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Effect of *Vitex agnus-castus L.* Extract in Carp Fish Infected with *Vibrio anguillarum*

Enas A. Khoris[1*], Eman M.El. El-Sherbeny[2], Aml M. Ragab[3]

[1]*Fish disease Department, Animal Health Research Institute (AHRI), Tanta provincial lab., Agricultural Research Center (ARC), Egypt.*
[2]*Pharmacology unit, Animal Health Research Institute (AHRI), Tanta provincial lab., Agricultural Research Center (ARC), Egypt.*
[3]*Bacteriology Department, Animal Health Research Institute (AHRI), Tanta provincial lab., Agricultural Research Center (ARC), Egypt.*

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***Correspondence:**

Corresponding author: Enas A. Khoris
E-mail address: enas_khoris@yahoo.com

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ABSTRACT

This work studied in vitro; the antibacterial activity of *Vitex agnus-castus* (VAC) extract against *V. anguillarum*, its minimal inhibitory and bactericidal concentrations (MIC and MBC) and detected its bioconstituents using gas chromatography-mass spectrometry (GC-MS) analysis besides evaluated its effect in vivo in carp either alone or combined with antibiotic to prevent or treat *V. anguillarum* infection. A total of 180 fish were divided into 6 groups; G1: negative-control, G2: *V. anguillarum* infected-control, G3: infected-(marbofloxacin (MAR) 10 mg/kg body weight), G4: infected-(VAC 1g/kg ration), G5: infected-(VAC 1 g/kg + MAR 10 mg/kg) all treatments lasted 7 days and G6: prophylactic treated-(VAC 1 g/kg/30 day) then challenged. Results of scanning electron microscope revealed changes in bacterial morphology and loss of flagella by VAC where its MIC and MBC were 1.95 and 15.63 mg/ml respectively. Growth performance and survival rate improved in all treated groups in this descending order (G6, G4 then G5). All treatments significantly increased total protein, albumin, globulin, superoxide dismutase and decreased aspartate and alanine aminotransferases activities, malondialdehyde, urea and creatinine than G2 and mostly near to G1 in this ascending order (G3, G4 then G5). In G6, VAC protected carp from the infection and improved growth, survivability and most of blood parameters. MAR residue in fish musculature needed 4 days as a withdrawal period to be less than the maximum residue limits (MRL), while combination of VAC with MAR decreased it less than the MRL from the 1st day post treatment. Finally, VAC had a considerable antibacterial activity against *V. anguillarum* in both prevention and treatment assays. It can be used either alone or adding with MAR.

Introduction

Common carp (*Cyprinus carpio L.*) belongs to *Cyprinidae*, is undoubtedly considers one of the most cultured fish in the world and represents about 10% of global annual fresh water aquaculture production (Bostock *et al*., 2010). Fish farming industry is considered one of the fastest growing and important sources of food production worldwide. Meanwhile, the intensive culture affected fish quality and safety and led to outbreaks of several bacterial diseases and severs economic losses to the aquaculture industry (Guidi *et al*., 2018). Vibriosis, one of these major prevailing bacterial diseases, causes high mortalities in fish and shellfish cultures worldwide. In Egypt it has economic and public health concerns among cultured fish (Abdelgayed *et al*., 2021). Its general signs include external skin lesions, hemorrhages, and septicemia; began with the crucial initial attachment of the bacterium to the host tissue by various virulence factors, followed by proliferation and invasion into the internal organ systems through blood circulation (Manchanayake *et al*., 2023). *Vibrio anguillarum*, Gram-negative bacterium, is an important member of pathogenic *Vibrio* spp. in fish aquaculture. It cangrow and proliferate efficiently under environmental stress conditions (Gao *et al*., 2018). Furthermore, bacterial diseases are one of the major issues at present owing to the ability of several bacterial strains to develop resistance to antibiotics through genetic mutations. In aquaculture industry, prevention of diseases is a major concern in order to abandon growth reduction and mortalities. Although there is different chemical-based therapeutics for controlling fish bacterial infections, many of them have been restricted in use due to their side-effects, creating drug-resistant bacteria, reduction of their efficacy against multidrug resistant bacteria, flesh residual, immunosuppression effect and environmental pollution (Guardiola *et al*., 2016).

As a fact, developing alternative antibacterial drugs to control or prevent bacterial outbreaks is an urgent need (Van Doan *et al*., 2019). Medicinal plants possess many therapeutic and antimicrobial properties with minimum side effects; where using their extracts is considered an effective and new approach in the fish rearing industry to overcome the bacterial pathogens as a return in the use of natural alternatives rather than chemical agents(Ahmadifar *et al*., 2019). Fish pharmacology is now changing quickly as aquaculture continues to expand; there is a necessitate for greater knowledge about the bioactive compounds of the medicinal plant treatments, assessment of their efficacy for prevention and treatment of diseases and subsequently their economic impact on fish (El-Sayed *et al*., 2019). Also, plant medicines could be used either as decoctions (individual) or concoctions (mixed) or in combination with other chemical drugs for effective management of health (Harikrishnan, 2003).

Vitex agnus-castus (VAC), genus Vitex (*Lamiaceae*), is one of the most important medicinal plants. It grows in different parts of the world, ranging from the Mediterranean regions, including Egypt, to Central Asia and Southern Europe (Kosovac *et al*., 2016). All parts of this plant have a different kind of active composites, where its essential oils and organic extracts revealed many promising properties including antibacterial, antioxidant, anticancer, and anticorrosion (Nyiligira *et al*., 2008; Asdadi *et al*., 2014), besides its effect on fish growth performance and survival rate of *Danio rerio* (Gholampour *et al*., 2020) and *Carassius auratus* (Rashmeei *et al*., 2022). Immunity and resistance against *A. hydrophila* infection in *Carassius auratus* were also examined (Rashmeei *et al*., 2020). The bacteriological activity of various VAC extracts were tested against different types of bacteria; *E. coli, B. pumilus, Pseudomonas sp., Enterobacter,* and *Streptococcus sp.*, where they showed various differences in their activities (Chiad *et al*., 2015). As well, VAC has anti-inflammatory, nutritious, diuretic, appetizing, carminative, relaxing and anti-flatulence properties (Gholampour *et al*., 2020). Its reproductive performance on *Carassius auratus* (Sahafi *et al*., 2018) and Xiphophorus helleri (Zamani

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et al., 2018) were also reported.

Fluoroquinolones (FQs) are synthetic antibiotics where fluorine atom enhanced their activities and broaden spectrum than quinolons. Hence, they effected on a variety of human and veterinary bacterial diseases and indicated in the treatment of local and systemic infections caused by a wide range of Gram negative and positive bacteria. They have low toxicity, excellent pharmacodynamic and pharmacokinetic characteristics, rapid bactericidal activity, lipophilicity, and relatively low minimal inhibitory concentrations (MIC) against target pathogens with a concentration-dependent effect (Poapolathep *et al*., 2020). Marbofloxacin (MAR) is a second generation of FQs developed for veterinary use only. It is approved by FDA for treatment of respiratory, urinary, skin, gastrointestinal and soft tissue infections. MAR as inhibitor of bacterial DNA gyrase, hindered its fitting; supercoiling, packing, replication and transcription followed by rapid bacterial cell death. Its characteristic oxadiazine ring broadens its bactericidal activity, reveals a significant postantibiotic effect, be active in both stationary and growth phases of bacterial replication and provides some pharmacokinetic advantages. MAR oral bioavailability approached 100% in several species with good systemic distribution and tissue penetration, longer elimination half-life (t1/2), a wide safety margin and few adverse effects (Plumb, 2015). It weakly bound to plasma proteins ranged from (<10% to around 30%). It had large volume of distribution in various physiological states and excreted mostly in the urine. In all species, non-metabolized MAR was the main component of the residues in tissues and excreta. The extent of biotransformation was very limited less than 5% of the administered dose with nonsignificant species differences in metabolism (EMEA, 2000; Veterinary Medicine Directorate, 2022).

Consistent with instructions of the World Health Organization, it is necessitate reducing the overuse and inappropriate use of antimicrobials. Antibiotics are permitted for use in food producing animal based on the recommended Maximum Residue Level (MRL) set by joint Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO), Codex Alimentarius Commission, and European Union legislation (FAO and WHO, 2020). Therefore, antibiotics withholding period must be observed until their residues are negligible or no longer be detected to assure the safe consumption of treated fish. Presence of these low amounts may lead to allergy, disruption of intestinal microbiota, emergence of drug-resistant bacteria, failure of human therapy in clinical situations, and chronic toxic effects with prolonged administration (Abdel Aziz *et al*., 2017). However, a few studies were conducted on the residue elimination of MAR in fish carp (Zhu *et al*., 2009), it is crucial from a health safety to monitor the presence of antibiotics in edible tissue. For instance, Yipel *et al*. (2017) found that 33.3% of detected fish species samples contained at least one antibiotic residue and 20% samples contained more than one antibiotic residue. Microbial resistance to FQs develops slowly via mutations in the bacterial chromosomal genes encoding DNA gyrase or topoisomerase IV, active transport of the drug out of the bacteria or plasmid resistance. Post emergence of many FQ-resistant strains, they show the same resistance mechanisms between animal and human and maintained bacteria resistance in populations (Farca *et al*., 2007). Hence, antimicrobial therapy of some diseases will be complicated besides increase therapeutic costs.

