IZ, 12.59 ± 0.4185 mm). Even at a 1:10 dilution, the oil continued to show an antibacterial effect against *Pseudomonas aeruginosa* (IZ, 8.37 ± 0.29 mm). The antibacterial potency of the NOF blend against a panel of pathogenic microorganisms was evaluated by measuring the MIC. The results are presented in Table 2. Natural herbal formulations exhibit antibacterial and antifungal activities. In particular, *Candida albicans* strains exhibited a higher sensitivity to the tested formulation (from 0.625 mg/ml to 1.25 mg/ml in terms of MIC).

Table 2: Antibacterial activity of NOF Formulation against bacterial and yeast strains expressed by MIC

Bacterial and yeasts strains	NOF (mg/ml)	Tetraciclyn (µg/ml)	Fluconazole (µg/ml)
Staphylococcus aureus ATCC-6538	2.5	0.006	ND
Staphylococcus epidermidis ATCC-12228	2.5	6.25	ND
Escherichia coli ATCC-8739	2.5	24.8	ND
Salmonella entericaserovar Typhimurium ATCC-14028	2.5	23.5	ND
Pseudomonas aeruginosa ATCC-9027	2.5	13.3	ND
Micrococcus luteus ATCC-10240	2.5	29.4	ND
Candida albicans ATCC-10231	1.25	ND	250
Candida albicans NCTC-885-653	0.625	ND	500

Efficacy of NOF in Improving Clinical Performance of Penaeus vannamei White Leg Shrimp AHPND or Early Mortality Syndrome (EMS) Bacterial Challenge Model. Challenged shrimp displayed clinical signs (such as anorexia, lethargic swimming, pale coloration of the body, and HP) at 9 h post-challenge. Typical gross signs of AHPND (gut with discontinuous content, an empty gut, or pale colored HP) were seen 21 h post-challenge. The gross signs started recovering after day 4-5 of challenge in the groups. Mortalities were started at 9 h post-challenge and seen clearly at 21-24 h post-challenge. The average cumulative mortality rate is presented in Figure 3. The highest survival rate was 86.7% in treatment group 1 (regular feed + 40 ppm NOF+EMS). Relative percent survival (RPS) is based on cumulative mortality (CM) and the best RPS recorded value was 86.7% for group 4 (regular feed + 40 ppm NOF +EMS), followed by group 2 (regular Feed + 80 ppm NOF +EMS) (76.7%). A comparison of the total Vibrio counts from samples were taken before the challenge, 3 h post-challenge, 6 h post-challenge, and 24 h post-challenge. All the challenged tanks showed the presence of Vibrio bacteria in the water after the challenge. The pick count was recorded at 6 h, decreasing at 24 h interval. HP and stomach samples were collected from moribund shrimp for PCR (*Vibrio parahaemolyticus* causing AHPND) were taken on day 2 after the challenge (48 h after the challenge). All treatment groups samples were PCR positive for EMS. The 40 ppm dose demonstrated a better relative percent survival value than the other doses. There was a 100% reduction in green Vibrio within 24 h of the challenge with NOF. The developed NOF shows potential as an anti-AHPND/EMS product based on a lab-scale trial, with laboratory results indicating that in a controlled environment, NOF containing natural oil extracts can prevent AHPND.



ANOVA (40 ppm compared to positive control)

Cum

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6670.375	1	6670.375	1410.934	.000
Within Groups	56.732	12	4.728		
Total	6727.106	13			

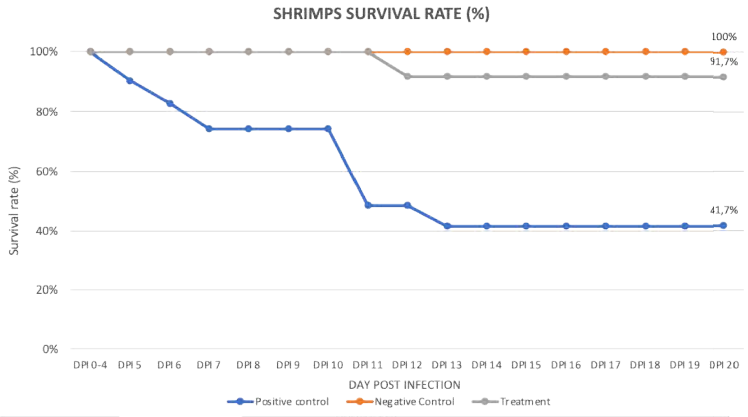
ANOVA (80 ppm compared to positive control

Cum

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3988.069	1	3988.069	647.838	.000
Within Groups	73.872	12	6.156		
Total	4061.940	13			

Figure 3. Survival rate of shrimp fed with regular Feed+NOF. Formula = 1- (% mortality in Treatment Group/% mortality in Positive Control) ×100.

