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Assessment of some medicinal plant's potential as anti-infectious agents for red Tilapia fish pathogens

Presented by

- AIT ALI SAID Ryma
- ALLAOUI Maroua

Jury members:

President	Dr. SOUANA K.	MCA
Examiner	Dr. ACHIR M.	MCA
Supervisor	Pr. AIT ABDERRAHIM L.	Professor
Co- Supervisor	Pr. TAIBI K.	Professor

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Abstract

Tilapia, a crucial species in global aquaculture, faces significant threats from microbial infections, leading to economic losses and potential transmission of zoonotic diseases. Excessive use of antibiotics to treat these infections has resulted in antibiotic resistance.

This study evaluates the antimicrobial effects of aqueous and ethanolic extracts of medicinal plants: *Rosmarinus tournefortii*, *Cedrus atlantica*, *Juniperus oxycedrus*, *Allium sativum*, and *Ocimum basilicum* against 13 pathogenic microorganisms, including strains affecting Tilapia: 2 strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli*, *Enterobacter cloacae*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and 2 strains of *Candida albicans*.

Subsequently, plants exhibiting the best antimicrobial activity were selected for in vivo testing on Tilapia infected with *P. aeruginosa*.

Results indicate that all tested plants possess antimicrobial activity against the tested microbial strains at varying concentrations. Garlic, however, showed no effect on the tested microorganisms. Generally, ethanolic extracts of all plants were more effective against the tested microorganisms at lower concentrations compared to aqueous extracts. Ethanolic extract of rosemary showed the lowest MIC values compared to extracts from other plants, while Atlas cedar's aqueous extract exhibited the lowest MIC values among the aqueous extracts.

Regarding in vivo experimentation, juniper, Atlas cedar, and rosemary were selected and tested as anti-infective and immunostimulant agents for Tilapia infected with *P. aeruginosa*. Hematological analyses indicated that Atlas cedar and juniper induce better resistance to infection compared to rosemary.

Keywords: Tilapia, aquaculture, natural products, infections, *Cedrus atlantica*, *Rosmarinus tournefortii*, *Allium sativum*, *Ocimum basilicum* L., antimicrobial activity, ethanolic extract, aqueous extract.

Resumé

Tilapia, une espèce essentielle dans l'aquaculture mondiale, est largement menacée par des infections microbiennes, ce qui entraîne des pertes économiques et une transmission potentielle de maladies zoonotiques. L'utilisation excessive d'antibiotiques pour traiter ces infections a conduit à la résistance aux antibiotiques.

Cette étude évalue l'effet antimicrobien des extraits aqueux et éthanolique de plantes médicinales : *Rosmarinus tournefortii*, *Cedrus atlantica*, *Juniperus oxycedrus*, *Allium sativum* et *Ocimum basilicum* contre 13 microorganismes pathogènes dont certains du Tilapia : 2 souches de *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli*, *Enterobacter cloacae*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* et 2 souches de *Candida albicans*. Par la suite les plantes présentant la meilleure activité antimicrobienne ont été sélectionnées pour les tester in vivo sur le Tilapia rouge infecté par *P. aeruginosa*.

Les résultats montrent que toutes les plantes testées possèdent une activité antimicrobienne contre les souches microbiennes testées à des concentrations différentes. L'exception est observée chez l'ail qui n'a eu aucun effet sur les microorganismes testés.

De manière générale, les extraits éthanoliques de toutes les plantes sont plus efficaces contre les microorganismes testés à des concentrations faibles par rapport aux extraits aqueux.

L'extrait éthanolique du romarin a montré les plus basses valeurs de CMI comparé aux extraits des restes des plantes, alors que l'extrait aqueux du cèdre a montré les valeurs les plus basses de CMI par rapport aux autres extraits aqueux.

Concernant l'expérimentation in vivo, le genévrier, le cèdre ainsi que le romarin ont été sélectionnés et testés comme agents antiinfectieux et immunostimulants du Tilapia rouge infecté par *P. aeruginosa*. Les analyses hématologiques ont montré que le cèdre et le genévrier induisent une meilleure résistance à l'infection comparée au romarin.

Mots clés : Tilapia, aquaculture, produits naturels, infections, *Cedrus atlantica*, *Rosmarinus tournefortii*, *Juniperus oxycedrus*, *Allium sativum*, *Ocimum basilicum* L., activité antimicrobienne, extrait éthanolique, extrait aqueux.

ملخص

يعتبر سمك البلطي نوعاً مهماً في الاستزراع المائي العالمي بسبب قيمته الاقتصادية العالية وشعبيته كغذاء صحي. ومع ذلك، يواجه سمك البلطي تحديات بيئية وصحية، بما في ذلك العدوى البكتيرية التي تؤدي إلى خسائر اقتصادية كبيرة وإمكانية نقل العدوى إلى الإنسان. أدى الاستخدام المفرط للمضادات الحيوية في علاج هذه العدوى إلى ظهور مقاومة المضادات الحيوية.

تقوم هذه الدراسة بتقييم التأثيرات المضادة للميكروبات لمستخلصات النباتات الطبية المائية والإيثانولية من: إكليل الجبل، وأرز الأطلس، والثوم، والعرعر، والريحان ضد 13 ميكروباً ممرضاً، بما في ذلك السلالات التي تؤثر على سمك البلطي: سلالتان من المكورات العنقودية الذهبية، المكورات المعوية البرازية، العصوية الشمعية، العصوية الرقيقة، الليستيريا المستوحدة، الإشريكية القولونية، المعوية كلواسيا، الأمعائية المدرة، السلمونيلا المعوية، الكليبيلا الرئوية، الزائفة الزنجارية، و سلالتين من المبيضات البيضاء.

تم اختيار النباتات التي تظهر أفضل نشاط مضاد للميكروبات لاختبارها في سمك البلطي الأحمر المصاب بالزائفة الزنجارية.

تشير النتائج إلى أن جميع النباتات التي تم اختبارها تمتلك نشاطاً مضاداً لسلالات الميكروبات المختبرة عند تراكيز متفاوتة. لم يظهر الثوم أي تأثير على الميكروبات المختبرة. بشكل عام، كانت مستخلصات الإيثانول من جميع النباتات أكثر فعالية ضد الميكروبات المختبرة عند التراكيز المنخفضة مقارنة بالمستخلصات المائية. أظهر مستخلص إكليل الجبل أقل قيم للحد الأدنى لتنشيط النمو مقارنة بالمستخلصات من النباتات الأخرى، بينما أظهر مستخلص أرز الأطلس الأدنى قيم لتنشيط النمو بين مستخلصات النباتات المائية.

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الكلمات المفتاحية: سمك البلطي ; الاستزراع المائي ;المواد الطبيعية ;العدوى الميكروبية ;الثوم ; العرعر ;إكليل الجبل ;الأرز الأطلسي ;الريحان

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LIST OF ABBREVIATIONS

FAO	Food and Agriculture Organization
DMSO	Dimethyl sulfoxide
Aq	Aqueous extract
Eth	Ethanolic extract
MIC	Minimum Inhibitory Concentration
<i>O. basilicum</i>	<i>Ocimum basilicum</i>
<i>C. atlantica</i>	<i>Cedrus atlantica</i>
<i>J. oxycedrus</i>	<i>Juniperus oxycedrus</i>
<i>R. tournefortii</i>	<i>Rosmarinus tournefortii</i>
<i>A. sativum</i>	<i>Allium sativum</i>

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INTRODUCTION

Because of the expanding global population, humanity faces numerous nutritional challenges. Aquaculture, the farming of aquatic organisms has become one of the fastest-growing sectors in global food production (Naylor et al., 2021). It plays a crucial role in meeting the rising demand for sea food (Ahmad et al., 2021) and has become a valuable component of national development and poverty reduction plans in many areas of the world (Prabu & Santhiya, 2016). This industry has become an increasingly important source of food, employment, and economic activity around the world

Among the various species cultivated in aquaculture tilapia become one of the most farmed fish due to his tolerance to the environment condition and to their rapid growth, ease of breeding, and his high nutritional value (Ashouri et al., 2023). However, tilapia is susceptible to various microbial infections including bacteria, fungi, parasite and virus leading to significant economic losses due to reduced growth, increased mortality, and treatment costs. Certain microbes that infect tilapia can be zoonotic where pathogens can be transmitted from fish to humans, posing a risk to public health (Haenen et al., 2023).

Recently, the popularity of natural products such as honey, minerals, and medicinal plants as an alternative to antibiotics and a natural immunostimulant is gaining in demand and importance in medical purposes and ecofriendly. The incorporation of some plants extracts for the prevention and treatment of microbial infections is likely to increase the effectiveness because some materials can work synergistically, so that the infection in the fish body can be controlled optimally (Hardi et al., 2019).

By integrating natural therapeutics into aquaculture practices, it is possible to mitigate the impacts of infections, reduce economic losses, and safeguard public health, ensuring the sustainability and resilience of the aquaculture industry (Troell et al., 2023).