According to literatures, limited studies investigated the antibacterial activity of VAC ethanolic extract and its efficacy to prevent or treat *V. anguillarum* infection in carp fish; either alone or combined with antibiotic to study their interaction. Hence, this study aimed to investigate in vitro; the sensitivity test of antibiotics and VAC ethanolic extract and their minimal inhibitory and bactericidal concentrations (MIC and MBC), use scanning electron microscope (SEM) to observe changes in the microstructure of bacteria and assure the antibacterial mechanism of VAC against *V. anguillarum*, besides detected the bioactive compounds of VAC extract using the gas chromatography mass spectrometry (GC-MS) device to provide insight into its potential constituents. Moreover, this study advanced knowledge about the in vivo effects of VAC and MAR either alone or combined on growth performance, survival rate, *V. anguillarum* count, non-specific immunity, antioxidants assay and some liver and kidney health indicators. This study also used high performance liquid chromatography (HPLC) to estimate MAR residue and its withdrawal time in carp musculature with and without the extract to detect VAC effect on it and avoid antibiotic residue problems for consumers.

Materials and methods

Plant materials and preparation of VAC ethanolic extract

The plant was identified according to Brickell and Zuk (1997). After complete grinding of the shade dried whole plant (fruit, leaves and stem bark), 20 grams of the milled sample were soaked in 100 ml absolute ethanol, for 48 h at room temperature in a rotator shaker. The initial suspension was separated through Whatman No.1 filter paper. The resultant extract was evaporated by vacuum pump, weighed and used for further phytochemical analysis, susceptibility test then in vivo fish experiment (Weniger, 1991; Ababutain and Alghamdi, 2021).

Detection of bioactive compounds of VAC extract using Gas Chromatography-Mass spectrometry (GC-MS)

Analysis was performed in the laboratory of the Scientific Research Center and Measurements (SRCM), Tanta University, Egypt. Sample was extracted using GC program acquisition parameters; instrument (Perkin Elmer model; clarus 560S). Column oven initial heating rate was 50°C/4 min, ramp1; 10°C/min to 150°C, hold 5 min, ramp2; 10°C/min to 280°C, hold 1 min, Volume= 1 µL, Inj= 280°C, Split ratio= 20:1, Delay of solvent= 3 min, Carrier gas= Helium, Transfer temperature= 280°C, Source temperature= 200°C, Scan; 50 to 620Da, Capillary column; Elite-5MS; 30 m 0.25mmID with 0.25um df and sampling flow velocity rate: 12.50000 pts/s. Preliminary recognition of the various constituents was performed by matching up their mass-spectra to the literature (MAINLIB, Pfleger and replib). Compounds quantitative data were computed from GC peak areas (Ababutain and Alghamdi, 2021).

Bacterial isolation and identification

Sampling

A total number of 50 random samples of cultured common carp fish (*Cyprinus carpio*) with natural signs of infection were freshly collected from Kafr El-Sheikh freshwater fish farms. Fish were transferred alive in plastic tanks with air blower; then freshly dead samples were kept in ice boxes and transported as soon as possible to Animal Health Research Institute- Tanta branch and subjected to clinical, postmortem and bacteriological examination.

Bacteriological examination

Samples from the internal organs of the examined fish were carried out under complete aseptic conditions then inoculated into tryptic soya broth with 2% NaCl and incubated at 28ºC for 24 h. A loopful of incubated broth was streaked on thiosulfate citrate bile salt sucrose agar (TCBS, Oxoid) and incubated at 28ºC/24-48 h. The growing colonies were picked up in pure form and reinoculated into trypticase soy agar for further identification.

Identification

Identification of all isolates was done by Gram staining property, morphological colony and biochemical characters according to Quinn *et al*. (2002); Austin and Austin (2007) and sensitivity to vibriostatic agent

O/129 of the isolates were recorded. The bacterial isolates were characterized by a commercial API 20E system (Biomérieux).

In vitro antibacterial activity screening tests

Antibacterial sensitivity test (AST)

AST was performed using the disk diffusion method on Mueller Hilton agar according to the guidelines and recommendations of CLSI (2015). Six standard discs namely amoxiclave AMC (30µg), gentamicin GEN (10µg), tetracycline T (30µg), chloramphenicol C (30µg), sulphamethoxazole-trimethoprim (Cotrimoxazole) COT (25µg) and marbofloxacin MAR (5µg) were utilized to represent six different families of commercial antibiotics: β-lactamases inhibitors, aminoglycosides, tetracyclines, phenicoles, folates inhibitors, and fluoroquinolones. Results were inferred as susceptible, intermediate or resistant by measuring the inhibition zone diameter (IZD) in millimeters (mm) (CLSI, 2015). MAR was sensitive ≥ 18 mm; intermediate 14-18 mm or resistant ≤ 14 mm (Farca *et al*., 2007).The multiple antibiotic resistance (MAR) index was determined (Krumperman, 1983), MAR-index >0.2 indicated a high-risk exposure towards these antibiotics.

The antibacterial activity of VAC extract was assessed using well diffusion method (Mattana *et al*., 2010), where stock solutions of 5 concentrations; 125, 62.53, 31.25, 15.63 and 7.81 mg/ml distilled water placed onto *V. anguillarum* inoculated agar then incubated at 28°C/24 h. Each antimicrobial assay was applied simultaneously in triplicate and mean values were calculated.

Minimum inhibitory concentration (MIC)

The MIC assay was employed by using micro-broth dilution test in a 96-well microplate using standard procedure of Zgoda and Porter (2001) and CLSI (2015). From our AST assay, marbofloxacin (MAR) was the effective antibiotic against *V. anguillarum* so; we used it in our experimental study to compare the VAC efficacy. For MIC assay, 125 µg/ml of MAR and 125 mg/ml of VAC extract were twofold serially diluted till 0.06 µg/ml and 0.06 mg/ml respectively. Both negative and positive control wells were performed. Referring to the results of the MIC assay a loopful, from each clear well was streaked on the specific media for *V. anguillarum* incubated at 28ºC/24 h, then observed for growth. MIC was the lowest concentration that showed no turbidity and minimum bactericidal concentration (MBC) was the lowest concentration did not show any growth. The experiment was performed in duplicate.

Scanning electron microscope (SEM)

SEM was used in conjunction with a few modifications to the technique reported by Roya *et al*. (2016). SEM was used to determine the influence of the used VAC extract on the morphology of *V. anguillarum*, which was added at MIC and 2MIC concentrations to working cultures. After 5 min and1 hour at 37°C to each concentration, 1 ml sections of each tube were collected and centrifuged for 10 minutes at 4000 rpm. Fix with 2.5% buffered gluteraldehyde in 0.1 M PBS pH 7.4 at 4˚C for 2 h. Following fixation in 1% osmic acid for 10 minutes, wash three times with PBS (10 minutes between each wash) (30min). Three PBS washes (10 minutes each), followed by 30 minutes of dehydration with an ascending sequence of ethyl alcohol concentrations (30, 50, 70, 90, and absolute alcohol) infiltrated with acetone. SEM samples were dried with liquid CO_2, supplied by SPI supplies®, using a critical point drying device; SPI-Module TM Vac/Sputter, mounted on aluminum stubs and gold-coated. Scanning electron microscope JEOL JSM-5200LV (SEM, Hitachi, Tokyo, Japan) was performed at Electron Microscope Unit, Tanta University.

In vivo assessment of the therapeutic and preventive effect of VAC extract

Fish and Experimental design

Fish

This study was done with cultured freshwater common carp (*Cyprinus carpio*) fish with a healthy appearance and normal behavioral reflexes (with initial body weight about 30 g/ fish) collected and brought from a private farm at Kafr El-Sheikh governorate, Egypt. Fish were transferred alive in plastic tank with air blower and transported to Animal health research institute, Tanta Lab., and kept for acclimatization under laboratory condition for 15 days prior to the experiment. Fish were placed in glass aquaria (70 × 40 × 30 cm), with temperature (26°C) controlled water, supplied with chlorine free tap water (Innes, 1966). Continuous aeration was maintained in each aquarium through an air compressor and air stones. Fish were fed twice daily at (9:30 AM. and 4:30 PM.) at a fixed feeding ratio of 3% of fish body weight (Eurell *et al*., 1978), which is measured weekly over the course of the experiment. Fish excreta were removed by manual siphoning and water was replaced with a rate of 30% every 2 days.

Experimental design

After 15 days of fish acclimatization, the internal organs of 10 fish were randomly selected and examined bacteriologically to ensure that there is no systemic infection of *V. anguillarum*. To evaluate the therapeutic and preventive effect of VAC extract. A total of 180 fish were grouped into 6 groups (six treatments, in triplicate, with 10 fish per aquarium) were reared in the experimental aquaria.

The groups were divided as follow; G1: negative control, in which a normal fish were injected intraperitoneally with 0.1 ml of 0.85% sterile saline at the first day of the experiment after acclimatization period, and fed on a basal commercial ration (without any supplementation). G2: positive control, in which fish were injected intraperitoneally with $1x10^7$ CFU/0.1ml *V. anguillarum* at the first day of the experiment and were fed the basal ration as previously mentioned. G3: fish were infected as previously and treated with MAR antibiotic at a dose of 10 mg/kg body weight for 7days. G4: fish were infected as previously and treated with VAC (1 g/kg ration for 7days). G5: fish were infected as previously and treated with MAR (10 mg/kg) and VAC (1 g/kg) for 7days. G6: fish were firstly supplemented with VAC (1 g/kg ration) for 30 days, and then fish infected with *V. anguillarum* as previously at the 30th day of the experiment. The experimentally infected fish were inspected daily post infection and the clinical signs, mortality rate and necropsy finding recorded for 15 days after the infection in both therapeutic and prophylactic treated groups. All treatments were given after the appearance of the infection symptoms at the 5th day post-infection (PI) and lasted for 7 successive days. Re-isolation of *V. anguillarum* pathogen from the liver and kidney of the moribund experimentally infected fish was done. Fish were treated with marbofloxacin, (Marbox TM 100 mg/ml) Ceva Sante Animale- France, used at a dosage of 10 mg/kg body weight according to Zhu *et al*. (2009); Qi *et al*. (2017). While VAC extract was used at a dosage of 1 g/kg ration according to Zamani *et al*. (2018). All treatments were added to a commercial basal ration according to method of Rodgers and Furones (2009) for medicated feed, which consider the most cost-effective and commonly used method in aquaculture production.