Effectiveness of NOF against Infectious Myonecrosis Virus in White Leg Shrimp (IMNV) Penaeus vannamei. The NOF, which was directly poured into the tank water, supplemented the protection level; this was done to reduce the gross sign during the stressed condition, especially when the feed consumption dropped. NOF was applied daily to maintain optimum protection levels. The NOF was applied to the experimental shrimp in the form of feed and as a liquid supplement for the first 14 days to develop a sufficient level of protection The gross sign started to appear on dpi 4 in the positive control group and reached 83.3% on dpi 14. In the treatment group, the gross sign occurred on dpi 12, and the highest gross sign recorded was 16.7%. The HP analysis and PCR test results showed that the positive control shrimp grade ranged from severe to light positive to IMNV, whereas the surviving shrimp in the experimental group were light positive, while negative control groups were negative for IMNV. This indicates that the blend oil mixed feed could reduce IMNV multiplication and thus the viral load in the shrimp body. A higher rate of cumulative mortality was recorded in the positive control group than in the experimental group. The first mortality was recorded on day 11 of the challenge in the experimental feed groups, showing the antiviral activities of the product compared to the positive control group, where mortality started on day 4 of the challenge. This shows that viral multiplication slowed down in the presence of NOF (Figure 4) and the survival rate in the treatment group was 91.7% compared to 41.7% in the control. It took a longer time for the IMNV to reach virulence levels in the treatment group compared to the control. The appearance of white muscles in IMNV-infected shrimp is a typical sign. White muscle appearance occurs due to the death of tissue in that region. Tissue regeneration occurs in non-stressed conditions, generally requiring 3-4 weeks. The chances of recovery are lower in stressed shrimp conditions. Moreover, there was a significant difference between the experimental and control groups.



ANOVA

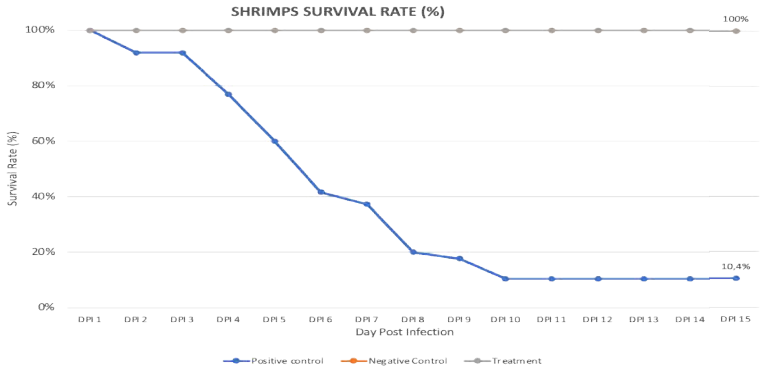
Cum

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15114.286	1	15114.286	2743.539	.000
Within Groups	66.109	12	5.509		
Total	15180.394	13			

Figure 4. Survival rate percentage of experimental and control shrimp.

The histopathological analysis of surviving shrimp from the treatment and positive control groups showed noticeable differences. The slides of the positive control group showed abnormal muscle in the positive control group, showing signs of necrosis and hemocyte infiltration, typical signs of IMNV infection. Likewise, the PCR analysis report showed that the positive control shrimp were positive for IMNV, whereas the treatment and negative control shrimp were negative for IMNV.