This study, aims to evaluate the antimicrobial activity of ethanolic and aqueous extracts of *Cedrus atlantica*, *Juniperus oxycedrus*, *Rosmarinus tournefortii*, *Allium sativum* and the ethanolic extract of aerial parts (stems and leaves) of *Ocimum basilicum* against some pathogenic microorganisms frequently encountered in fish infections. Afterwards, the plants showing the best antimicrobial activity will be tested in vivo, in tilapia fish infected with the bacteria *Pseudomonas aeruginosa*.

LITERATURE REVIEW

1. Aquaculture

Aquaculture is the science of cultivating, breeding, and enhancing aquatic organisms that are economically important (Hutu, 2020), plays a significant role in global efforts to eradicate hunger and malnutrition. By providing fish and other aquatic products rich in protein, essential fatty acids, vitamins, and minerals, aquaculture addresses nutritional needs. Additionally, it boosts development by enhancing incomes, providing employment opportunities, and maximizing the benefits of resource utilization (Subasinghe et al., 2009).

A key branch of aquaculture is fish farming, where fish are commercially raised in tanks or enclosures primarily for food purposes. Among the most frequently cultivated species in fish farms are tilapia, salmon, carp, and catfish (Araújo et al., 2022).

2. Tilapia

Tilapia (Fig. 1) is an important aquatic animal for food security, with a global annual production reaching 5.5 million tons in 2020 (Paimeeka et al., 2024). And it became the second-most farmed fish in the world, after carp, it originates from Africa and the Middle East, and has become extensively farmed worldwide, making it the most prevalent fish species in aquaculture. Its cultivation spans across more than 120 countries globally. In 2020, global production of tilapia surpassed 6 million tons, with Asia serving as the primary contributor. Key tilapia-producing nations include China, Indonesia, Egypt, Brazil, the Philippines, Bangladesh, Vietnam, Thailand, and Colombia (Charo-Karisa, 2024).



Figure 1 — Red tilapia.

Tilapia is a mild-flavored fish that provides a good amount of protein and important minerals. It contains vitamins and minerals such as choline, niacin, vitamin B12, vitamin D, selenium, and

phosphorus. Additionally, Tilapia is a good source of omega-3 fatty acids, which are essential for various bodily functions and overall health (Islam et al., 2021).

Among aquacultured fish, tilapias are preferred due to their fast growth (Sewaka et al., 2019). Lower trophic level, and adaptability to both saltwater and freshwater environments in tropical and subtropical regions. Primarily herbivorous, tilapia feed on phytoplankton and other aquatic vegetation, but they also accept lipids and proteins from plant or animal pellets. Ecologically, tilapia contributes to the circulation of nutrient metabolites, which is essential for production (Caruso et al., 2024).

Tilapia is a common name used for various cichlid species belonging to three genera, with more than 70 species identified. These genera include *Oreochromis*, known for maternal mouthbrooding behavior, *Sarotherodon* which comprises paternal and biparental mouthbrooders and *Tilapia*, characterized as substrate incubators. Some popularly cultured tilapia species include Nile tilapia (*Oreochromis niloticus*), Blue tilapia (*Oreochromis aureus*), Mozambique tilapia (*Oreochromis mossambicus*), and red (*Oreochromis* spp.) (FAO, 2018).

Red tilapia is a freshwater fish typically refers to hybrids between two or possibly more species of tilapia, selectively bred for a red color morph. The term "red" generally encompasses various color variants resulting from the absence or reduction of normal black pigmentation on the skin compared to the wild-type fish (Hilsdorf et al., 2002). Red tilapia was first developed in Taiwan, Province of China, during the late 1960s. Initially, it emerged as a genetic mutation resulting from the crossbreeding of female reddish-orange Mozambique tilapia (*O. mossambicus*) with ordinary male Nile tilapia (*Oreochromis niloticus*) (Lu et al., 2022). Following this breakthrough, various countries, including the USA, further refined the red tilapia strains using different genetic lines, thereby altering the original strain's genetic composition (Lingam et al., 2021).

Red tilapia, scientifically known as *Oreochromis* sp., is gaining popularity among producers for several reasons. Its attractive color, enhanced marketability, and notable tolerance to high salinity in certain strains make it increasingly sought after in aquaculture (Vadhel et al., 2017).

Efforts are underway in Algeria to boost Red Tilapia production through local reproduction and aquaculture initiatives. Notably, the Cosider aquaculture farm in Babar municipality in Khenchela province has seen an increase in production from 25 tons in 2021 to 40 tons in 2022, with plans to further expand to 250 tons by the end of 2023. The positive outcomes of these endeavors have prompted the adoption of Tilapia farming in other regions

particularly El Oued, Ghardaia, and Ouergla, contributing to the reinforcement of local production and enhancing food security measures (Mounia, 2023).

2.1 Morphological characteristics

Red Tilapia exhibits a streamlined body shape, displaying hues that range from gray, albino, pink, to red-orange (Moralee et al., 2000). In most cases, the morphological characteristics of the Red Tilapia are intermediate (snout shape, mouth width, head length, etc.) between the species used in this crossbreeding.

The Red Tilapia shares a similar overall profile to the "bluegill". Its body is notably more compressed compared to other tilapia species and is uniformly red with a pinkish underside, turning reddish-white after death.

Red Tilapia has a small head and medium-sized eyes. Their morphological traits include an anal fin with 3 spines and 9 or 10 rays, a rounded caudal fin, and a dorsal fin with 15 to 18 spines and 10 to 13 rays. They possess 16 to 22 gill rakers on their first arch, and their lateral line is interrupted. Their body is adorned with cycloid scales (Red Tilapia, 2021).

2.2 Tilapia reproduction process

The reproductive cycle of Tilapia begins with male individuals seeking out a suitable territory, where they proceed to excavate and construct a nest. The male tilapia then assumes the responsibility of guarding and caring for this nest. Mature female Tilapia deposits their eggs within this nest, which the male fertilizes. Subsequently, the female retrieves the fertilized eggs, holding them in her mouth for the incubation period until the absorption of the yolk sac completes (Pedrazzani et al., 2023).

2.3 Optimal environmental conditions for red tilapia

Tilapia exhibit growth and reproductive capabilities even in environments with salinity concentrations reaching up to 36 parts per thousand. They demonstrate high tolerance to low levels of dissolved oxygen (DO). Typically, their optimal growing temperatures fall within the range of 22°C (72°F) to 29°C (84°F) (Towers, 2019). It can tolerate pH levels ranging from 3.7 to 11, but their optimal growth occurs within the pH range of 7 to 9 (El-Hack et al., 2022).

2.4 Infections in Tilapia

Tilapia culture is challenged by various infectious diseases, predominantly those caused by microbial bacterial, fungal and parasitic pathogens, these pathogens can spread rapidly in high-density aquaculture settings, leading to high mortality rates, this isn't just a heartbreaking loss of fish for farmers, it translates to severe economic burdens (Debnath et al., 2023).

For example; Egyptian aquaculture governorates namely Kafr El Sheikh, Beheira and Sharqia were affected with summer mortality syndrome (this syndrome is related to different bacteria including *Aeromonas*, *Vibrio*, *Streptococcus*, *Pseudomonas*, *Enterococcus*, and *Edwardsiella* spp.) with an average mortality rate of 9.2% and a potential economic impact of around US\$100 million (Ali et al., 2020) .

Additionally, these infectious diseases pose potential threats to public health due to their zoonotic potential (Haenen et al., 2023), a zoonosis is an pathogen that can be transmitted from animal including fish to human (Ziarati et al., 2022) causing different disease and infection that can lead to death , for example in Osaka, Japan in 1950 a bacteria *Vibrio parahaemolyticus* caused an outbreak, involving 272 patients and causing the death of 20 people after the ingestion of Shirasu, a semi dried juvenile sardine (Sanches-Fernandes et al., 2022), according to (Hounmanou et al. 2019) each year in the united stat 260,000 people get sick from contaminated fish what make Fish the most commonly implicated food category in outbreaks.

2.4.1 Bacterial infections

The most common bacteria that infect tilapia are:

a. Streptococcosis

It is a major cause of damage to the global aquaculture industry, particularly in tilapia culture. Additionally, it can be transmitted from fish to humans. *Streptococcus* species are Gram-positive, spherical or ovoid, non-spore-forming, non-motile, and facultative anaerobic bacteria. There is a notable incidence of *S. iniae*, *S. agalactiae*, and *S. dysgalactiae* infections, which are typically observed during hot and dry seasons. Infection by *Streptococcus* leads to various clinical signs, which include hemorrhages at the gill plate, loss of appetite, spine displacement, hemorrhages in the eye, corneal opacity, hemorrhages at the base of the fins and in the opercula, and dark body coloration. The most prominent signs are uni-lateral or bi-lateral exophthalmia, also known as 'pop eye' (Fig. 2) (Abdelsalam et al., 2017).