The experiment was carried out in accordance with the guidelines set by the Egyptian Ethics Committee and the NIH of Research Ethics Committee for environmental and clinical studies at Animal Health Research Institute (AHRI) for the Care and Use of Laboratory Animals.

V. anguillarum infective dose, challenge test and survival percentage

After the period of acclimatization all fish of G2, G3, G4 and G5 were injected intraperitoneal with 0.1 ml PBS containing (1x10^7CFU) *V. anguillarum* strain. With the onset of infection symptoms, *V. anguillarum* was re-isolated to confirm that infection was due to the bacterial infection. Post treatment with MAR and VAC in G3, G4 and G5; observation of surviving fish was extended to 1 week after the end of the treatment period. Meanwhile in G6, after 30 days of VAC extract feeding; all fish of this group were injected intraperitoneal (I/P) with 0.1 ml PBS containing (1x10^7CFU) *V. anguillarum* strain, and mortalities was recorded for 7 days until mortalities had stopped and the observation of surviving fish extended up to 2 weeks. The pathogenicity was proved by challenge and the bacterium was re-isolated from the dead fish and moribund fish. The percent of survival was calculated as follow, survival %= 100 × final fish number/ initial fish number.

Growth parameters

Weight of all fish in all groups was recorded at the first and the last day of feeding trials to determine the growth performance using these formulas.

Specific growth rate (SGR)= 100 × [(Ln final fishweight) - (Ln initial fish weight)]/days fed.

Weight gain (%)= (final fish weight-initial fish weight)/initial fish weight×100.

Feed conversion ratio (FCR)= feed intake (g)/weight gain (g).

Sampling

Blood samples

Blood samples of all groups were taken from the caudal vein (5 fish/group) twice; post treatment (PT) of G3, G4 and G5 and at the end of the experiment a week post-challenge of the prophylactic treated G6. Samples were taken in clean dry centrifuge tube without anticoagulant for serum biochemical investigations included hepatic health indicators: aspartate and alanine aminotransferase (AST and ALT) enzymes activities (Reitman and Frankel, 1957), total proteins (TP) (Doumas *et al*., 1981), albumin (Reinhold, 1953). Globulin was detected by subtraction albumin value from TP and albumin/globulin ratio was detected by dividing both values. Antioxidant and lipid peroxidation status; superoxide dismutase (SOD) (Nishikimi *et al*., 1972) and malondialdehyde (MDA) (Ohkawa *et al*., 1979; Yagi, 1984) besides, urea (Batton and Crouch, 1977) and creatinine (Houot, 1985) were also estimated. All testes were determined using commercial kits (Spectrum, BioSystems and Biomed Companies, Egypt) in accordance with the manufacturer's instructions.

Liver specimens

For *V. anguillarum* count, liver specimens were taken from all treated groups (3 fish/group) post treatment period to evaluate their efficacy.

Musculature samples

For marbofloxacin residue detection, musculature samples of antibiotic treated groups with and without the extract, G3 and G5 respectively (3 fish/group) were collected at the 1st and the 4th day PT.

Marbofloxacin residue analyzed by high performance liquid chromatography (HPLC)

Analysis was performed in Animal Health Research Institute using HPLC (Agilent Technologies, 1200 Series Japan). According to Abdel Aziz *et al*. (2017), MAR extraction was carried out according to Ding *et al*. (2013); three grams of tissue homogenized and 15 ml extraction solution (0.015 mol/L perchloric acid and 0.015 mol/L phosphoric acid in water-methanol (50:50 v/v)) was adjoined. The samples were then hydrolyzed in water bath at 50°C/90 min, cooled to room temperature and centrifuged at 5000 rpm /10 min. Then 50 µl of supernatant solution was put in to autosampler vial for analysis as injection volume. During analysis (12% acetonitrile; 0.75% formic acid and 0.4% triethylamine) was used as a mobile phase, where liquid chromatographic conditions flow rate was 1mL/min., column temperature was 50°C, and UV detector was 295 nm. Residues quantification was worked out from area under curves extrapolated automatically by the software. According to ICH (2005), linearity is identified by the squared correlation coefficient (r2) = 0.99. Post using 5 replicates of MAR standard solutions; acceptance relative standard deviation (RSD) was ≤ 1%. The tissue samples of fish musculatures are spiked by adding known quantities of MAR, then they scrutinized against standard solutions of the same concentrations. The accuracy is then calculated from the test results as a percentage recovery (Senyuva *et al*., 2000). The standard calibration curve was prepared from different concentrations between 25-500 ppb using blank fish musculature.

Water samples

Water samples were taken every week to determine the water quality parameters (temperature using digital thermometer, dissolved oxygen using digital oxygen meter (HI-9142, HANNA Instruments, China), PH using PH meter (HANNA Instruments, Research Model 211 Digital pH Meter, China), and a portable digital multi-meter (Crison Model MM41, China) for measuring the levels of total ammonia, unionized ammonia (NH3), nitrite (NO_2) and nitrate (NO_3) in the water (USA, Virginia Company, lot. No. 201134). All water quality parameters considered within the acceptable ranges according to the recommended standard guidelines (APHA, 1998).

Statistical analysis

Results were analyzed using IBM SPSS 22 software package for Windows (IBM SPSS Inc., USA). Statistical analysis of data was conducted using the one-way analysis of variance (ANOVA) and Duncan's post hoc tests to evaluate significant differences among the groups at a significance level (P value < 0.05). Results are expressed as means±-standard error (SE).

Results

Detection of active compounds of VAC extract by GC-MS

The chemical constituents chromatogram of the VAC extract peaks was detected using gas chromatography joined to mass spectrometry (GC-MS) (Fig 1). In Table 1, the principal and slight components of the VAC extract identified by GC-MS according to area % of peaks at specific retention time (RT). The major compounds (Peak 24: 10.396 %) were Oleic acid / Hexadecanoic acid/ 10-Undecenoic acid, octyl ester and Pentadecanoic acid. The second most abundant compounds (Peak 26: 6.185 %) were l-(+)-Ascorbic acid 2,6-dihexadecanoate / Eicosanoic acid / 1-Hexadecanol, 2-methyl- in addition to 2-Myristynoyl pantetheine. The third major peak (Peak 37: 5.811 %) represented cis-Vaccenic acid. Where Peak 33: 5.340 %, Peak 23: 5.244 % then Peak 28: 4.590 % represented Palmitic acid, Octadecanoic acid and Erucic acid respectively. Other vital fatty acids, esters and other compounds constituted an important portion of the extract were also detected as Phenol, Myristic acid / n-Butyl ricinoleate / Triarachine / Trichloroacetic acid / à-D-Gluco pyranoside, O-à-D-glucopyranosyl/ á-D-Glucopyranose, 4-O-á-D-galactopyranosyl- and Dodecanoic acid, 3-hydroxy.

Table 1. Gas chromatography-mass spectrometry (GC-MS) analysis clarified the composition of *Vitex agnus-castus* extract.