Study on Efficacy of NOF in Improving Clinical Performance of Penaeus vannamei White Leg Shrimp in White Spot Syndrome Virus (WSSV) Challenge Model. Cumulative mortality, gut content, shrimp activity, and mortality rate were recorded daily. The Survival rate on day 10 of the challenge is as follows *Negative control* - Initial population: 48; Cumulative Mortality: 1; Gut content full: 46; Gut content empty: 1; Molting: 1; Non active shrimps: 0. *Positive control* - Initial population: 48; Cumulative Mortality: 40; Gut content full: 0; Gut content empty: 8; Molting: 1; Non active shrimps: 8. *Treatment group (NOF)*- Initial population: 48; Cumulative Mortality: 0; Gut content full: 47; Gut content empty: 0; Molting: 1 and Non active shrimps: 0 (Figure 5).



ANOVA

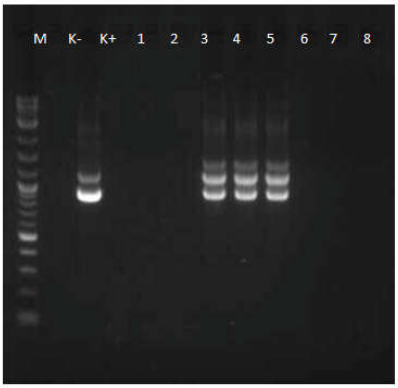
Cum

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37083.429	2	18541.714	4180.396	9.75E-25
Within Groups	79.837	18	4.435		
Total	37163.266	20			

Figure 5. Survival rate percentage of experimental and control shrimp.

The remaining shrimp from all groups were tested for the presence of WSSV by PCR. WSSV was positive in the positive control, whereas the negative control and NOF groups were negative for WSSV (Figure 6).

Impact of NOF in Productivity and Blood Parameter on Calves During Growth and Transportation Period. The effect of NOF on calves during the growth period, and the productivity of calves fed with NOF supplementation did not differ from that of the control ($P > 0.05$). However, at the end of the experiment, the final weight of calves supplemented with NOF had a bodyweight 6.85% (8.8 kg) higher than that of the control (Table.3).



Lane	Note
M	Marker
K-	Marker Negative
K+	Marker Positive
1	Negative Control - 1
2	Negative Control - 2
3	Negative Control - 3
4	Positive Control - 1
5	Positive Control - 2
6	Positive Control - 3
7	NOF 9 D -1
8	NOF 9 D -2
9	NOF 9 D -3

Figure 6. PCR results of the WSSV-infected control, negative control, and NOF group shrimp.

Table 3. Effect of NOF on calves bodyweight (kg) during transportation and recovery period.

Treatment	Period			Average
	Before transportation	After transportation	Recovery	
Control	70.08±10.07	65.52±10.31	79.62±11.23	$71.74±11.85^{x}$
NOF	73.58± 9.07	69.90± 9.40	83.25±10.47	$75.59±10.98^{y}$
Average	$71.83± 9.54^{b}$	$67.71± 9.90^{a}$	$81.44±10.77^{c}$	-

a,b,c - mean value with different superscript letters within a row differ significantly ($P < 0.05$)
x, y: mean value with different superscript letters within a column differ significantly ($P < 0.05$)

Dietary NOF yielded a higher bodyweight ($P < 0.05$) than the control group. The NOF-fed calves had increased bodyweights up to 5.6% (4 kg) compared to the control. Bodyweight after transportation was lower than before the transportation condition and after the recovery period. There was no interaction between the period and treatment ($P > 0.05$). Dietary intake of NOF did not affect bodyweight loss during transportation and gain of the recovery period ($P > 0.05$); however, the treatment reduced bodyweight loss by 1.39 kg compared to the control. This study showed that supplementation with NOF had a significant effect on uric acid and albumin content ($P < 0.05$); however, no difference was observed in calcium, phosphor, and total protein between the NOF supplementation and control. The levels of uric acid and albumin increased during NOF supplementation before transportation. Hematology evaluation indicated no effect of dietary NOF; however, the level of leucocytes increased ($P < 0.05$). After transportation, the NOF supplement decreased uric acid ($P<0.05$); however, it did not influence calcium, phosphor, total protein, and albumin levels. NOF significantly increased hematocrit, MCV, and MCH and decreased MCH. Hemoglobin, leucocytes, erythrocytes, neutrophils, and lymphocytes were not influenced by NOF supplementation ($P > 0.05$). Dietary NOF significantly affected final bodyweight ($P < 0.05$) on day 122; however, it did not affect daily gain ($P > 0.05$). Dietary NOF increased bodyweight by 7.78% (12 kg) during the feedlot period. The addition of NOF, which contains EO, optimizes the digestion process by increasing the fermentation activity in the rumen, especially with the reduction of methanogenesis and energy waste; increases the production of microbial protein; and reduces ammonia concentration. Selecting EOs in ruminant feed additives should be able to boost rumen microflora proliferation, increase the amount of propionate, and reduce the production of acetate and methane without changing the total amount of VFA. In conclusion, the addition of NOF (2 kg/ton feed) increased the health status and