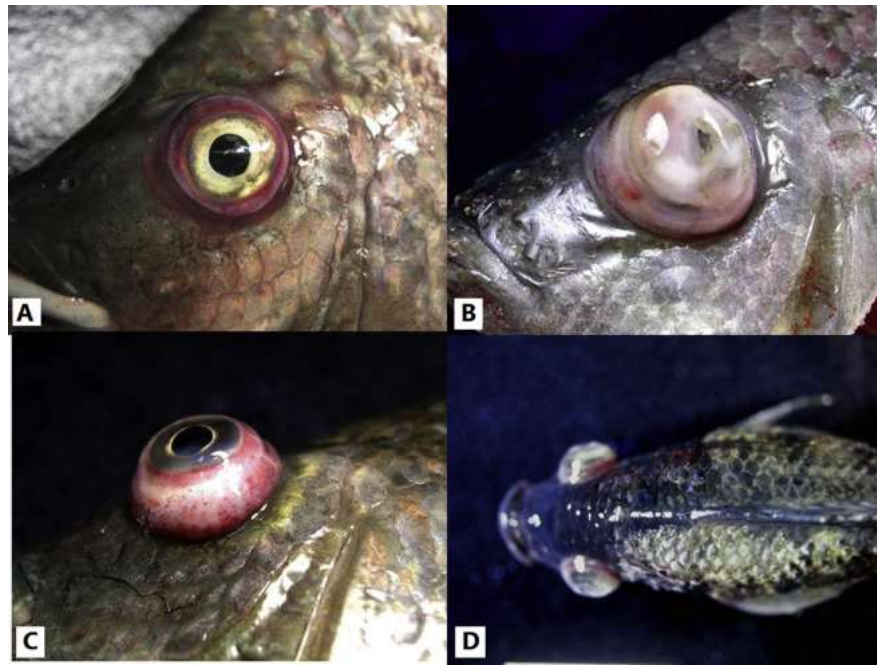


Figure 2 — Pathological features of *Streptococcus agalactiae* infection in tilapia, (A) showing conjunctival hemorrhage, (B) eye hemorrhage and cataract, (C) exophthalmia and hemorrhage, (D) bilateral exophthalmia and skin hemorrhage (Wang, Maekawa and Chen, 2022).

b. Aeromonas

Aeromonas bacteria, particularly *Aeromonas hydrophila* and *Aeromonas sobria*, are among the most common pathogens infecting wild and cultured tilapia. They are characterized as gram-negative, flagellated, facultative aerobic, non-spore-forming, catalase and oxidase positive organisms.

In infected tilapia with *A. hydrophila*, typical symptoms include circular or elliptical skin erosion, 'rotten skin', and dorsal fin deterioration. The early stages of infection manifest as hemorrhagic lesions on the body surface. The disease tends to occur in low-volume, high-density aquaculture systems of tilapia during the rainy season and colder months when water temperatures decrease, leading to high mortality rates. Bacterial isolation commonly reveals the presence of Aeromonas in various organs such as the liver, kidney, spleen, gall bladder, and opaque eyes of infected fish (Fig. 3) (Lema B, et al 2021).



Figure 3 — *Oreochromis niloticus* naturally infected with *Aeromonas hydrophila* showing skin hemorrhage (Aboyadak, 2015).

c. *Francisella noatunensis* subsp

Francisella noatunensis subsp, it is one of the main causes of the mortality in tilapia and other species found in both warm and cold waters. *Francisella noatunensis* subsp. has a zoonotic potential it can be transmitted from fish to humans.

They are Gram-negative bacteria, strictly aerobic, facultatively intracellular, and characterized by immobile coccobacilli. Among the most species that infect tilapia are *F. tularensis*, *F. novicida*, and *F. noatunensis* (Ortega et al., 2016).

This bacterium infects various organs including the kidney, spleen, liver, heart, gastrointestinal tract, and gonads. Tilapia infected with *Francisella* exhibit the presence of numerous white granulomas on their gills and internal organs, including the spleen, liver, kidney, and intestine (Fig. 4). Francisellosis can result in mortality rates ranging from 50 % to 60 % in cultured tilapia, typically occurring during cooler seasons (Nguyen et al., 2015).

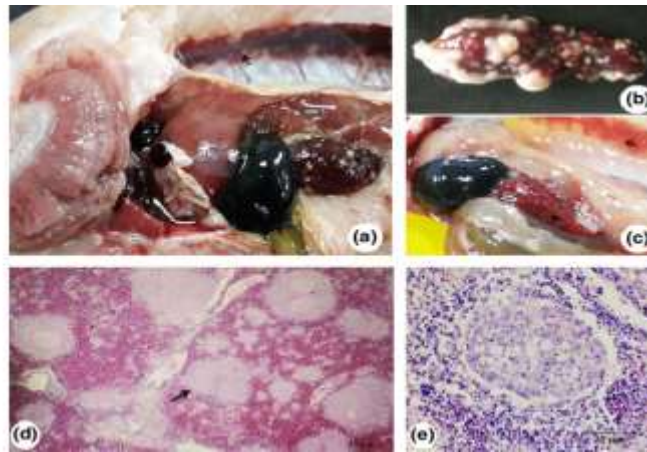


Figure 4 — Red tilapia infected with *Francisella noatunensis* subsp. *orientalis* showed white nodules in the internal organs (arrows) of naturally infected fishes (a, b) and experimentally infected fish (c). Haematoxylin and eosin-stained sections of the spleen show the granulomatous lesions (d), typical granuloma with central necrosis surrounded by epithelioid macrophages (e) (Nguyen et al., 2015).

d. Flavobacteria

It is one of the common bacteria that infect tilapia farmed fish. Flavobacteria is a gram-negative rod-shaped bacteria with no spore forming and strictly aerobic. It affects different fish species culturing in cold or warm water including *Oreochromis* spp. (Wahli and Madsen, 2018). This bacteria in tilapia affect the skin, gills, muscles and is found rarely in the internal organs, in particular *Flavobacterium columnare* that cause columnaris that affect fish in hatcheries and grow out system with mortality of 10%–70%. It spreads rapidly and is associated with culture condition especially stress, the signs of this infection is discoloration, respiratory discord, skin erosion and gill necrosis (Fig. 5) (Waśkiewicz and Irzykowska, 2014).

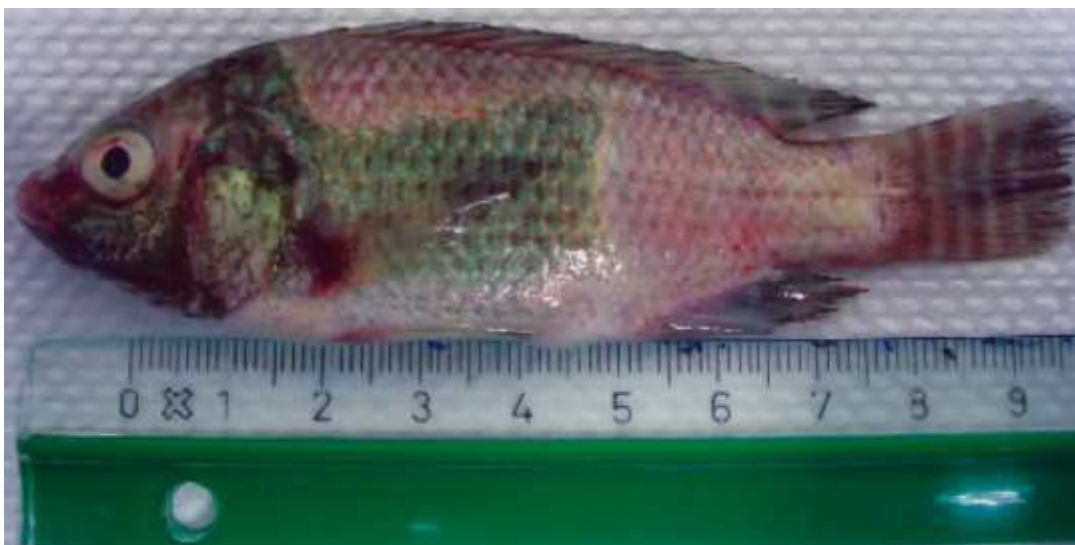


Figure 5 — *F. columnare* infection symptoms in tilapia (Global Seafood Alliance, 2024).

e. *Vibrio*

Vibrio is one of the bacteria that can cause a serious problem to the cultured tilapia. It is a Gram-negative rod-shaped bacterium that do not form spores, facultative anaerobes and have a polar flagellum. It can affect fresh water and salty water fish.

Vibrio is responsible of the vibriosis fish disease, it affects the kidney, liver, brain and spleen with a high mortality. The condition of culture like the water quality, temperature ..., has an important impact on the susceptibility of tilapia to vibriosis.

There is different vibrio species that can infect tilapia including *Vibrio vulnificus*, *Vibrio harveyi* and *Vibrio mimicus*, the symptoms of the infection are dark coloration, exophthalmia, skin ulcers and external haemorrhagic areas, a pale liver with haemorrhagic lesions and oedematous brain (Fig. 6). *Vibrio* is considered one of the zoonotic bacteria that can be transmitted from fish to human (Haenen et al., 2023).



Figure 6 — Artificially infected Nile tilapia showing hemorrhagic, ulceration and lesions on the skin surface, rot fins, and exophthalmia on the abdominal parts (Ogundiran MA, et al 2021).

f. *Pseudomonas*

Pseudomonas are gram negative bacteria with a rod shape that live in aerobic condition, it can infect tilapia in winter and spring with a high mortality at low water temperature.