Peak	Retention time (RT)	Compounds	Area %
1	3.19	Propane, 2,2- diethoxy-/ Ethoxycitronellal/ Silane, triethyl- /Trichloroacetic acid, decyl ester / Cyclohexane, ethyl- / Cycloheptane, methyl- / 2-Pentanone, 4-hydroxy-4-methyl-	0.57
2	5.05	2-Pentanone, 4-hydroxy-4-methyl-/ 3-Heptanol, 4-methyl-	0.48
3	7.96	9,12,15-Octadecatrienoic acid/3-Dimethylsilyloxytetradecane /1-Methoxy-3-(2-trimethylsilyloxyethyl) nonane/D-Fructose, diethyl mercaptal, pentaacetate	0.25
4	8.30	d-Mannitol, 1-O-heptyl-/ Dimethyl(1-cyclopentylethoxy) silane /Decanoic acid, 3-hydroxy-, methyl ester	0.48
5	9.51	Lactose/ D-Glucose, 4-O-à-D-glucopyranosyl-/ Galacto-heptulose/ DL-Arabinose	0.29
6	9.78	à-D-Glucopyranoside, O-à-D-glucopyranosyl-/ Desulphosinigrin/d-Glycero-d-ido-heptose	0.45
7	10.42	Oleic acid/ 9-Acetoxynonanal/ 10-Octadecenal	0.37
8	11.52	á-D-Glucopyranose, 4-O-á-D-galactopyranosyl-/ Oleic acid	0.42
9	11.57	Undecanoic acid/ Nonanoic acid/ Tetradecanoic acid/ Tridecanoic acid	0.51
10	12.49	Dodecanoic acid, 3-hydroxy-	0.26
11	13.07	Oleic acid / 9-Octadecenoic acid (Z)-, hexyl ester/ 1-Hexadecanol, 2-methyl-	0.36
12	13.28	Dodecanoic acid, 3-hydroxy-/ 2-Dodecenoic acid / trans-2-Dodecenoic acid	0.26
13	13.33	Heptadecanoic acid, heptadecyl ester / Oleic acid / 4-Octadecenal	0.46
14	13.58	Oleic acid / Hexadecane, 1,1-bis(dodecyloxy)- / Octadecanoic acid	0.36
15	13.70	2-Myristynoyl pantetheine / 7-Methyl-Z-tetradecen-1-ol acetate / 2H-Pyran, tetrahydro-2-(12-pentadecynyloxy)-	0.25
16	13.88	Oxacyclotetradecan-2-1/ 3-Trifluoroacetoxypentadecane / n-Hexadecanoic acid	0.36
17	14.03	Dodecanoic acid, 3-hydroxy- / l-Gala-l-ido-octose	0.73
18	14.41	Oleic acid / 2-Hexadecanol / 10-Undecenoic acid, octyl ester	0.27
19	14.62	Oleic acid / Z-8-Methyl-9-tetradecenoic acid / Erucic acid	1.15
20	14.83	Oleic acid / Eicosanoic acid / l-(+)-Ascorbic acid 2,6-dihexadecanoate	0.87
21	15.02	10-Undecenoic acid, octyl ester / Erucic acid /9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	0.75
22	15.61	2-Myristynoyl pantetheine	3.78
23	15.86	Oleic acid / n-Hexadecanoic acid / Octadecanoic acid / Eicosanoic acid/	5.24
24	17.34	Oleic acid / Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester / Pentadecanoic acid / 10-Undecenoic acid, octyl ester	10.40
25	17.94	Oleic acid / Octadecanoic acid / Isopropyl Palmitate	2.20
26	18.11	1-Hexadecanol, 2-methyl- / 2-Myristynoyl pantetheine /Eicosanoic acid / l-(+)-Ascorbic acid 2,6-dihexadecanoate	6.19
27	18.92	Oleic acid / n-Hexadecanoic acid	3.37
28	19.31	Erucic acid	4.59
29	19.93	Z-8-Methyl-9-tetradecenoic acid / 12-Hydroxydodecanoic acid	1.8
30	20.15	Oleic acid / Pentadecanoic acid / 2-Tridecenoic acid, (E)-	1.85
31	20.63	Heptadecanoic acid, heptadecyl ester / trans-2-undecenoic acid	0.29
32	23.18	Myristic acid / Tetradecanoic acid	0.48
33	25.89	Palmitic acid/ n-Hexadecanoic acid / l-(+)-Ascorbic acid 2,6-dihexadecanoate	5.34
34	26.06	Octadecanoic acid / Oleic acid	0.73
35	26.29	Oleic acid	0.32
36	26.40	Oleic acid/ 7-Methyl-Z-tetradecen-1-ol acetate	0.28
37	27.77	cis-Vaccenic acid / trans-13-Octadecenoic acid	5.81
38	28.00	Octadecanoic acid / Oleic acid	2.89
39	29.22	2-Hexadecanol / Heptacosane / Ethanol, 2-(octadecyloxy)-	0.28
40	29.37	1-Hexadecanol, 2-methyl- / Oxirane, [(hexadecyloxy)methyl]-	0.84
41	29.41	Z-8-Methyl-9-tetradecenoic acid / Erucic acid / Dodecanoic acid, 3-hydroxy-	0.73
42	29.54	Oleic acid / Z-8-Methyl-9-tetradecenoic acid	0.26
43	31.05	Hexadecanoic acid	2.35
44	31.1	Hexadecanoic acid / Dodecyl cis-9,10-epoxyoctadecanoate	3.51
45	31.92	n-Butyl ricinoleate / Triarachine / Dodecyl cis-9,10-epoxyoctadecanoate/ Hexadecanoic acid	0.46
46	32.08	Hexadecanoic acid / Dodecyl cis-9,10-epoxyoctadecanoate	2.54
47	32.36	Hexadecanoic acid / Dodecyl cis-9,10-epoxyoctadecanoate	1.02
48	32.43	Dodecyl cis-9,10-epoxyoctadecanoate/ Hexadecanoic acid/ n-Butyl ricinoleate	2.97
49	32.65	Hexadecanoic acid / Dodecyl cis-9,10-epoxyoctadecanoate	1.28
50	32.78	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl- /Hexadecanoic acid / Dodecyl cis-9,10-epoxyoctadecanoate	0.26

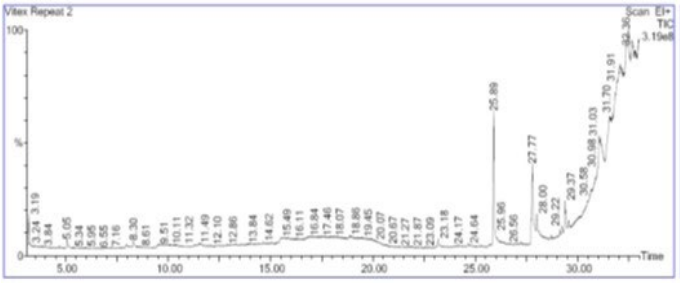

Fig. 1. Chromatogram of *Vitex agnus-castus* ethanolic extract revealed GC-MS analysis and showed various peaks with various areas (represent many compounds in different amounts) obtained at different retention times.

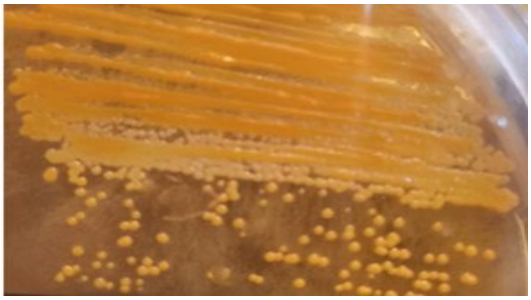

Fig. 2. *V. anguillarum* colonies showed yellowish color on TCBS medium with a range of (2-3 mm in diameter) with yellow pigmentation.

Bacterial isolation and identification

In this study, six *V. anguillarum* isolates were isolated from 50 common carp (*Cyprinus carpio*) fish samples. The colonies were yellowish color on TCBS medium with a range of (2-3 mm in diameter) with yellow pigmentation (Fig. 2), Gram negative small curved rods, motile on microscopy, catalase and oxidase positive, indol positive, reduced nitrate to nitrite, ferment lactose, mannitol, sucrose, and glucose without gas production and sensitive to vibriostatic agent O/129. All of the identified *V. anguillarum* isolates were confirmed with API 20E (bioMe´rieux®).

In vitro antibacterial activity screening tests

AST and MIC tests

Our AST of *V. anguillarum* revealed its resistance to the used antibiotics amoxiclave, gentamicin, tetracycline, chloramphenicol, and sulphamethoxazole-trimethoprim (Cotrimoxazole). Meanwhile, it was sensitive to marbofloxacin with MIC 0.98 µg/ml and MBC 3.90 µg/ml. Therefore, multiple antibiotic resistance (MAR) index was 0.83.

VAC extract antibiogram showed marked antibacterial activity in all of the used concentrations against *V. anguillarum* with IZDs (22±0.33, 20±0.33, 17±0.00, 15±0.66, and 14±0.00) mm corresponding to 125, 62.53, 31.25, 15.63 and 7.81 mg/ml (Fig. 3). MIC and MBC were 1.95 and 15.63 mg/ml respectively.

Table 2. Effect of *Vitex agnus-castus* (VAC) ethanolic extract and marbofloxacin (MAR) on growth performance and survival rate (Mean±SE) n=30.

Items	G1	G2	G3	G4	G5	G6
Initial weight (g)	30.78±0.01 a	30.79±0.4 a	30.65±0.12 a	30.79±0.02 a	30.70±1.2 a	30.68±1.03 a
Final weight (g)	33.80±1.5 b	31.95±1.66 a	33.25±1.34 b	35.15±1.22 c	34.80±1.66 b	53.88±1.4 d
Weight gain (g)	3.02±1.32 b	1.16±1.03 a	2.60±0.02 a	4.36±1.32 c	4.1±1.16 c	23.20±0.01 d
WG%	9.81±0.9 b	3.76±1.02 a	8.48±1.52 b	14.16±1.8 c	13.35±0.9 c	75.61±1.2 d
SGR(%/day)	1.42±1.3 c	0.75±0.6 a	1.14±1.01 b	1.85±1.02 c	1.71±1.02 c	1.86±0.01 d
Feed consumption (g feed/ fish)	6.59±1.02 b	6.50±0.76 a	6.52±0.49 a	6.64±0.04 b	6.62±0.55 b	33.61±1.06 c
FCR	2.18±1.33 a	5.60±1.29 c	2.50±1.73 b	1.52±1.6 a	1.61±1.54 a	1.44±1.3 a
Survival %	100	56.7	80	80	83.3	86.7

The various letters in the same row indicate statistically significant differences when (P<0.05).
G1: negative-control; G2: infected-control; G3: infected-(MAR); G4: infected-(VAC); G5: infected-(MAR + VAC); G6: VAC prophylactic treated then challenged.

Table 3. Effect of *Vitex agnus-castus* (VAC) ethanolic extract and marbofloxacin (MAR) on some liver biochemical parameters (Mean±SE) n=5.

G	Periods	ALT (u/l)	AST (u/l)	Total protein g/dl	Albumin (g/dl)	Globulin (g/dl)	A/G ratio
G1	PT	40.09±1.58 a	45.11±1.08 a	2.82±0.04 cd	1.09±0.03 cd	1.72±0.06 b	0.64±0.03 a
	AEE	49.27±1.00 a	49.27±1.57 ab	3.05±0.04 bc	1.25±0.04 abc	1.80±0.08 a	0.70±0.05 a
G2	PT	91.12±0.97 d	121.10±2.12 d	2.12±0.03 a	0.69±0.02 a	1.43±0.05 a	0.48±0.03 a
	AEE	63.04±1.25 d	73.90±1.07 d	2.71±0.09 a	1.08±0.05 a	1.63±0.06 a	0.67±0.04 a
G3	PT	65.55±1.18 c	64.89±1.23 c	2.35±0.03 b	0.79±0.00 ab	1.56±0.02 ab	0.50±0.01 a
	AEE	52.94±1.12 bc	52.02±1.36 b	2.95±0.15 ab	1.16±0.02 b	1.79±0.18 a	0.66±0.08 a
G4	PT	61.60±1.69 c	62.85±1.21 c	2.49±0.04 b	0.92±0.04 bc	1.57±0.03 ab	0.59±0.03 a
	AEE	51.37±1.25 abc	51.03±1.12 ab	3.41±0.07 d	1.27±0.04 bc	2.14±0.08 b	0.60±0.03 a
G5	PT	55.79±0.93 b	57.34±0.98 b	2.68±0.02 c	1.01±0.02 bcd	1.67±0.04 ab	0.60±0.02 a
	AEE	50.68±0.93 ab	48.16±0.71 a	3.24±0.03 bcd	1.37±0.03 cd	1.87±0.02 ab	0.73±0.02 a
G6	PT	42.19±1.30 a	46.49±1.18 a	2.96±0.09 d	1.19±0.05 d	1.77±0.14 b	0.70±0.05 a
	AEE	54.60±1.04 c	59.12±0.60 c	3.35±0.10 cd	1.39±0.02 d	1.95±0.10 ab	0.72±0.03 a

The various letters in the same colon of the same period indicate statistically significant differences when (P<0.05).
G1: negative-control; G2: infected-control; G3: infected-(MAR); G4: infected-(VAC); G5: infected-(MAR + VAC); G6: VAC prophylactic treated then challenged, PT= Post treatment, AEE= at experimental end.