reduced bodyweight loss during transportation. Thus, NOF improved the economic value after the 122-day feedlot program.

Efficacy of NOF and NOF Liquid on Productivity of Broiler Chicken as Antibiotic Replacement Strategy. The effect of NOF and NOF liquid addition on chicken productivity can be seen through several indicators, such as body weight gain, final bodyweight, mortality, and FCR. Data from the results of testing the efficacy of NOF and NOF liquid on broiler chicken productivity are presented in Table 4.

Chicken feed consumption showed no significant difference between the chicken groups administered NOF or NOF liquid and the control group. Chicken feed consumption given by NOF had a value of 70.55 g/bird, given NOF liquid with a value of 71.84 g/bird, while the control chicken group had a feed consumption value of 68.80 g/bird. Feed consumption in the two treatment groups tended to be higher than that in the control group. This is indicated by a higher final weight compared to that of the control group. Data on broiler chicken water consumption showed that broilers with NOF in the feed had higher drinking water consumption than control chickens and chickens fed NOF liquid.

Table 4. Effects of NOF and NOF liquid on the productivity of broiler chickens.

	Group		
	Control	**NOF**	**NOF Liquid**
Feed consumption (g/chicken/day)	68.80 ± 6.00^{a}	70.55 ± 3.33^{a}	71.84 ± 3.23^{a}
Water consumption (mL/chicken/day)	190.01 ± 16.72^{b}	224.27 ± 10.80^{a}	186.32 ± 8.26^{b}
Body weight gain (g)	1457.00 ± 8.15^{b}	1555.50 ± 3.25^{a}	1570.00 ± 3.15^{a}
Final weigh gain (g)	1496.20 ± 7.95^{b}	1594.70 ± 3.17^{a}	1609.10 ± 3.07^{a}
Mortality (%)	10.00	5.63	8.75
FCR	1.65 ± 0.14^{a}	1.59 ± 0.07^{a}	1.60 ± 0.07^{a}

Note: different superscripts on the same line show significantly different results ($p < 0.05$)
Note: Different superscripts on the same line show significantly different results ($p < 0.05$)

The water consumption of the NOF group chicken was 224.27 mL/bird, 186.32 mL/bird for the NOF liquid group, and 190.01 mL/bird for the control group. These results indicate a phenomenon in which chickens that receive the liquid NOF treatment have a lower drinking water consumption; moreover, the feed consumption tends to be higher than the control. Conversely, chickens given NOF treatment in feed consumed more drinking, and feed consumption tended to be higher than the control. The final weight of broilers harvested after 4-5 weeks is in the range of 1.2-1.9 kg/bird; the body weight gain of broiler chickens reached 48.65 g/bird/day. These results indicate that the final bodyweight of chickens administered NOF and NOF liquid preparations significantly differed ($p < 0.05$) from that of the control group, where the results of the two treatment groups were better than those of the control group. The average final bodyweight in the NOF group was 1594.70 g/bird and 1609.10 g/bird for the NOF liquid group, while the average final bodyweight in the control group was 1496.20 g/bird. NOF liquid through drinking water increased body weight gain by 6.76% and 7.75% compared to control chickens. In addition, the administration of NOF in feed and NOF liquid through drinking water also increased the final weight of chickens by 6.58% and 7.54%, respectively, compared to control chickens. Measurement results showed that adding NOF in feed and NOF liquid through drinking water can increase the bodyweight growth and final bodyweight of broiler chickens. In addition to body weight gain and final bodyweight, efficiency in this study is indicated by the FCR. The FCR value is a comparison between feed consumption and body weight gain over a certain period. The high value of feed conversion indicates that more feed is needed to increase