Pseudomonas fluorescens, *P. putida* and *P. aeruginosa* are often isolated from the infected tilapia, and the infection shows different symptoms like exophthalmia and dark skin coloration, nodular lesions and focal necrosis in different internal organ liver, kidney spleen (Fig. 7) (Haenen et al., 2023).



Figure 7 —Tilapia fish infected by *Pseudomonas aeruginosa* (Naena, 2020).

g. Klebsiella pneumoniae

According to Vaneci-Silva et al. (2022) *Klebsiella pneumoniae* caused a high mortality in juvenile Nile tilapia in Brazil, the clinical signs of the infection are lethargy, anorexia, subcutaneous haemorrhages, urogenital bleeding, and ascites (Fig. 8).

K. pneumoniae is described as a gram-negative, non-motile bacterium that is becoming increasingly resistant to antibiotics (Ashurst & Dawson, 2023).



Figure 8 — Tilapia fish infected by *K. pneumoniae* (Vaneci-Silva et al., 2022).

h. Enterobacter sp

Enterobacter sp are a gram negative bacteria that reported as a living opportunistic pathogens in fish, *Enterobacter* exhibited pale internal organs such as liver and kidney, and caused anemia, intestinal congestion, ulceration of the anus accompanied by mucus and bleeding in its infected external organs (Hardi et al., 2018), and cause a high mortality rate (43, 33%) (Aly et al., 2012), the most common *Enterobacter* species that infect tilapia are *Enterobacter cloacae* and *Enterobacter cancerogenus* (Ahmed, 2019).

i. *Enterococcus faecalis*

Is one of the most bacteria that can infect tilapia, the clinical signs of this infection are anorexia, detached scales, dark skin pigmentation, slight exophthalmia, and redness on the skin and base of the fins leading to a high mortality rate (Fig. 9) (Zahran et al., 2019).

E. faecalis is are gram-positive cocci that are grouped in chains and have resistance against commercial antibiotics (Rahman et al., 2017).



Figure 9 — *E. faecalis* infection in tilapia shows bilateral opacity and sign of asphyxiation (Rahman et al., 2017b).

j. Other bacteria that infect tilapia

Listeria monocytogenes is an important pathogen, often associated with fish (Zakrzewski et al., 2023). It is a gram-positive facultatively aerobic rod-shaped bacteria (Duze et al., 2021).

According to Fernandes et al. (2018) studies have identified a 10.4 % prevalence of *Salmonella* in fresh fish in Iran, with the detection of five different serotypes: *S. Typhimurium*, *S. Enteritidis*, *S. Typhi*, *S. paratyphi* B, and *S. newpor*.

Escherichia coli is a gram-negative bacillus known to be a part of normal intestinal flora (Mueller & Tainter, 2023). However, this bacterium can also be pathogen to fish (Trang et al., 2023).

Table 1 summarizes other most frequently encountered bacteria that infect Tilapia fish.

Table 1 — Other common bacteria that infect Tilapia.

Bacteria	Characteristics	Symptoms	Zoonotic potential	References
<i>Staphylococcus epidermidis</i> <i>Staphylococcus aureus</i>	gram-positive cocci bacteria facultative anaerobe	White nodules and lesions in the spleen and anterior kidney	+	(Haenen et al., 2023)
<i>Edwardsiella</i> <i>E. tarda</i> <i>E. ictaluri</i> <i>E. anguillarum</i>	short-rod, Gram-negative, facultatively anaerobic	40%–90% mortality white spots appearing on the spleen and head kidney	+	Olga L. M. Haenen et al. (2023)
<i>Lactococcus garvieae</i>	Gram-positive, ovoid cocci bacteria, non-spore forming, facultatively anaerobic	Skin erosion and scale detachment , dark skin pigmentation	+	Egger et al. (2022)
<i>Aerococcus viridans</i>	Gram-positive, facultatively anaerobic	Spiral movement, loss of scales	+	Elgohary et al. (2020)
<i>Mycobacterium marinum</i>	Gram-positive, aerobic, pleomorphic	Uncoordinated swimming, abdominal swelling, loss of weight, skin ulceration, white nodule formation as granuloma in liver, kidney	+	El-Sayed (2019)
<i>Nocardia</i>	Gram-positive rod-shaped bacteria aerobic	Skin ulcers, and red spots. Brownish or haemorrhagic gills, abscess inside the operculum, a greyish	+	Olga L. M. Haenen et al. (2023)

		or haemorrhagic liver with white nodules , mortality is mostly chronic and may in cases reach 30%		
<i>Chlamydia spp.</i>	Gram-negative, anaerobic coccoid or rod-shaped	Tiny white cysts in the gill or skin epithelium	+	(El-Sayed, 2020)

2.4.2 Fungal infections

a. *Saprolegnia* spp

It's a type of fungi that cause Saprolegniasis disease in fish (Fig.10), the clinical symptoms of this infection are white cottony patches on the infected fish mainly skin, eyes, mouth and gills. It also causes erosion in the fish body, systemic mycosis of the liver, spleen, eyes and kidney and a high mortality. The most prevalent species is *Saprolegnia parasitica* and *Saprolegnia ferax* (Smith, 2019). The saprolegniasis disease also can be caused by *Achlyaspp* (El-Sayed, 2019).



Figure 10 — Tilapia infection by *saprolegnia* spp. (Microbiología, 2016).

b. *Branchiomyces* spp.

It is a genus of branchiomycetes that cause *Branchiomyces* (Fig. 11), the common species that cause this infection are *Branchiomyces sanguinis* and *Branchiomyces demingrants*. The infected fish suffers from a rapid movement of the gill operculum, damage on the gills tissue and

a massive mortality. The poor quality of the water is one of the main causes of this infection (Choudhury et al., 2014).



Figure 11 — Contaminated fish with *branchiomyces* (Goodwin, 2014).

c. *Candida albicans*

According to Zayed et al. (2016) *Candida albicans* can cause erosion with congestion of external surface of fish and darkening of skin and eye opacity, the combination between *C. albicans* and *S. iniae* shows the highest mortality rate (Oda, Tohamy and Massoud, 2016).

2.4.3 Viral infections

a. Tilapia lack virus

TiLV is a negative sense single stranded RNA virus belonging to the family *Amnoonviridae*, the virus infects the vital organ of the fish causing mortality up to 90%.

The pathological signs of the infection are gross lesion, corneal opacity, loss of appetite, pale coloration of the body, skin erosion, congestion of the spleen and kidney and foci of gliosis (Fig. 12) (Aich et al., 2022).

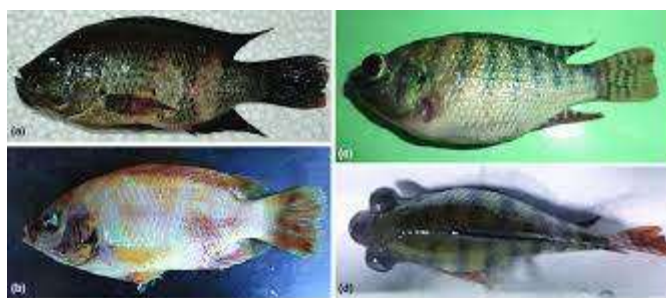


Figure 12 — TiLV infected Nile Tilapia and red tilapia (Dong et al, 2017).

2.4.4 Parasitic infections

a. *Alitropus typus*

It is a parasitic isopod that causes important damages to the aquaculture and causes 50 to 100% mortalities within 2 to 7 days. These parasites feed by sucking the blood of their host. The observed signs of the infection are degenerative lesions in the gills of infested fish, including destruction, detachment, hyperplasia and fusion of the primary and secondary gill lamellae (Fig. 13). Two common parasites are cymothoid isopods *Nerocilaorbegnyi* and *Rencilathresherorum* (El-Sayed, 2019).



Figure 13 — *Alitropus typus* infect fresh water fish (Bhakta et al., 2021).

b. *Lernaea* sp.

Commonly known as anchor worms, are a type of parasite that cause a varying loss in tilapia culture. The head of lernaea deeply embed in fish skin and muscles causing inflammatory lesion and reduction in the fish growth (Fig. 14) (Hossain et al., 2013).



Figure 14 — *Lernaea cyprinacea* infecting *Oreochromis mossambicus*. (B) Skin damage from *L. cyprinacea* infection, before drought. (C) Uninfected *O. mossambicus*, during drought. (Welicky et al., 2017).

2.5 Co-infection in Tilapia

Co-infection is the presence and the interaction of two or multiple different pathogens in a host either as simultaneous or as secondary concurrent infection and it can potentially affect the host's immunological responses, clinical outcomes, survival, and disease control efficacy. *A. hydrophila* and *Edwardsiella tarda* is an example of bacterial co-infection that cause pigmentation loss, exophthalmia, eye opacity and swelling at abdomen with a significant mortality. Another co-infection between parasite and bacteria *F. columnare* and *Myxobolus tilapia* causes a mass mortality (Abdel-Latif et al., 2020).

According to Basri et al. (2020) the most common co-infection in tilapia is TiLV and *A. hydrophila* and *Streptococcus agalactiae* causing a high mortality, deformation of kidney and gross lesions (Fig. 15).