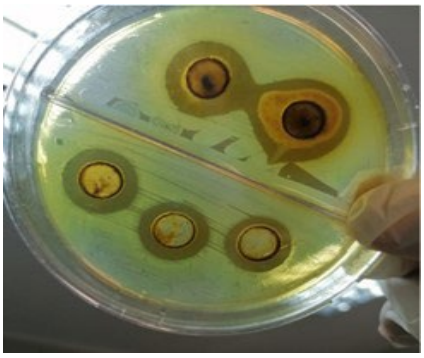

Fig. 3. *Vitex agnus-castus* extract antibiogram showed marked antibacterial activity against *V. anguillarum* with IZDs (22±0.33, 20±0.33, 17±0.00, 15±0.66, and 14±0.00) mm corresponding to 125, 62.53, 31.25, 15.63 and 7.81 mg/ml concentrations.

Scanning electron microscope (SEM)

As shown in Fig. 4, not treated bacteria were used as a control (Fig.4a), it had a curved rod-shape and single polar flagellum. SEM was used to examine changes in bacterial cells morphology, which treated with MIC dose of VAC extract after 5 min and1 hr respectively (Fig. 4 b, c) and cells treated with 2MIC dose of VAC extract after 5 min and1 hr respectively (Fig. 4 d, e). VAC changed their morphology and lost some of properties (loss of flagella). Compared with normal bacterial cells, the majority of the treated bacteria were irregular and shriveled to varying degrees. Treated bacteria with either MIC or 2MIC dose exhibited varying degrees of distortion. Morphological changes were detected on bacterial cell wall treated with 2MIC within 1 h, which was significantly more effective than MIC within 5 minutes.

In vivo evaluation of the therapeutic and preventive effect of VAC extract

Clinical signs and postmortem lesions

Group 1 did not show any abnormalities or mortalities during the experiment. Experimentally infected fish with *V. anguillarum* (Fig. 5) showed a typical external and internal sign in the first week of the infection similar to those caused by natural infection such as loss of reflexes, anorexia, abdominal distention (Fig. 5 a), hemorrhagic patches on different parts of the body, around mouth and around the vent (Fig. 5 b, c) and skin erosions and ulcerations (Fig. 5d). Moreover, postmortem lesions were ascites, edema, liver paleness, congestion, and enlargement (Fig.5 e, f), congestion with dark coloration of spleen and congestion of kidney (Fig. 5 g, h). In order to confirm the causative agent of fish infection *V. anguillarum* were re-isolated from skin lesions, posterior kidney, liver and spleen of moribund and dead fish.

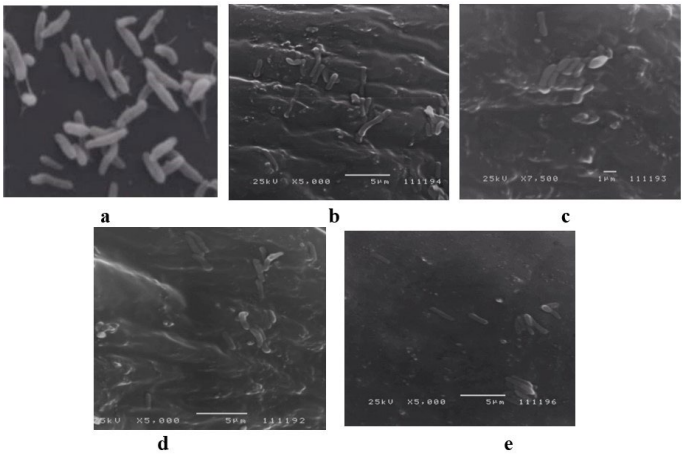

Fig. 4. Scanning electron micrographs showed (a): untreated bacteria, (b and c): treated with MIC dose of *Vitex agnus castus* extract after 5 min and1 hr respectively, (d and e): treated with 2MIC dose of *Vitex agnus castus* extract after 5 min and1 hr respectively.

Growth performance and survival rate

In Table 2, the best growth performance parameters and decreased FCR were shown in this descending order G6, G4 then G5 and all treated groups resulted in improved survival rates when compared with the infected control G2. Noting that increasing the period of VAC feed supplementation in fish diets up to 30 days, led to more increase in growth performance and survival rate.

V. anguillarum count

Post treatment, liver specimens of infected control G2 revealed (3x 10^2±0.57) cfu/g; meanwhile no bacteria were re-isolated from all treated groups G3, G4 and G5. Our results indicated the efficacy of the VAC to prevent systemic infection as MAR, with no antagonistic interaction when used together.

Serum biochemical parameters

In Table 3, biochemical analysis post treatment and at the end of the experiment revealed that infected control G2 had a significant increase in the enzymes activities AST and ALT, while TP, albumin and globulin levels were significantly decreased than negative control G1. Regarding

Table 4. Effect of Vitex agnus-castus (VAC) ethanolic extract and marbofloxacin (MAR) on serum superoxide dismutase (SOD), malondialdehyde (MDA), urea and creatinine (Mean ± SE) n=5.

G	Periods	SOD (U/ml)	MDA (nmol/ml)	Urea (mg/dl)	Creatinine (mg/dl)
G1	PT	274.81±3.56 b	20.46±0.94 b	7.42±0.23 a	0.09±0.01 a
	AEE	285.67±5.78 b	23.01±0.85 b	8.94±0.18 a	0.11±0.01 b
G2	PT	243.88±3.72 a	32.87±0.62 d	13.59±0.37 e	0.20±0.00 d
	AEE	258.52±4.23 a	26.70±0.77 c	11.96±0.14 c	0.16±0.00 c
G3	PT	273.23±1.86 b	25.42±0.38 c	11.44±0.30 d	0.15±0.00 c
	AEE	276.59±5.55 b	24.95±0.65 bc	9.87±0.22 b	0.07±0.01 a
G4	PT	301.47±1.84 c	26.25±0.37 c	9.83±0.25 c	0.13±0.01 b
	AEE	286.98±4.06 b	20.32±1.09 a	9.24±0.31 a	0.06±0.00 a
G5	PT	293.67±4.26 c	24.09±0.85 c	8.65±0.28 b	0.11±0.00 b
	AEE	285.48±3.37 b	20.89±0.63 a	8.98±0.14 a	0.07±0.01 a
G6	PT	318.62±7.57 d	17.83±0.92 a	7.14±0.28 a	0.08±0.01 a
	AEE	335.79±7.20 c	21.97±0.79 a	9.95±0.16 b	0.10±0.01 b

The various letters in the same colon of the same period indicate statistically significant differences when (P<0.05).
G1: negative-control; G2: infected-control; G3: infected-(MAR); G4: infected-(VAC); G5: infected-(MAR + VAC); G6: VAC prophylactic treated then challenged; PT: Post treatment; AEE: at experimental end.

treated groups there were significant changes among groups in various degrees. However, all treatments significantly improved the majority of the detected parameters than G2 and turned towards normal levels of G1 mostly in this ascending order G3, G4 then G5. Prophylactic treated G6 at the end of the experiment post challenge showed a significant increase in the enzymes activities AST and ALT, TP, albumin, and globulin levels than negative control G1, where the enzymes' activities significantly decreased than infected control G2. Throughout the study no significant change was recorded in A/G ratio among all groups.

Fig. 5. Experimentally infected *Cyprinus carpio* fish with *V. anguillarum* showing; (a): abdominal distention and ascites, (b): hemorrhagic patches on different parts of the body, around mouth, (c): hemorrhagic vent, (d): skin erosions and ulcerations, (e and f): ascites, oedema, liver paleness, congestion, and enlargement, (g): paleness and enlargement of liver and congestion with dark coloration of spleen and (h): congestion of liver and kidney.

Antioxidants and kidney function tests

In Table 4, antioxidant biomarkers and kidney function evaluation tests post treatment and at the end of the experiment revealed that infected control G2 had a significant increase in the MDA, urea and creatinine levels where SOD levels were significantly decreased than negative control G1. Treated groups revealed significant changes in various degrees. All treatments significantly increased SOD levels and decreased MDA, urea and creatinine levels than G2 and turned towards normal levels of G1 mostly in this ascending order G3, G4 then G5. Prophylactic treated G6 at the end of the experiment post challenge showed a significant increase in the SOD and urea levels without affect creatinine while decreased MDA levels than negative control G1. Meanwhile, G6 showed a significant increase in the SOD and a significant decrease in the MDA, urea and creatinine levels than infected control G2.

Marbofloxacin residues

As regards to standard curve, MAR standard concentrations of 25, 50, 100, 250 and 500 µg/kg were spiked in homogenized blank samples of fish musculature then followed by the extraction procedure to give their corresponding peak responses as showed in Table 5. The calibration curve was calculated by linear regression; y = 0.093 + x - 0.374 where y refers to the area under peak and x refers to the concentrations of MAR (Fig. 6 a-g). Linearity existed within the range between 25-500 µg/kg with squared correlation coefficient (R2) = 0.998.