the bodyweight per unit weight. The administration of NOF and NOF liquid in the chickens showed lower FCR values of 3.63% and 3.03% compared to controls. The FCR values of the NOF and NOF liquid treatment groups were 1.59 and 1.60, respectively, while the FCR value in the control group was 1.65. The administration of NOF and NOF liquid in broiler chickens resulted in mortality rates of 5.63% and 8.75%, respectively, while mortality in the control group was 10.00%. Chicken mortality also decreased in the chicken groups given NOF and NOF liquid by 43.70% and 12.50%, respectively, compared to the controls (Table 4). Meanwhile, at weeks 3 and 4, chickens with NOF in feed and NOF liquid administration through drinking water had higher body weight gains than the control group. At week 5, the growth of the experimental chickens in all treatment groups had the same value. The average body weight gains in the treatment group given NOF preparations every week were 124.19, 286.71, 404.01, 369.99, and 370.65 g/bird. The average body weight gains in the treatment group given NOF liquid preparations each week were 126.80, 292.94, 387.96, 393.55, and 368.66 g/bird. The control group only experienced successive body weight gains every week; 132.53, 272.23, 363.53, 353.97, and 334.74 g/bird.

Through the consumption of feed and water when associated with the phenomenon of final chicken weight, the NOF and NOF liquid group chickens had a greater final bodyweight compared to the control group chickens. Feed and water consumption that is not significantly different can produce a better final weight values than the control group. This shows that the adding NOF and NOF liquid can improve the efficiency of nutrient absorption of feed, resulting in a better final bodyweights. Proximate analyses were conducted to determine the water content, crude protein, crude fat, BETN, and ash in broiler chicken meat on the chest, thighs, and wings. Water content in meat has a pattern that is inversely proportional to the fat content in meat. This means that meat with high water content has a low fat content. For water content, the crude protein, crude fat, and BETN in meat (chest, thigh, and wings) for all treatments had relatively similar values. Breast meat ash in the chicken group given liquid NOF was lower than that in the control group. Likewise, the ash content of experimental chicken thighs given NOF and NOF liquids was lower than that of the controls. In contrast, the ash content of the experimental chicken wings in all treatments was the same. Ash contents in the breast for the control, NOF, and NOF liquid were 1.57%, 1.37%, and 1.29%, respectively, while the thighs had ash contents of 1.23%, 1.10%, and 1.12%, respectively. The NOF and NOF liquid preparations resulted in decreased ash content in broiler chicken meat. A decrease in ash content in meat resulted in an increase in the organic matter content.

CONCLUSIONS

1. NOF compounds, including anethole, anisylacetone, borneol, phenylethyl alcohol, polypropylene glycol, and tripropylene glycol could be potent suppressors of four major potential pathogenic mechanisms of *V. parahaemolyti*cus-induced AHPND in *Penaeus vannamei* shrimp, including $PirA^{vp}$ toxin interaction with *Penaeus vannamei* shrimp APN- and ALP-receptors, $PirA^{vp}$ toxin homodimer, and $PirA^{vp}/PirB^{vp}$ heterotetramer formation.
2. The NOF mixture showed moderate antimicrobial effects against six bacterial strains (*Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Salmonella enterica serovar Typhimurium, Pseudomonas aeruginosa,* and *Micrococcus luteus*) and two fungal *Candida albicans* strains in vitro. We observed a prominent suppression of proinflammatory cytokine production by LPS-primed PBMCs in the presence of NOF. Therefore, our results suggest that NOF may serve as a promising pharmaceutical agent with combined anti-inflammatory, antimicrobial, and antifungal action.
3. A bioassay trial was conducted to determine the efficacy of the developed product, NOF, as an anti-AHPND. The obtained results demonstrated that the survival rate was 43.3% in the

positive control group, 76.7% in the 80-ppm NOF group, and 86.7% in the 40-ppm NOFgroup at dpi 8; no mortality was recorded in the negative control. The trial results showed that the developed NOF had a significant effect against AHPND-Vibrio *parahaemolyticus* under controlled conditions.