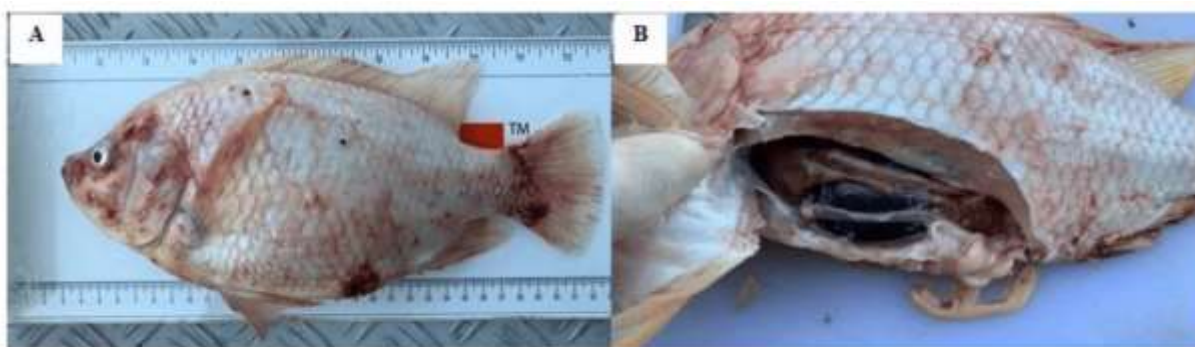


Figure 15 — Clinical signs and gross lesions of red hybrid tilapia naturally co-infected by Tilapia Lake Virus, *Aeromonas hydrophila* and *Streptococcus agalactiae* (Basri et al., 2020).

2.6 Management and control of the infection

Infections, especially bacterial ones, are usually treated with antibiotics like Sarafloxacin to treat vibriosis infection, Oxytetracycline for *Aeromonas hydrophila*, *A. sobria* and *Pseudomonas*, Lincosamides for *Staphylococcus* species, Amoxicillin for *Streptococcus* (Debnath et al., 2023). Nevertheless, antibiotics resistance has been observed in the major bacteria specially in *Streptococcus* and *Aeromonas* (Mawardi et al., 2023).

Antibiotic resistance in bacteria, residues in fish tissue, immune system inhibition, and harm to the beneficial microbial flora prompted scientists to investigate alternate techniques to microbial infection management in tilapia farming (Okeke et al., 2022).

Researchers are currently exploring new treatment using natural product to combat antibiotic resistance and improve Tilapia culture.

3. Natural products

Nature offers a vast array of substance with a healing property that can treat different infections including tilapia infection, it can be from plant, animals, microorganism and minerals (Criollo-Mendoza et al., 2023).

Natural products are commonly utilized to enhance health and resist diseases of human, livestock, and poultry. In recent decades, their use has also expanded significantly in fish aquaculture, is increasingly favored over antibiotics and chemicals due to their outstanding therapeutic efficacy, low toxicity, minimal side effects, diverse mechanisms of action, and reduced drug resistance (Zhang et al., 2022).

3.1 Plant as a treatment to tilapia infection

These last years, scientists focused on exploring new products from nature to treat tilapia infection and improve the resistances against diseases. There is different experiment that proved that using plant in aquaculture can have a therapeutic effect and avoid losses because of their richness in secondary metabolites and phytochemical compounds that have antimicrobial activities covering flavonoids, tannins alkanoides and phenolic compounds (De Oliveira et al., 2020).

Different plant parts can be used like seeds, leaves, root, rhizome, tree bark, fruit, bulbs and it can be used in different preparation as an extract, oil or powder.

Garlic *Allium sativum* shows a good result in controlling fungal infections in Nile Tilapia against *Saprolegnia parasitica* (Aly et al., 2023). Garlic extract contains Allicin that's an active molecule that can kill bacteria and can clear the blood from its toxins, it also can be used to insure immune system in tilapia fish (Lestari et al., 2021).

The essential oil of clove basil (*Ocimum gratissimum*) and ginger (*Zingiber officinale*) improved the immune response against *S. agalactiae*, the main compound that is found in the essential oil of clove basil is 1,8-cineole, also known as eucalyptol which has an antimicrobial and anti-inflammatory activity. In ginger the main compound was citral that has a strong antimicrobial property (Brum et al., 2017b).

Ethanolic extract of Rosemary (*Rosmarinus officinalis*) leaf powder shows an antibacterial activities against bacteria that infect tilapia including gram negative such as *Enterobacter spp.*, *Enterobacteriaceae*, *Serratia marcescens*, and *A. hydrophilia* and this is due to the compound that countians carnosic and rosmarinic acids and carnosol (Naiel et al., 2020) methanol and acetone extracts of clove (*Syzygium aromaticum*) also shows an anti-bacterial activity, inhibited the growth of *E. faecalis* and the mortality of tilapia fish artificially infected with *E. faecalis* (Rahman et al., 2017).

3.2 Animal product as a treatment for fish infection

According to Asely et al., (2014) adding honey bee pollen to the tilapia fish (*Oreochromis niloticus*) diets can enhance non-specific immunity and control *A. hydrophila* infections in tilapia, that's because its rich in proteins, essential amino acids, oils, fatty acids, minerals, enzymes and co-enzymes, carbohydrates and flavonoids, carotenoids and phytosterols in another study Kefa-originated forest honey determined that it has an ability to cure *Aeromonas hydrophila*-infected carp *Cyprinus carpio* from the concentration of 25 to 75% that's due to the

biological compounds with anti-bacterial , anti-oxidant , anti-inflammatory and immunostimulant activities (Salosso et al ., 2020).

3.3 Minerals product as a treatment for fish infection

The sodium bentonites have proved his efficacy to strengthen the catfish *Heteropneustes fossilis* immune system and reduce the mortality against *Aeromonas hydrophila* by adding this type of clay to the fish diet (Jawahar et al., 2018).

Sulfur could replace the antibiotics and improve the resistance against *Edwardsiella ictaluri* in olive flounder (*Paralichthys olivaceus*) and this due to the activities that it contain including antimicrobial and immunostimulant activities , in addition, sulfur-containing compounds found in garlic are antimicrobial components (Park et al., 2021).

Research has shown that using different concentration of sodium chloride (salt) can be effective against parasitic infection *trichodiniasis* in tilapia and it can eliminate the parasite while causing less stress to the fish (García-Magaña et al., 2019).

3.4 Microorganism product that treat fish infection

According to Cavalcante et al., (2020) adding symbiotics (probiotics and prebiotics) to the fish diet can ameliorate immune response against *Aeromonas hydrophilia* , commercial probiotic DBA (*Bifidobacterium* sp., *Lactobacillus acidophilus* and *Enterococcus faecium*) and prebiotics MOS and chitosan was added to the fish feed and protection level with percentage of 40% was observed.

METHODOLOGY

1. Aim of the study

This study aimed to assess the antimicrobial activity of selected plant extracts against some pathogenic bacterial and fungal species. Then, test these plants as anti-infectious and immunostimulant agents to treat red Tilapia fish infection caused by *Pseudomonas aeruginosa*.

2. Material and methods

2.1 Materials

2.1.1 Biological material

a. Plant material

Five plant species were selected and tested in this work based on previous studies (Fig. 16). Basil *Ocimum basilicum* was collected from the region of El Oued (east Algeria), Atlas cedar *Cedrus atlantica* was collected from Theniet El hed, Tissemsilt (west Algeria), Juniper *Juniperus oxycedrus* and Rosemary *Rosmarinus tournefortii* were collected from El Fayja, Tiaret (west Algeria) and garlic *Allium sativum* was obtained as a powder from a local herborist in Tiaret.

These plants were dried at room temperature under shed. Then, the leaves were separated and ground using a blender.



Figure 16 — Plant species used in this study. a) *A. sativum* powder, b) *J. oxycedrus*, c) *C. atlantica*, d) *O. basilicum*, e) *R. tournefortii*.

b. Microbial species

Thirteen microbial species were tested in this study including 11 bacteria and 2 fungi. These include 5 Gram positive bacteria: two strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, and 6 Gram negative bacteria: *Escherichia coli*, *Enterobacter cloacea*, *Listeria monocytogenes*, *Salmonella enteritidis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The tested fungi are two strains of *Candida albicans*.

These microbial species were checked for purity by microscopic observation after Gram staining for bacterial species and simple staining for fungal species.

2.2 Methods

2.2.1 Preparation of plant aqueous and ethanolic extracts

The aqueous and ethanolic extracts of the selected plants were prepared following a modified version of the method described by Ghedadba et al. (2014). Briefly, 10 % (w/v) of each plant powder was mixed in distilled water for the aqueous extract and in 70 % ethanol solution for ethanolic extract. The mixtures were macerated for 24 hours. After maceration, the extracts were filtered and evaporated using a rotary evaporator (Fig. 17). To complete the drying process the extracts were placed in an oven at 37°C until complete drying. The resulting extracts were placed in vials and stored in a refrigerator at 4°C. Percent yield of all extracts is calculated using the following formula:

$$\text{Yield\%} = \text{Weight of the dry extract} \times 100 / \text{Initial weight of the dry plant.}$$



Figure 17 — Steps of plant aqueous and ethanolic extracts preparation.