Concerning to MAR residues (ppb) in fish musculature, it showed a gradually decrease up to the 4th day of the withdrawal period to be (110.57±3.81 then 79.8±1.14) in MAR treated G3 and (88.9±1.70 then 61.3±0.65) in MAR and VAC treated G5 corresponded to 1st and 4th days post the last dosage of drug respectively as illustrated in curves of Fig. (6 h-k). MAR stayed within evident limit till the 4th day in fish musculature. Results revealed that MAR residue needed 4 days as a withdrawal period to be less than the maximum residue limits (MRL), while combination of VAC extract with MAR decreased MAR residues less than the MRL from the 1st day post treatment.

Water quality parameters

During the experiment the water quality parameter mean values±SE were: temperature (26±1°C), pH (7.5±0.2), dissolved oxygen (6±0.8) mg/L, total ammonia (0.026±0.01) mg/L, unionized ammonia (NH3) (0.02±0.01) mg/L., Nitrite (No2-N) (0.04±0.01) mg/L and nitrate (No3) (1.3±0.4) mg/L. All water quality parameters were within the acceptable ranges.

Discussion

Appropriate application of efficient therapeutic products is of vital importance for controlling of fish primary and secondary bacterial infections. Meanwhile, the extensive use of FQs in aquacultures, increased their residues and bacterial resistance (Cheng *et al*., 2014). Hence, strict regulations on the application of antibiotics and chemotherapeutics in aquaculture are increased by time (Lim *et al*., 2013). Exploration of antibiotic-like compound, herbal plant-based biopharmaceuticals can act as a natural medication for prophylaxis and treatment of infection. Consequently, the present investigation studied the efficacy of using VAC extract in treatment and prevention trials alone or with MAR against V. anguilarrum infection and detected the interaction between its bioactive constituents and MAR residue.

It is essential to quantify and qualify the active compounds of VAC that contribute to its antibacterial activities. Applying of mass spectrometry (MS) device provides abundant information for the structural elucidation of the compounds. Therefore, the combination of chromatography and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable (Altemimi *et al*., 2017). Previous studies investigated the various constituents of VAC in disconnected leaves or fruit extracts. Meanwhile, in our study the whole dry plant ethanolic extract were analyzed for its therapeutic efficacy and pharmacological activities using GC-MS-technique. The principal recorded compounds (Fig. 1, Table 1) were Oleic acid / Hexadecanoic acid / l-(+)-Ascorbic acid 2,6-dihexadecanoate / Eicosanoic acid / cis-Vaccenic acid / Palmitic acid / Octadecanoic acid

Table 5. The concentrations of marbofloxacin standard spiked tissues (µg/kg) and their corresponding peak response determined automatically using HPLC chromatogram system.

Retention time (RT) (min)	Level	Amount (µg/kg)	Area	Concentration (µg/kg)	Recovery (%)
11.39	1	25	2.14	26.94	107.75
	2	50	4.55	52.77	105.53
	3	100	9.12	101.78	101.78
	4	250	21.85	2.38E+2	95.28
	5	500	46.73	504.84	100.97

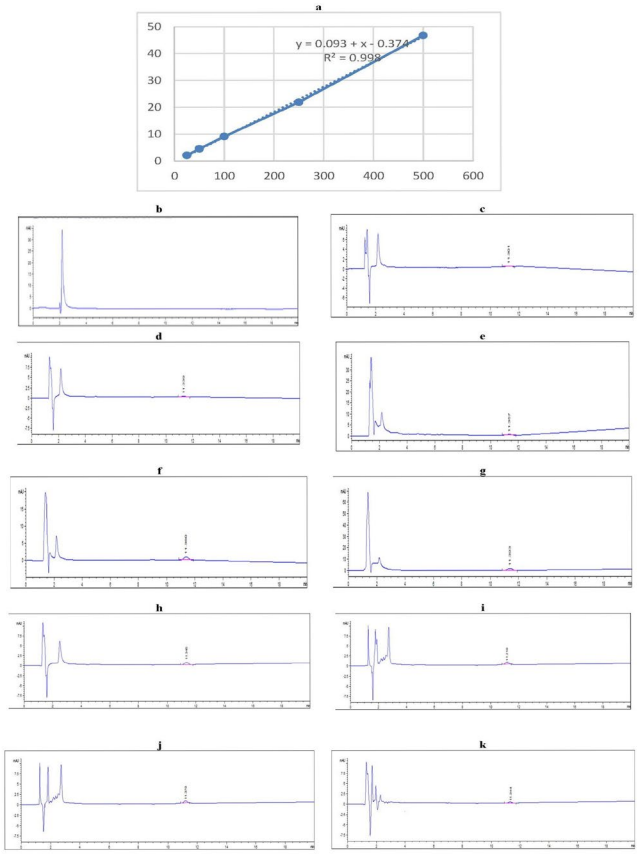

Fig 6. (a) Showing the standard curve linearity of marbofloxacin (MAR) in blank fish musculature from (25–500 ppb), where y refers to the area under peak, x refers to the concentrations of MAR, and r2 refers to squared correlation coefficient. (b) Showing the chromatogram of control blank curve, while (c–g) showing the chromatogram of MAR standard curve at different concentrations (25, 50, 100, 250, 500 ppb) respectively. (h) Showing chromatogram of MAR in G3 = 110.57 ppb at the 1st day PT. (i) Showing chromatogram of MAR in G5 = 88.9 ppb at the 1st day PT. (j) Showing chromatogram of MAR in G3 = 79.8 ppb at the 4th day PT and (k) Showing chromatogram of MAR in G5 = 61.3 ppb at the 4th day PT. Where; PT: Post-treatment, G3: infected-(MAR), G5: infected-(MAR + *Vitex agnus-castus* extract).

and Erucic acid. Other compounds constituted an important portion of the extract were also detected as Phenol, Myristic acid / n-Butyl ricinoleate / Triarachine / Trichloroacetic acid / à-D-Glucopyranoside, O-à-D-glucopyranosyl/ á-D-Glucopyranose, 4-O-á-D-galactopyranosyl- and Dodecanoic acid, 3-hydroxy. These naturally bioactive ingredients related to vital fatty acids (FAs), fatty alcohol, antioxidants, phenolics and others where these essential nutrients possess a wide range of important biological activities as a growth promoter, broad spectrum antimicrobial, anti-inflammatory, antioxidant and free radical-scavenging properties. Our findings coincide to some extent with Farzaei *et al*. (2018) and Keikha *et al*. (2018) who found 36 compounds in ethanoilc and aqueous VAC leaf extract, while the main were α-Pinene, isoterpinolene, caryophyllene, azulene, terpinen-4-ol and α-Terpineol. Also, Ababutain and Alghamdi (2021) identified 43 compounds in methanol and 47 in ethanol VAC leaf extracts, where 4,5-Dichloro-1,3-dioxolan-2-one and 1H-Indene, 2,3-dihydro-1,1,2,3,3-pentamethyl were the highest concentrations in ethanol extract. Other phytochemical compounds belong to different bioactive chemical group such as polyphenols, fatty acids, terpenes, terpenoids, steroids, aldehydes, alcohols, and esters. As well Jokic *et al*. (2017) found that the major compounds in VAC fruits were sesquiterpene hydrocarbons trans-farnesene (15.6-23.4%), trans-caryophyllene (13.6-18.6%) and bicycloger-macrene (8.5-11.6 %) along with oxygenated monoterpenes-terpinyl acetate (7.6-13.5%) and 1, 8-cineole (2.6-12.2%). These discrepancies in the VAC phytoconstituents may be attributed to the various analytical method techniques and its factors, plant parts, origin, cultivation seasons, conditions, methods of extraction and type of solvent, leading to broad ranges of pharmacological compounds. In this context, Jokic *et al*. (2017) reported that different processing pressure and temperature influenced the percentage and chemical composition of the resulted compounds.

In this study, six *V. anguillarum* isolates were isolated from 50 naturally infected common carp (*Cyprinus carpio*) fish samples. Since the clinical signs, postmortem pictures and destructive effects in collected samples are claimed for the pathogenesis of *V. anguillarum* in the target fish and its virulence factors (Frans *et al*., 2011).

Our AST of *V. anguillarum* revealed its resistance to the used antibiotics amoxiclave, gentamicin, tetracycline, chloramphenicol and sulphamethoxazole-trimethoprim (Cotrimoxazole). Meanwhile, it was sensitive to marbofloxacin with MIC 0.98 µg/ml and MBC 3.90 µg/ml. Therefore, our multiple antibiotic resistance (MAR) index was 0.83. Concerning to similar antibiotic resistance patterns among various pathogenic Vibrio strains, Mohamad *et al*. (2019) recorded that MAR index ranged between 0.06 and 0.56, particularly against polypeptides, ampicillin, penicillin, and streptomycin. Where, 75% of the isolates have alarming rate of MAR index (higher than 0.20). As well, Sotomayor *et al*. (2019) found that 50%, 45% and 5% of the strains resistant at the same time to 2, 3 and 4 antibiotics, respectively. The average MAR index was 0.23, where less efficient antibiotics were penicillin; oxytetracycline and tetracycline. Meanwhile furazolidone, ciprofloxacin, chloramphenicol, norfloxacin, nalidixic acid, florfenicol, fosfomycin and enrofloxacin inhibited the growth of most of the strains; nevertheless, most of these products are not authorized for use in aquaculture. Meanwhile, Marimuthu *et al*. (2012) found that chloramphenicol and ampicillin showed 25mm IZD, where gentamicin had 12 mm. In the same context, Lee *et al*. (2018) reported antibiotic resistant profiles of various *Vibrio* isolates to be ampicillin (88%), amikacin (64%), and kanamycin (50%). A notable resistance pattern can be observed to the third generation cephalosporins (cefotaxime 52% and ceftazidime 28%). In contrast, high susceptibility rate was seen to chloramphenicol (93%), tetracycline (90%), imipenem (85%), levofloxacin (85%), gentamicin (84%), sulfamethoxazole/trimethoprim (80%), nalidixic acid (78%), oxytetracycline (72%), and ampicillin/sulbactam (70%). These discrepancies among studies could be explained by various *Vibrio* sp. resistance mechanisms under antibiotic selection pressure, aquaculture environment, seasons, fish species or type of collected samples. Generally, the global increase of antibiotic resistant bacteria warrants a continuous monitoring. Recently, the Food and Agriculture Organization (FAO) have drawn action plans to increase awareness and promote far-sighted use of antimicrobials (FAO, 2015). Therefore, antibiotics not recommended as a control strategy at production level. It is necessary for manufacturer to consider safer alternative strategies for the control of pathogenic bacteria.