4. Provision of NOF 2-kg/ton feed and NOF liquid 0.5-mL/L drinking water in broiler chickens was effective in increasing the productivity and health of broiler chickens, characterized by a higher increase in final weight and lower mortality compared to control group chickens. NOF 2-kg/ton feed and NOF liquid 0.5-mL/L drinking water were proven safe (non-toxic to chickens) based on liver function (SGPT and SGOT), kidney function (urea and creatinine), and PA and HP descriptions from the liver and kidney.
5. The trial results on the IMNV of *Penaeus vannamei* shrimp showed that the developed blended oil extract formulation had a significant effect against IMNV under controlled conditions. The developed product is user-friendly as it is mixed and incorporated into the shrimp feed in specific amounts to have antiviral properties.
6. The study aimed to evaluate the impact of NOF in productivity and blood parameters on calves during their growth and transportation period, demonstrating that the addition of NOF (2-kg/ton feed) increased the health status and tended to reduce bodyweight loss during transportation. The NOF improved the economic value after the 122-day feedlot program.
7. The NOF efficacy results of the clinical performance of white leg shrimp (*Penaeus vannamei*) in the WSSV challenge model showed that the developed blended oil extract formulation had a significant effect against WSSV in controlled conditions. The developed product was incorporated into shrimp feed in specific amounts to obtain antiviral properties.

LIST OF PUBLICATIONS

Journal Articles

1. Babikian HY, Jha RK, Haliman RW, Halalludin B, Srisombat S, Davtyan T, Babikyan Y. Essential oil blend as a safe and effective disinfectant strategy for shrimp hatcheries. // Int. J. Fish. Aquat. Stud. 2021; 9(2):112-118.
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ԲԱԲԻԿՅԱՆ ՀԱՅԿ ՀՈՎՍԵՓԻ

ԲՈՒՍԱԿԱՆ ՀԱՄԱԿՑՎԱԾ ՑՆԴՈՂ ԵԹԵՐԱՅՈՒՂԵՐԻ ՀԱԿԱԲԱԿՏԵՐԱՅԻՆ, ՀԱԿԱՎԻՐՈՒՍԱՅԻՆ ԵՎ ԻՄՈՒՆՈՄՈԴՈՒԼՅԱՏՈՐ ՀԱՏԿՈՒԹՅՈՒՆՆԵՐԻ ՈՒՍՈՒՄՆԱՍԻՐՈՒԹՅՈՒՆԸ *IN VIVO* ԵՎ *IN VITRO*

ԱՄՓՈՓԱԳԻՐ

Հանգուցային բառեր՝ բուսական ծագման ցնդող եթերայուղեր, *Penaeus vannamei* ծովախեցգետնի սուր հեպատոնեկրոզի կամ վաղ մահացության համախտանիշ, IMNV և WSSV վիրուսներ:

Ներկայացված ատենախոսական աշխատանքը անդրադարձ է այնպիսի արդիական բժշկակենսաբանական խնդրի, ինչպիսին է բուսական ծագման կենսաբանական ակտիվ նյութերի, տվյալ դեպքում՝ ցնդող եթերայուղերի հակաբակտերային, հակավիրուսային և իմունոմոդուլյատոր հատկությունների ուսումնասիրությունը: Ժամանակակից հակաբիոտիկաթերապիան ունի ցածր էֆեկտիվություն և շատ սահմանափակված է՝ կապված հակաբիոտիկների նկատմամբ կայուն միկրոօրգանիզմների շտամերի տարածվածությամբ: Միկրոբների կողմից կայունության մեխանիզմները հեշտությամբ են ձեռք բերվում, ինչպես *de novo* մուտացիաների առաջացման, այնպես էլ շրջակա միջավայրի այլ միկրոբներից գեների հորիզոնական տեղափոխման արդյունքում: Կիրառական բժշկության համար նոր, բարձր էֆեկտիվությամբ, հակամանրէային, հակավիրուսային և իմունոմոդուլյատոր հատկություններով օժտված դեղամիջոցների մշակումը ու կիրառումը, որոնք ընդունակ են ճնշել պաթոգենության գործոնների էքսպրեսիայի պրոցեսները, ինչպես նաև դեղամիջոցների նկատմամբ կայունության առաջացումը հանդիսանում են ժամանակակից մանրէաբանության և մոլեկուլաբջջային կենսաբանության արդիական խնդիրներ: Կապված վերոհիշյալի հետ՝ մեծ հետաքրքրություն է ներկայացնում պոտենցիալ հակամիկրոբային ազդեցությամբ օժտված բուսական համակցված ցնդող եթերայուղերի խառնուրդի (NOF, Lavisul, Pinus, Jasminum, Cytrus, Prunus, Viola, Gardenia, Cocos - Rosa, Eucalyptus) հակաբակտերային, հակավիրուսային և իմունոմոդուլյատոր հատկությունների ուսումնասիրությունը վիրուսների, պոլիռեզիստենտ բակտերիալ շտամերի վրա *in vivo* և *in vitro*: Աշխատանքում կիրառվել են դասական և ժամանակակից իմունաբանական, բջջակենսաբանական, մանրէաբանական, վիրուսաբանական, կենսաքիմիական, պաթոմորֆոլոգիական, հյուսվածքաբանական, մոլեկուլակենսաբանական, վիճակագրական և կենսաինֆորմատիկայի մեթոդներ:
Ներկայացված աշխատանքում առաջին անգամ ուսումնասիրվել է NOF խառնուրդի հակամանրէային և հակաբորբոքային ազդեցությունը մարդու ծայրամասային արյան մոնոնուկլեարների հար- և հակաբորբոքային ցիտոկինների արտադրության վրա *in vitro* պայմաններում: Գնահատվել է NOF-ի հակաբակտերիալ արդյունավետությունը *Penaeus vannamei* ծովախեցգետնի սուր հեպատոնեկրոզի կամ վաղ մահացության համախտանիշի մոդելում *in vivo*: Որոշվել է բուսական յուղերի հակավիրուսային

արդյունավետությունը IMNV վիրուսով հարուցված *Penaeus vannamei* ծովախեցգետնի միոնեկրոզի, ինչպես նաև WSSV վիրուսով պայմանավորված սպիտակ բծերի համախտանիշի մոդելներում: Գնահատվել է NOF-ի ազդեցությունը հորթերի տեղափոխման ժամանակահատվածում աճի, վերարտադրողականության և արյան պարամետրերի վրա և հավերի մոտ որպես հակաբիոտիկների օգտագործման այլընտրանքային փոխարինման ռազմավարության արդյունավետությունը: Կատարված հետազոտությունների արդյունքները ցույց են տվել, որ NOF խառնուրդը օժտված է հակամանրէային ազդեցությամբ 6 բակտերիալ (*Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Salmonella enteric serovar Typhimurium, Pseudomonas aeruginosa, Micrococcus luteus*) և 2 սնկային՝ *Candida albicans* ստանդարտ լաբորատոր շտամերի նկատմամբ: Բացահայտվել է դրա հակաբորբոքային վառ արտահայտված ակտիվությունը՝ LPS-խթանված ծայրամասային արյան մոնոնուկլեարների կողմից հարբորբոքային ցիտոկինների արտադրության ակնհայտ ճնշումով: Ապացուցվել է NOF-ի հակասեպտիկ ակտիվությունը *Penaeus vannamei* ծովախեցգետնի *Vibrio parahaemolyticus* բակտերիաներով պայմանավորված հեպատոպանկրեատիկ համախտանիշի զարգացման դեպքում, որտեղ կենդանիների ապրելիությունը փորձնական խմբում կազմել է 64,7-76.5%: Ծովախեցգետինների IMNV վիրուսային միոնեկրոզի ուսումնասիրության արդյունքները ցույց են տվել, որ պրեպարատը ցուցաբերում է հավաստի *in vivo* հակավիրուսային ակտիվություն: Կերերի մեջ ներառված NOF-ի բարձր արտահայտված հակավիրուսային արդյունավետությունն ապացուցվել է նաև ծովախեցգետնի WSSV վիրուսով հարուցված սպիտակ բծերի համախտանիշի կլինիկական ընթացքի, կենդանիների ապրելիության և վիրուսի ռեպլիկացիայի ցուցանիշների հավաստի բարելավմամբ: Չափազանց մեծ հետաքրքրություն են առաջացնում NOF-ի՝ որպես հակաբիոտիկներ օգտագործման այլընտրանքային փոխարինման ռազմավարության արդյունավետության մեծածավալ ուսումնասիրության արդյունքները, որտեղ հստակ ցույց է տրվել հորթերի և հավերի ապրելիության, աճի, վերարտադրողականության և այլ ցուցանիշների վրա պատրաստուկի խթանող ազդեցությունը:

БАБИКЯН АЙК ОВСЕПОВИЧ

ИЗУЧЕНИЕ АНТИБАКТЕРИАЛЬНЫХ, ПРОТИВОВИРУСНЫХ И ИММУНОДУЛЯТОРНЫХ СВОЙСТВ РАСТИТЕЛЬНЫХ ЛЕТУЧИХ ЭФИРНЫХ МАСЕЛ *IN VIVO* И *IN VITRO*

РЕЗЮМЕ:

Ключевые слова: летучие эфирные масла растительного происхождения, острый гепатонекроз или синдром преждевременной смерти креветок *Penaeus vannamei*, вирусы IMNV и WSSV.

Представленная диссертация является обращением к такой актуальной медико-биологической проблеме, как изучение антибактериальных, противовирусных и

иммуномодулирующих свойств биологически активных веществ растительного происхождения, в данном случае летучих эфирных масел. Современная антибактериальная терапия имеет низкую эффективность и весьма ограничена из-за распространенности устойчивых к антибиотикам штаммов микроорганизмов. Механизмы резистентности микробов включают как генерацию *de novo* мутаций, так и горизонтальный перенос генов от других микробов. Разработка и применение для практической медицины новых высокоэффективных противомикробных, противовирусных и иммуномодулирующих препаратов, способных подавлять экспрессию факторов патогенности, а также развитие лекарственной устойчивости, являются актуальной проблемой современной биологии и микробиологии. В связи с изложенным большой интерес представляет изучение антибактериальных, противовирусных и иммуномодулирующих свойств смеси эфирных масел растений (NOF - Lavisul, Pinus, Jasminum, Cytrus, Prunus, Viola, Gardenia, Cocos - Rosa, Eucalyptus) с потенциальным антимикробным действием на вирусы, полирезистентные штаммы бактерий *in vivo* и *in vitro*. В работе использованы классические иммунологические, клеточно-биологические, бактериологические, вирусологические, биохимические, патоморфологические, гистологические, молекулярно-биологические, статистические и биоинформатические методы.

В настоящей работе впервые изучено антибактериальное и противовоспалительное действие NOF на провоспалительные цитокины, продуцируемые мононуклеарными клетками периферической крови человека. Оценивалась антибактериальная эффективность NOF на модели острого гепатонекроза или синдрома ранней смерти у креветок *Penaeus vannamei in vivo*. Определена противовирусная эффективность растительных масел при индуцированном вирусом IMNV микозе креветок *Penaeus vannamei*, а также при синдроме белых пятен вируса WSSV. Оценивалось влияние NOF на рост, репродукцию и показатели крови у цыплят в качестве альтернативной стратегии замены антибиотиков у телят. Результаты исследований показали, что NOF обладает антимикробной активностью в отношении 6 бактериальных (*Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Salmonella enteric serovar Typhimurium, Pseudomonas aeruginosa, Micrococcus luteanus*) и 2 грибковых – *Candida albicans* стандартных лабораторных штаммов. Его выраженная противовоспалительная активность проявляется в подавлении продукции провоспалительных цитокинов ЛПС-стимулированными мононуклеарными клетками периферической крови. Антисептическая активность NOF была продемонстрирована при развитии гепатопанкреатического синдрома, вызванного бактерией *Vibrio parahaemolyticus* креветок *Penaeus vannamei*, где выживаемость животных опытной группы составила 64,7-76,5%. Результаты исследования вирусного мионекроза IMNV у креветок показали, что препарат показал достоверную противовирусную активность *in vivo*. Высокая противовирусная эффективность NOF в кормах была продемонстрирована достоверным улучшением клинического течения синдрома белых пятен, вызванного WSSV креветок, выживаемости животных и скорости репликации вируса. Большой интерес представляют результаты широкомасштабных исследований эффективности NOF как альтернативной стратегии замещения применения антибиотиков, которые наглядно показывают стимулирующее действие препарата на выживаемость, рост, воспроизводство и другие показатели у телят и кур.