2.2.2 Antimicrobial activity

Antimicrobial activity of plants refers to their ability to kill or inhibit the growth of microorganisms (Vaou et al., 2021). The agar dilution method was used in this study and relies on the incorporation of different concentrations of the tested substance into the melted agar to determine the minimal inhibitory concentration MIC (Fig. 18). Bacterial suspensions are prepared at 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml) and then inoculated onto Petri dishes containing the Mueller Hinton agar containing the diluted plant extracts. The plates are then incubated for 24h at 37°C. If growth is observed as microbial colonies on the agar, this means that the test substance does not have inhibitory effect against the tested microorganisms and vice versa (Balouiri et al., 2016).



Figure 18 — Steps of the agar dilution method.

2.2.3 In vivo experiment

a. Fish feed preparation

After the antimicrobial activity assessment, plants that show better activity against the tested microorganisms will be selected for in vivo application to test their potential antiinfectious and immunostimulatory activity against *P. aeruginosa* infected Tilapia fish.

For this purpose, each selected plant powder will be incorporated, separately to the standard feed of the fish (delivered by the ONAB; office national de l'aliment de bétail) at a concentration of 12 % (Fig. 19).

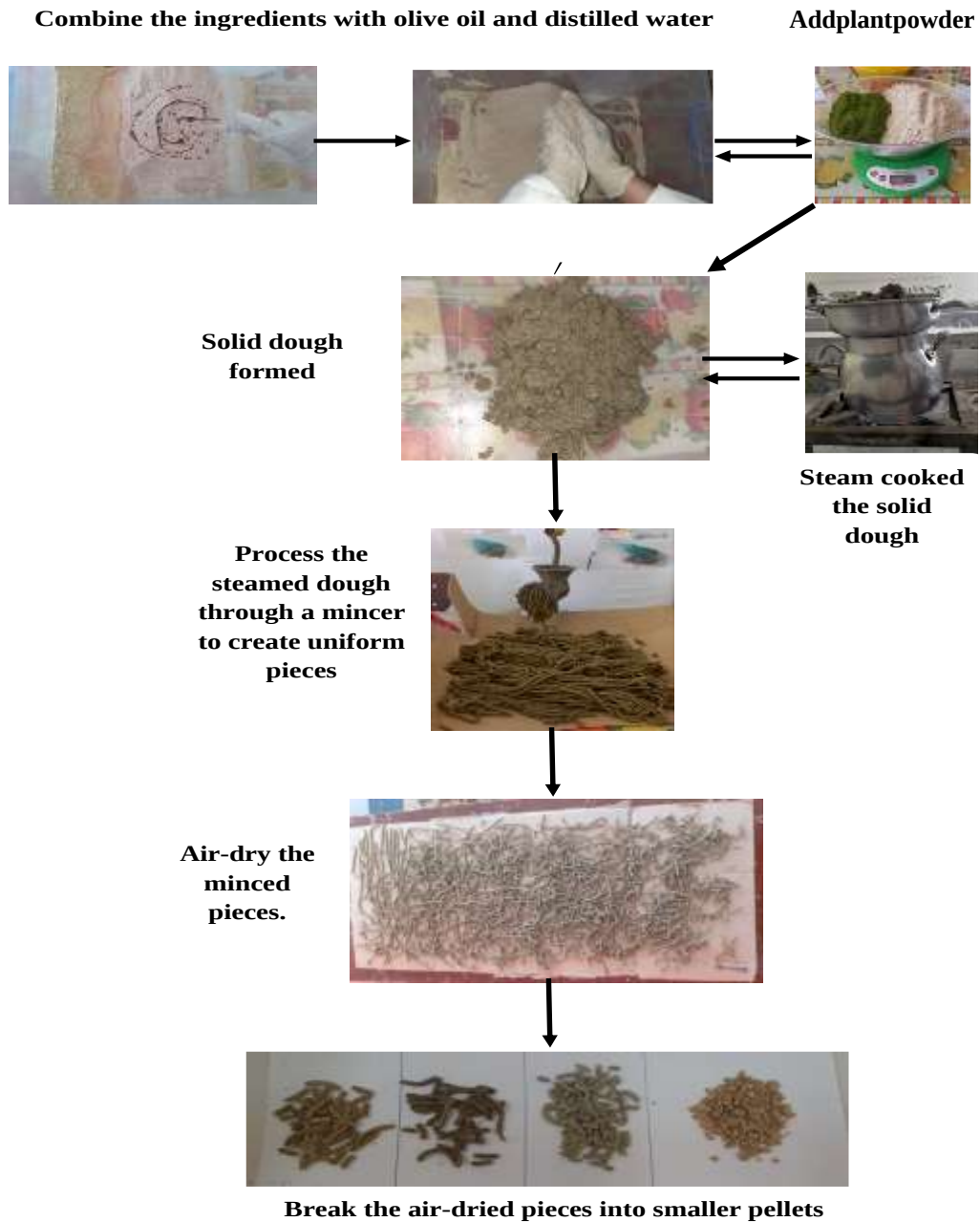


Figure 19 — Experimental fish feed preparation.

b. Challenge test

Healthy tilapia fish are divided in tanks containing 3 fish each. The control tanks will consist of:

- 1 Fish fed with the standard feed with no treatment and not infected.
- 2 Fish fed with the standard feed and infected with *Pseudomonas aeruginosa* by an intraperitoneal injection of 0.2 ml of a bacterial suspension containing approximately 1.5×10^8 cell/ml.

- 3 Fish fed with the standard feed and infected with *Pseudomonas aeruginosa* by an intraperitoneal injection of 0.2 ml of a bacterial suspension containing approximately 1.5×10^8 CFU/ml and treated after 7 days with drops of methylen blue solution.

The test tanks will consist of:

- 1 Fish fed with standard feed and infected with a suspension of *P. aeruginosa* (1.5×10^8 cell/ml). After 5 days infection, fish will be fed with the experimental feed containing the selected medicinal plants for 10 days before sacrifice.
- 2 Fish fed with the experimental feed containing the selected plants for 7 days, then infected with a suspension of *P. aeruginosa* (1.5×10^8 cell/ml).

All the fish are fed twice a day either with standard feed or experimental feed during the experiment period (15 days). They are monitored daily to report mortality, behavioral alterations and clinical signs of infection.



Figure 20 — In vivo experimental design.

At the end of the experiment, blood samples are taken from fish to evaluate the hematological parameters related to the infection (Fig. 21).



Figure 21 — Blood sample collection.

RESULTS and DISCUSSION

Results

1. Plant extracts yields

Quantifying the extract yield studied is an important step in determining the efficacy of the extraction method. The aqueous and ethanolic extracts of the selected plants in this study are represented in figure 22

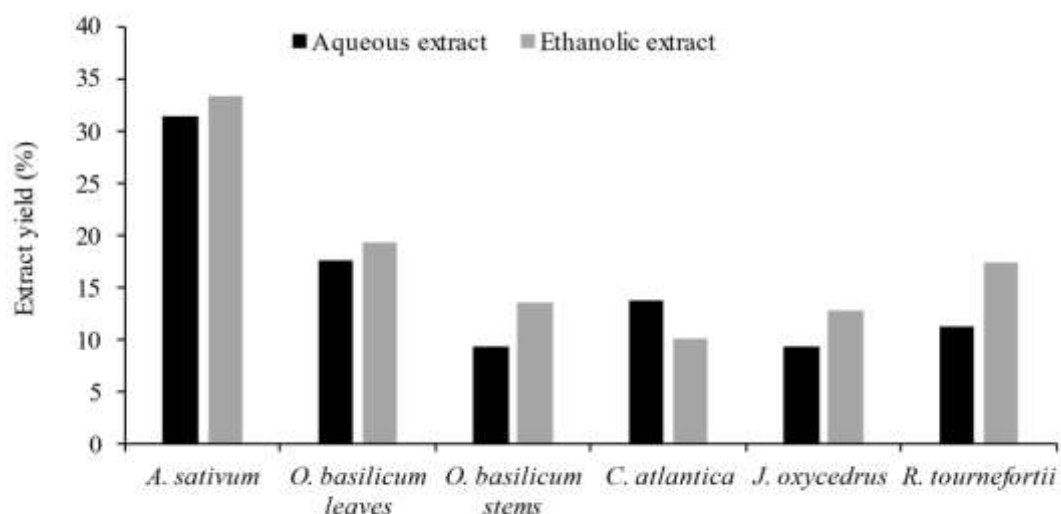


Figure 22 — Yields of the aqueous and ethanolic extracts of the tested plants.

The obtained results regarding plant extracts yields demonstrate that the ethanolic extracts presented slightly higher yields compared to the aqueous extracts except for *C. atlantica*.

2. Microscopic observation of tested microbial species

The microscopic observation of the tested microbial species demonstrates their purity (Fig. 23).