Concerning to marbofloxacin, Farca *et al*. (2007) found that MAR susceptibility rate decreased from 71% to 58% through two years in various field strains. To achieve the effective antimicrobial plasma concentration, MIC considered as one of the best pharmacodynamic parameters for predicting the antimicrobial effect of FQs. Meanwhile, there are few reports on the MICs of MAR against fish susceptible pathogens (Poapolathep *et al*., 2020). Therefore, MICs of MAR against Gram-negative strains could be taken in consideration (Marín *et al*., 2009). According to MIC values of other FQs members listed by CLSI (2015) against *Vibrio* spp., our MAR MIC value 0.98 µg/ml located in their sensitive range (1-2 µg/ml). Various MAR MIC values (0.016-0.56) µg/ml were reported for several bacteria (Meunier *et al*., 2004). For Enterobacteriaceae sp., it was ≤ 0.3 µg/ml, while for P. aeruginosa it equaled to 1 µg/ml (Lees *et al*., 2006), or in the range of 0.25-64 mg/L, where MIC 90= 16 mg/L (Jerzsele and Pásztiné-Gere, 2015). For E. coli, it was 0.536 µg/ml (EMEA, 2000); while for *A. hydrophila* it was 0.2 µg/ml (Zhiqiang and Lin, 2005). Qi *et al*. (2017) found that post pharmacokinetic analysis of oral dose of 10 mg/kg body mass; MAR could effectively control susceptible microorganisms involved in most common infections in tilapia. As well, post oral dose of 10 mg/kg b. wt, the concentration-dependent bactericidal effect of MAR was confirmed for levels lower than 4 MIC (Shan *et al*., 2015), where its MICs and MBCs against *A. hydrophila* were 0.05 and 0.125 µg/ml in serum, besides 0.032 and 0.064 µg/ml in broth respectively.

Our VAC extract antibiogram showed marked antibacterial activity from 125 to 7.81 mg/ml against *V. anguillarum* with IZDs ranged from (23±0.33 to 14±0.00) mm (Fig. 3). Its MIC and MBC were 1.95 and 15.63 mg/ml respectively. Regarding to the effect of the extraction solvent on the extract antibacterial activities, Ababutain and Alghamdi (2021) found that using ethanol solvent had a stronger extraction capacity that revealed a significant bacterial inhibitory activity even at low concentrations better than methanol and water extracts, that signify the importance of choosing the appropriate solvent to extract high quantities of effective antibacterial inhibiting phytochemicals. Also effective antimicrobial activity of VAC essential oil (Ghannadi *et al*. 2012; Gonçalves *et al*. 2017; Souto *et al*. 2020) was reported compared to chloramphenicol and amoxicillin against various Gram-negative bacteria (*Salmonella* sp. and *Pseudomonas* sp.), where IZD and MIC values ranged from 9.0 to 15.0 mm and 15.6 to 200 µg/ml respectively. Ghannadi *et al*. (2012) and Bouyahya *et al*. (2017) attributed the major antibacterial activities of VAC to its volatile oil constituents besides other main components flavonoids, coumarins, terpenes and phenolics such as luteolin and chlorogenic acid. Where

they inter the microbial cell, affect cellular activity due to its interaction with the membrane enzymes and proteins and reverse the flow of protons (Stojković *et al*., 2011). Similar compounds were reported in our GC-MS assay. Interestingly, Ahmad *et al*. (2010) found that separate active principles showed no antibacterial activity as the whole extract indicated that the major components act synergistically with its minor ones to amplify their activity. Another antimicrobial activity explanation attributed to the lipophilicity of VAC constituents which permitted it to disseminate easily across the bacterial membranes and disrupt its metabolic pathways or organelles (Gonçalves *et al*., 2017). However authors recommended further studies to confirm the in vivo safety of these extracts. Other Vitex sp. manifested good growth inhibition against Gram negative bacteria at 0.50-8.00 mg/ml (Nyiligira *et al*., 2008) and displayed a significant IZD on *Vibrio* sp. (Marimuthu *et al*., 2012; Zheng *et al*., 2015).

Our scanning electron microscope (SEM) results (Fig. 4) confirmed the antibacterial activity of VAC extract. Invasion and pathogenesis of *Vibrio* sp. depend mainly on bacterial motility, attachment besides other virulence factors (Frans *et al*., 2011), therefore the recorded flagella destruction in our SEM analysis considered an important mode of VAC bactericidal effect. The flagellin A, one of the five-flagellin subunits identified in *V. anguillarum*, is required for efficient invasion of rainbow trout fish (McGee *et al*., 1996). Furthermore, removing the conserved C terminus of flagellin A resulted in a decreased virulence of approximately three logs when fish were infected intraperitoneally and decreased motility by 50 % (Milton *et al*., 1996). In another study, Ababutain and Alghamdi, (2018) attributed the bactericidal effect of VAC extracts to many theories as its adhesion to the surface membrane of microbes, penetration into bacterial cells and disruption of intracellular structures and biomolecules damage, production of reactive oxygen species (ROS) causing oxidative stress and cytotoxicity in cells or disruption of cell signal transduction pathways (Anandalakshmi, 2021; Berrani *et al*., 2021).

The present study proved that VAC extract has an effective role in increasing growth performance; It can be related to the presence of beneficial phytochemical compounds in VAC extract, which is the main reason for raising digestive enzyme activity, appetite, and the population of vitamins producing microbiota in the gut (Dawood *et al*., 2014; 2016). Additionally, VAC contains some valuable bioactive compounds including phenolics and total flavonoids (Latoui *et al*., 2012; Pío-Leon*et al*., 2014). Plus, anthocyanins which are reported as a major pigments which are also considered as a potential antioxidant agent (Pío-Leon *et al*., 2014). Several researchers suggested that there is a positive correlation between the growth performance in fish and antioxidant status (Rempel and Schlenk, 2008; Taghavizadeh *et al*., 2020). This is in addition to stimulating fish resistance, survival rates and treatment of common carp fish challenged with *V. anguillarum* infection with an efficiency equivalent to the efficiency of using antibiotic as a treatment; this is due to its direct effect on the bacterial cell plus its effective role in raising the immune response of fish. Also, it is found that increasing the period of feeding trial of VAC extract leading to more increase in growth performance and survival rate. This result in agreement with the results of Zamani*et al*. (2018) who reported that VAC extract was effective in raising growth performance and survival rates in Xiphophorus helleri fish. And Gholampour*et al*. (2020) showed that VAC extract was effective in successful growth performance in Zebra fish, also the growth indices were affected meaningfully with the increasing of VAC doses. Also Rashmeei*et al*. (2020) proved that the dietary supplementation of VAC extract stimulates immunity and resistance of gold fish (*Carassius auratus*) challenged with *A. hydrophila* infection. And Rashmeei*et al*. (2022) also found that the dietary supplementation of VAC fruit extract could enhance the fish growth indices and feed utilization in goldfish fed with different levels of VAC extracts in comparison with the control group. However, our results disagree with Gholampour *et al*. (2020) who recorded that the effect of VAC was not significant on the survival rate of Zebra fish.

Regarding to *V. anguillarum* count post treatment, liver specimens of infected control G2 revealed ($3x10^2$±0.57) cfu/g; meanwhile no bacteria were re-isolated from all treated groups G3, G4 or G5. Our results indicated the efficacy of the VAC to prevent systemic infection as MAR, with no antagonistic interaction when used together. These in vivo results coincided with our in vitro antibacterial activity AST and SEM results and with Daniele *et al*. (2005) that VAC had no drug interactions or adverse events.

Regarding to our serum biochemical analysis (Tables 3 and 4), results revealed that post treatment and at the end of the experiment, infected control G2 had a significant increase in AST and ALT activities, MDA, urea and creatinine levels besides a decrease in TP, albumin, globulin and SOD levels than negative control G1. Meanwhile, all treatments significantly improved most of these parameters to be near to G1 in this ascending order G3, G4 then G5 through increased TP, albumin, globulin and SOD levels besides decreased AST, ALT, MDA, urea and creatinine than G2. Prophylactic treated G6 at the end of the study post challenge showed a significant increase in the AST, ALT, TP, albumin, globulin, SOD and urea levels without affect creatinine while decreased MDA levels than G1. *V. anguillarum* infection hindered TP and albumin production which occurred in a relative to vascular permeability increase, histamine release, liver damage, anorexia and nonspecific proteolysis. Additionally, maintaining a sustained infection-inflammatory response is heartily and can be considered as a stress (Vargas *et al*., 2018). *V. anguillarum* septicemia mainly affects the spleen, kidney and liver as target organs using the proteolytic activity of its protease to penetrate, colonize and damage tissues (Manchanayake *et al*., 2023). Similarly, Chaudhary *et al*. (2021) reported that *V. anguillarum* infection caused a significant decrease in biochemical constituents of muscles (TP, lipids, carbohydrates, RNA and DNA) indicated low physiological condition, stress and metabolic cycle alteration. Furthermore, Younes *et al*. (2021) recorded considerable biochemical and histopathological destructive changes in liver, kidney and spleen tissues post *V. anguillarum* infection within Egyptian cultivated fish that may be attributed mainly to its virulence factors throughout the infection, colonization, adhesion and subsequently vital organ obliteration such as down-regulation of the energetic metabolism, biosynthesis and production of enzymes as lecithinase, lipase, lipopolysaccharides, metalloprotease, proteases, hemolysins, siderophores, caseinase, gelatinase, haemaglutinin, cytotoxin (Lages *et al*., 2019; Mahrous *et al*., 2020; Younes *et al*., 2021).