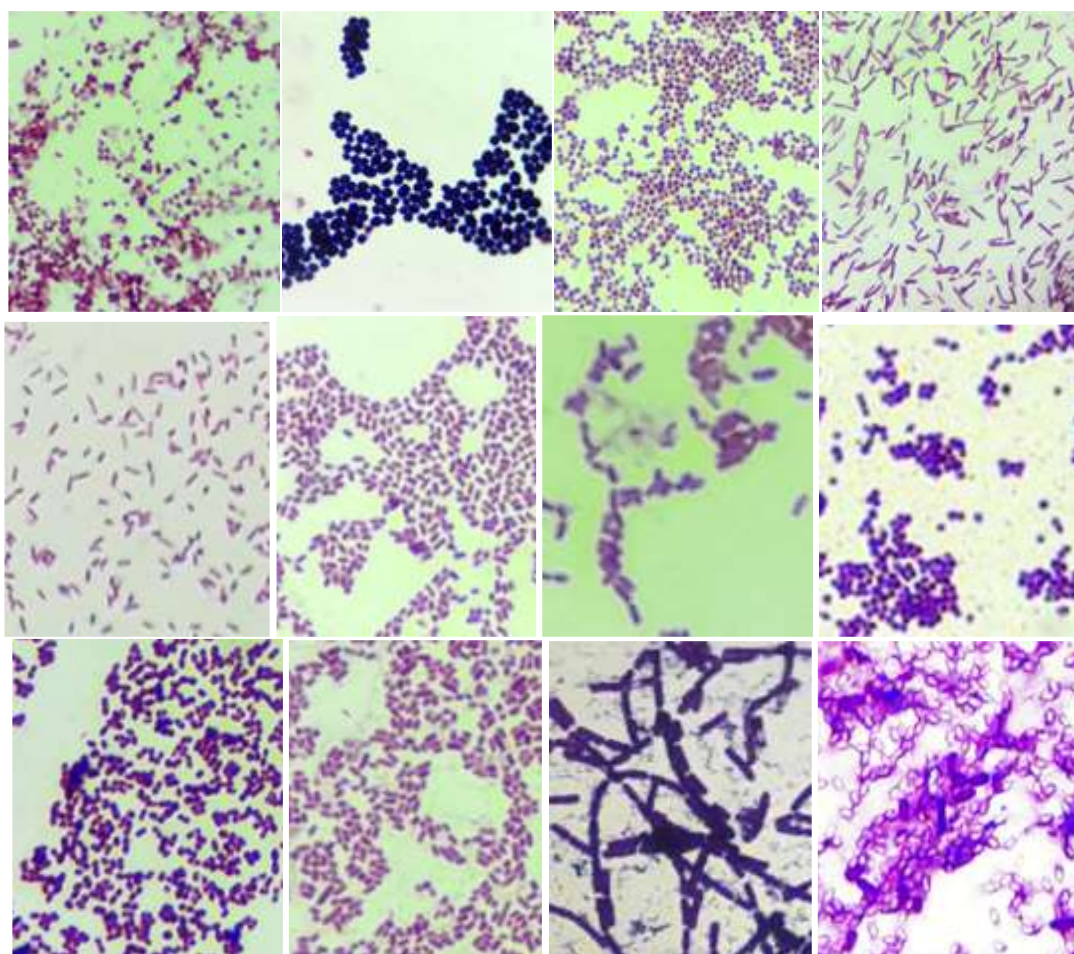


Figure 23 — Microscopic observation of the tested microbial species. *E. coli*, *C. albicans*, *L. monocytogenes*, *P. aeruginosa*, *S. enteritidis*, *K. pneumoniae*, *S. aureus*, *E. faecalis*, *E. cloacea*, *B. cereus*, *B. subtilis*.

3. Antimicrobial activity

The obtained results regarding the antimicrobial activity of the tested medicinal plants generally demonstrated that the ethanolic extracts have the better inhibitory effects against the tested microbial strains, reflected by lower MICs values, compared to the aqueous extracts.

All the tested strains shows a resistance to *A. sativum* extracts even at a concentration of 50 mg/ml

The ethanolic extract of *R. tournefortii* demonstrated the best inhibitory action compared to the other tested plants. Whereas, the aqueous extract of *C. atlantica* demonstrated relatively a better inhibitory action compared to the aqueous extracts of the other plants tested.

The ethanolic extract of *R. tournefortii* showed the lower MICs values compared to the aqueous extract. Furthermore, we notice that the aqueous extract had no inhibitory action against the two strains of *S. aureus* as well as on one strain of *C. albicans* (Fig. 24).

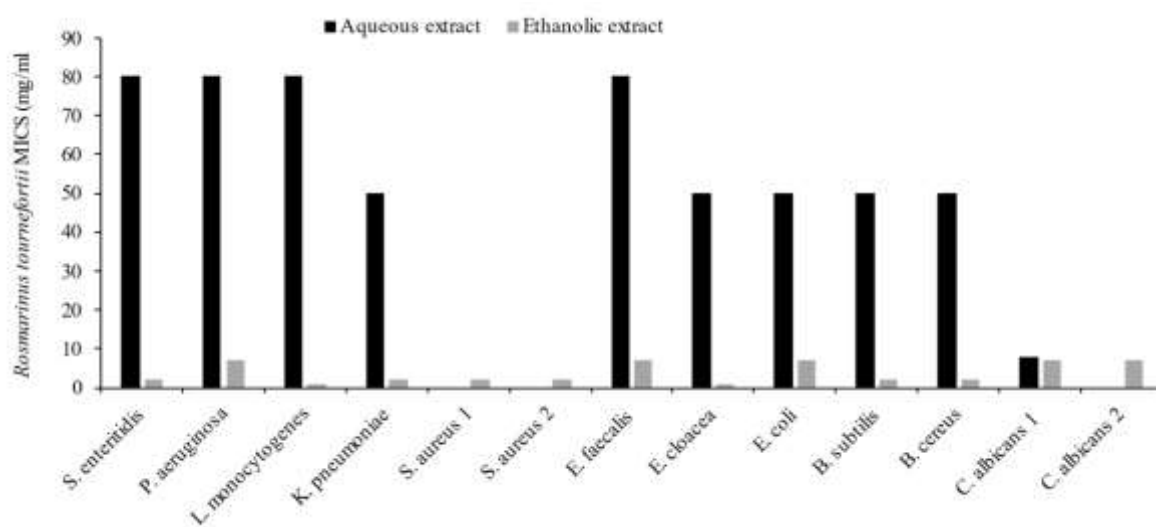


Figure 24 — MICs of the ethanolic and aqueous extracts of *R. tournefortii* against the tested microbial species.

Regarding the antimicrobial activity of *C. atlantica* the ethanolic extract showed higher inhibitory effect compared to the aqueous extract except for *P. aeruginosa*, *E. faecalis* and *E. coli* where the MICs values are high even compared to the aqueous extract. In addition, the aqueous extract showed no effect against the two strains of *C. albicans* (Fig. 25).

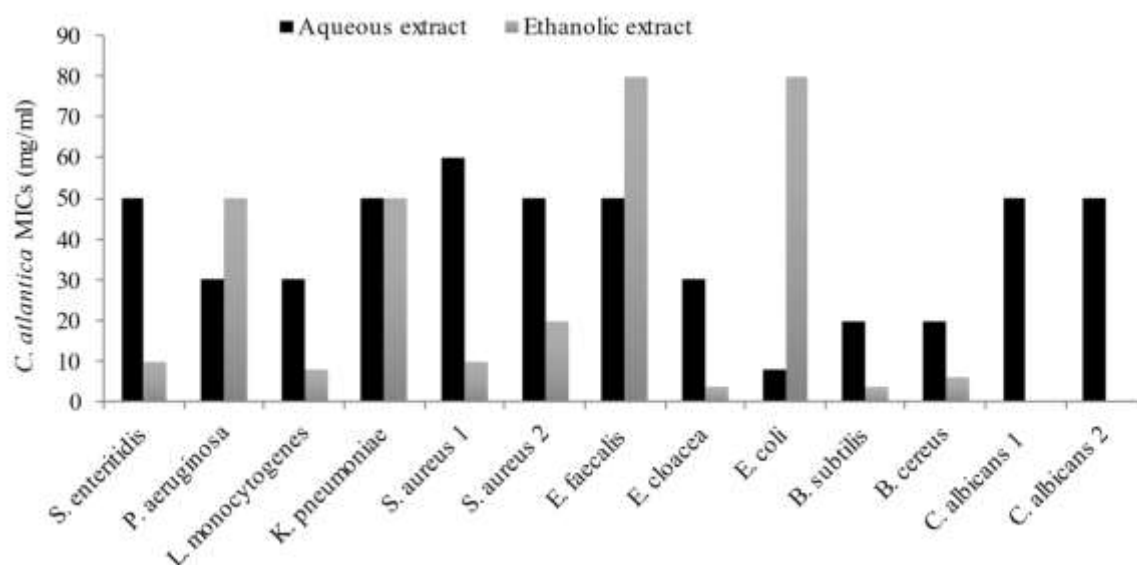


Figure 25 — MICs of the ethanolic and aqueous extracts of *C. atlantica* against tested microbial species.

Moreover, the two strains of *C. albicans* showed resistance to the ethanolic extract of *J. oxycedrus*, whereas *S. enteritidis* and one strain of *C. albicans* showed resistance to the aqueous extract of this plant (Fig. 26).