About treated groups, the significant elevation in TP than G2 further indicates that fish became immunologically well-built, its utilization as an energy source to recompense increased energy demand during remedial period besides it is needed to construct, repair tissues and sustain increase in physiological activity. Coincide to our findings, EMEA (2000); Coşkun *et al*. (2019) and Patel *et al*. (2020) recorded that MAR (10 mg/kg b. wt/day) for 13 weeks had no adverse effect on liver function parameters, while it affected renal function through increased BUN and creatinine levels (Coşkun *et al*., 2019). As reported, kidney was considered as the target organ of MAR, where it had the highest MAR residues due to its slow elimination (Shuai-peng *et al*., 2014). Conversely, significant changes in liver and kidney functions were reported by Mousa *et al*., (2011) post treatment with MAR (14.4 mg/kg/day) for 5 days. Discrepancies in parameters among studies may be attributed to sampling time, therapeutic period and dosage besides fish species, age, sex and habitat. Concerning to VAC extract treated G4, our findings coincide with VAC safety assessment reports of EMA (2009; 2018), where no serious adverse events were reported on similar biomarkers at 480 mg/14 days. As well, in acute or subacute toxicity studies (2000 mg/kg single dose or 1000 mg/kg/ 4 weeks repeat dose) respectively; all behavior, general condition, histopathological, biochemical and urine analytical parameters remained unaffected. In other in vivo assays, Farzaei *et al*. (2018) reported that crude VAC extract prevented nonalcoholic fat liver disease where its LD50 exceeded 5 g/kg. Similar hepatoprotective effect of Vitex negundo leaves ethanolic extract (250 mg/kg) was reported by Zheng *et al*. (2015) via decreasing the levels of ALT, ALP, and production of reactive oxygen species (ROS) and lipid peroxidation (MDA). As a fact, the importance of antioxidants concerted mainly to avoid oxidants; the ubiquitous in biological systems that cause significant damage to proteins, nucleic acids and increased peroxidation of polyunsaturated fatty acids leads to cell membranes dysfunction (Gill *et al*., 2012). In this context, VAC employs a range of enzymatic (SOD) and non-enzymatic antioxidants (ascorbic acid) that inhibit extra ROS generation released under stress; the key factor that can promote lipid peroxidation of biomembranes (Zoufan *et al*., 2018).

In MAR-VAC treated G5, most of the detected parameters showed the best results that indicated their synergistic interaction or additive effect to counteract the infection's negative impacts. As well, prophylactic treated G6 assured the efficacy of VAC extract to protect carp from *V. anguillarum* infection. VAC inhibited pro-inflammatory cytokines, cyclooxygenase-2 activity and leukotriene production (EMA, 2018; Farzaei *et al*., 2018; Souto *et al*., 2020). Its ascorbic acid, besides many phenolics play dynamic roles in reducing inflammation (Altemimi *et al*., 2017). Where, its flavonoids can trigger and affect superoxide anion activity which is important to prevent and control against in vivo microbial infections (Bilen *et al*., 2020). Also in vivo administration of VAC for 5 days protected from lipopolysaccharide (LPS)-induced organ toxicity through its antioxidant activity (Farzaei *et al*., 2018; Souto *et al*., 2020). Furthermore, it improved the immune system, removing body free radicals, and so forth through its phytoconstituents synergistic effect (Saklani *et al*., 2017). Therefore, VAC may be important as; nutraceuticals, biopharmaceuticals, food additives and possible cost-effective natural antioxidant.

For MAR residues (ppb) in fish musculature, MAR treated G3 and MAR-VAC treated G5 showed a gradually decrease up to the 4^{th} day of the withdrawal period to be (110.57±3.81 then 79.8±1.14) and (88.9±1.70 then 61.3±0.65) respectively correspond to 1st and 4th days post the last dosage of drug (Fig. 6h-k). Hence, MAR residue needed at least 4 days

as a withdrawal period to be less than the maximum residue limits (MRL) that considered 100 ppb; to be safe with no health hazard on consumers. Combination of VAC extract with MAR decreased its residues less than the MRL from the 1st day post treatment (PT). Usually, to protect consumers' health, MRL for antibiotics in food producing animal are established worldwide by several regulatory agencies, including the European Union (EU), the United States Food and Drug Administration (FDA), as well as the Codex Alimentarius and the European Medicines Agency (EMEA) (EC, 2010; CODEX, 2014; Rezk *et al*., 2015) where, concentrations above the MRL are improper for human consumption. Meanwhile MRLs of MAR residue in fish musculature not reported, it reaches up to 150 ug/kg at bovine, porcine muscle, liver and kidney (EC, 2010). European commission has set the approved administrative MRLs for FQs in animal tissue at a range 10-100 µg/kg. MAR Acceptable Daily Intake (ADI) is 4.5 µg/kg b. wt (EMEA, 2000).

Quinolones were one of the most frequently detected antibiotics in aquaculture farms, indicating its wide allocation; subsequently it has high level of residues than others (Thiang *et al*., 2021). Turnipseed *et al*. (2019) added MAR and orbifloxacin to the scope of analytes monitored in aquaculture products. As detected by Song *et al*. (2017), FQs represented 95.69% of all fish samples, where MAR represented (11.21%) with average residual level in crab exceeded the MRL (100 µg/kg), indicating potential consumption risk that require further consideration. As well, Yipel *et al*. (2017) recorded that 12% of examined fish had MAR residues. FQs residue levels ranged between 15 and 1270 µg/kg in which 17.3% of samples exceeded the MRL, indicated the lack of antibiotic withdrawal time knowledge besides their public health concern. Moreover, Shengguang *et al*. (2015) detected MAR at a rate of 4.2% of grass carp samples; where no residues were detected in small fish contrary to larger fish (179.6 µg/kg), which signified its body accumulation. Conversely, Cheng *et al*. (2014) not detected MAR residue in studied fish musculatures with limit of detection (LOD) = 37.5 µg/kg. Optimistically, the low occurrence of antibiotics in farm fish suggested responsible management of aquaculture where the samples were collected (Guidi *et al*., 2018). Similar pattern of results was recorded by El-Sayed *et al*. (2019) in MAR treated catfish, where it needed at least 4 days post dosing of 10 mg /Kg b. wt /5 days to avoid all tissues residues. Qi *et al*. (2017) found that oral dose of 10 mg/kg body mass MAR has t1/2 = 22.67 h in tilapia. Moreover, EMEA (2000) found that MAR residues in 2 rat studies, turned down to 16-23 µg/kg and 27-44 µg/kg in muscle, 4 days PT with 2 mg/kg b. wt/day for 5 days. Then they were below 8.5 µg/kg post 8 days in most tissues. Comparing with other species, MAR residue depletion in broilers post oral dose (5 mg/kg/day for 3 days) showed that kidney needed 4 days, while liver and muscle needed 3 days as withdrawal time (Yang *et al*. 2014). While Abdel Aziz *et al*. (2017) found that MAR residues in rabbit pectoral muscles post intramuscular dose (2 mg/kg b. wt for 5 days) were 77±3 and 19±4 µg/kg at 1st and 3rd day PT respectively, while it not detected at the 5th day PT. Meanwhile, in thigh muscle it was 61.5±2 µg/ kg at 1st day only and not detected at the 3th day PT. Differences in the frequency and concentration of reported MAR residues may be attributed to several factors; degrees of tissue penetration in different species, age, size, pathological status or even inter individual variation, besides changes in pharmacodynamics and pharmacokinetics of MAR in different fish species, drug formulation, period, dosage, routes of administration, metabolism and analytical techniques as well as changes in environmental factors.

Conclusion

Recently medicinal plants including VAC play a key role in drug discovery as a vital source of non or less toxic, cost-effective, easily accessible, and safe natural resources of alternative antimicrobials. Based on our GC-MS analysis, therapeutic properties of VAC were attributed to its bioactive ingredients enclosed vital fatty acids, fatty alcohol, antioxidants, phenolics among others. Post evaluated the in vitro and in vivo antimicrobial activity of VAC ethanolic extract against *V. anguillarum* in both prevention and treatment assays either alone or combined with MAR; it could be concluded that VAC possess a bactericidal activity as MAR against *V. anguillarum* to be a good choice as an effective natural antibiotic and antioxidant. It combated *V. anguillarum* challenge, increased growth indices and survivability, decreased lipid peroxidation and oxidative stress besides improved liver and kidney functions. MAR residue in fish musculature needed at least 4 days as a withdrawal period to be less than the MRL. VAC showed no antagonistic interaction with MAR; it had additive effect through increasing survivability and decreasing MAR residues less than the MRL from the 1st day post treatment. Further research are needed to investigate the pharmacokinetic properties of MAR combined with VAC in fish to explain the discrepancy in its residues with/ without the extract depends on pharmacokinetic-pharmacodynamic (PK /PD) relationships.

Conflict of interest

The authors declare that they have no conflict of interest.

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