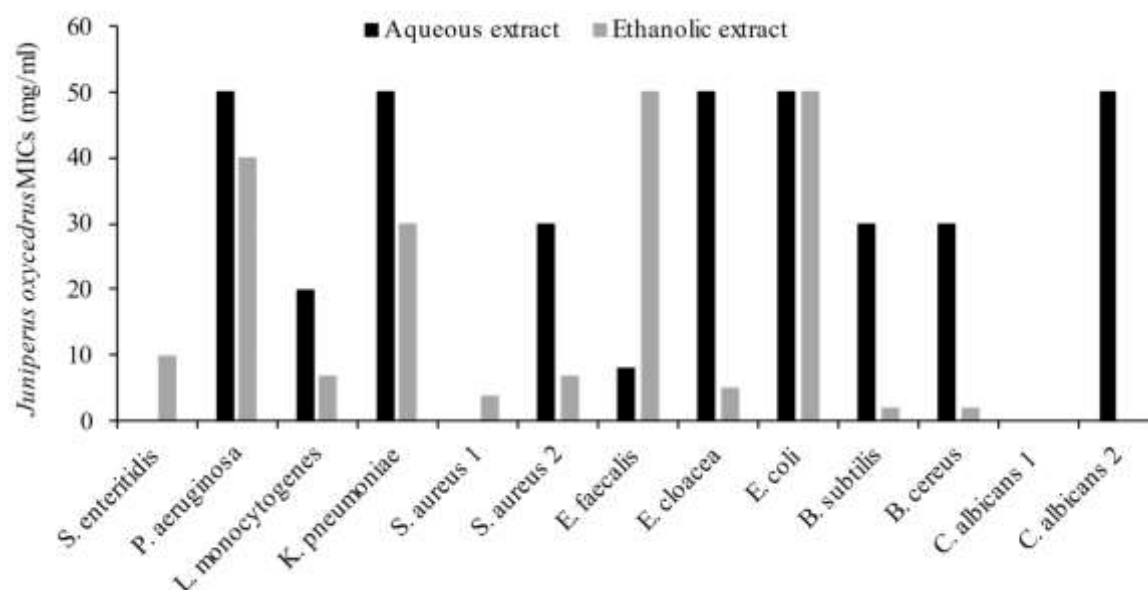


Figure 26 — MICs of the ethanolic and aqueous extracts of *J. oxycedrus* against the tested microbial species.

A previous study showed that the aqueous extract of *O. basilicum* has no antimicrobial effect. Hence only the ethanolic extract was tested for both leaves and stems. We notice that the ethanolic extract of the stems has no inhibitory effect against the two strains of *C. albicans*. As well, the ethanolic extract of the leaves has no effect on *E. coli*. In addition, we observe that the ethanolic extract of stems had better action against the tested microorganisms compared to the leaves (Fig. 27).

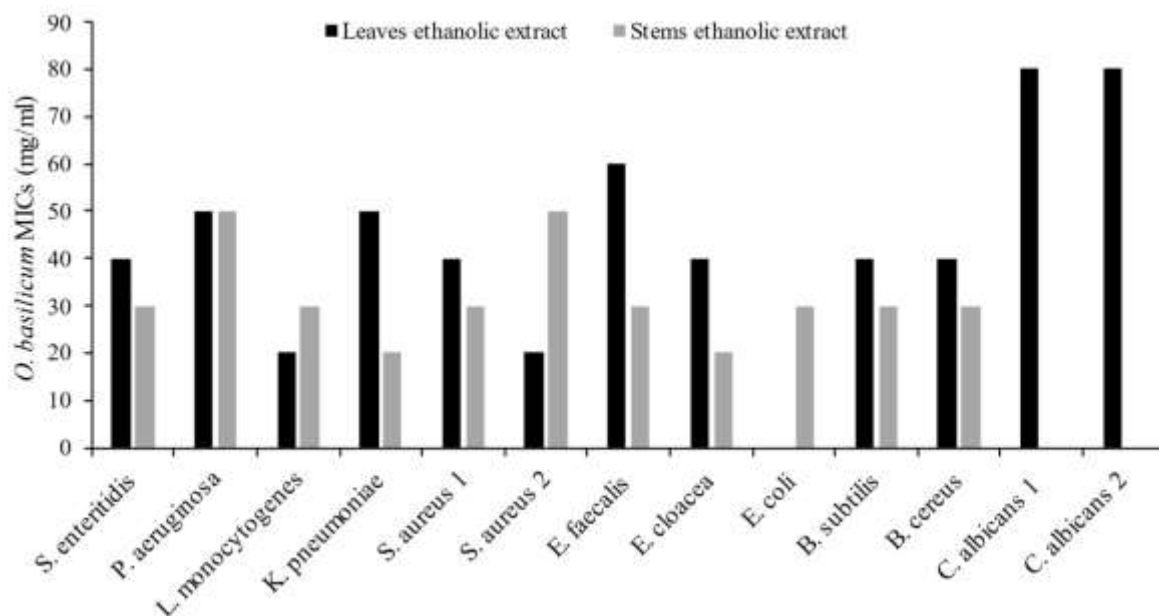


Figure 27 — *O. basilicum* MICs of the ethanolic extracts of leaves and stems against the tested microbial species.

4. In vivo experiment

Based on the obtained results regarding the antimicrobial activity, three plants were selected for in vivo assay, these are *R. tournefortii*, *C. atlantica*, and *J. oxycedrus*.

After the injection of Tilapia fish with *P. aeruginosa* suspension, symptoms of the infection started after 3 days in certain fish (fed with rosemary) reflected by dark spots on the skin (Fig. 28) but no symptoms were seen on fish fed with *C. atlantica* and *J. oxycedrus*. We noticed that the movement of infected fish fed with standard feed become slower and they had difficulty breathing

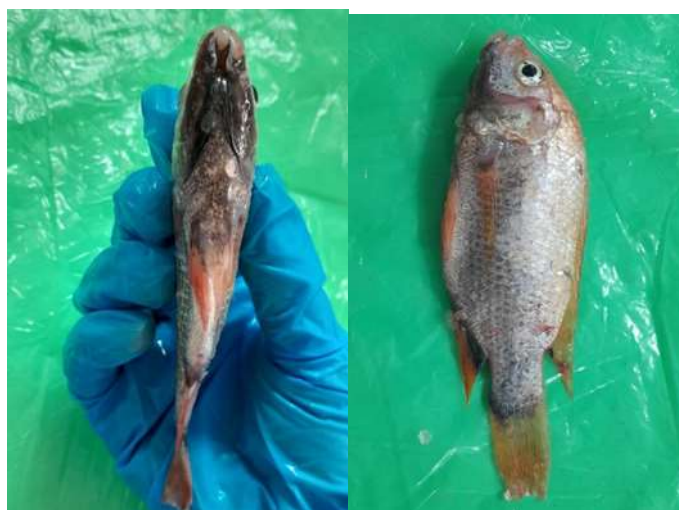


Figure 28 — Tilapia fish infected by pseudomonas aeruginosa and fed with rosemary shows dark spots on the skin.

After 3 days the infected fish that started feeding on the experimental diet showed active movements.

* After 5 days, the fish fed with rosemary were all dead that is why the experiment continued without that group.

After 10 days the infected fish with no did not receive any supplementary diet (negative control) shows a noticeable eye damage (Fig. 29).



Figure 29 — Tilapia fish infected with *Pseudomonas aeruginosa* shows eyes damages.

4.1 Hematology

Table 2 demonstrates the results obtained after hematology analysis of the blood samples collected from fish. Unfortunately, due to blood clotting, we could not analyze all blood samples.

We notice that the number of white blood cells (WBCs) is higher in fish fed with *C. atlantica* before infection with *P. aeruginosa* compared to the other groups. Whereas, the group fed with *J. oxycedrus* before infection showed the lower number of WBCs.

Neutrophils counts were higher in fish infected with *P. aeruginosa* then fed with *J. oxycedrus* compared with the others groups, in contrast, the group infected with *P. aeruginosa* and fed with *R. tournefortii* had significantly lower neutrophil counts

Lymphocytes count was notably elevated in fish fed with *C. atlantica* and infected with *p. aeruginosa* comparing to the group fed with *J. oxycedrus* and infected with *P. aeruginosa* that had much lower lymphocyte counts

Monocytes count was almost the same in the positive Control Tilapia treated with Methylen blue, the fed fish with *C. atlantica* and infected with *P. aeruginosa*, and the infected fish with *P. aeruginosa* then fed with *R. tournefortii* compared with the infected fish with *P. aeruginosa* fed with *J. oxycedrus* and the fed fish with *J. oxycedrus* infected with *P. aeruginosa* that shows a lower monocyte count

RESULTS AND DISCUSSION

Basophil counts were elevated in fish treated with fed *C. atlantica* and infected with *P. aeruginosa* compared with the group infected with *P. aeruginosa* and fed with *J. oxycedrus* had a lower basophil counts

Eosinophil counts were extremely elevated in fish fed with *C. atlantica* than infected with *P. aeruginosa* and the fish infected with *P. aeruginosa* and then fed with *J. oxycedrus*. The group fed with *J. oxycedrus* and infected with *P. aeruginosa* also showed elevated eosinophil counts comparing to the positive control tilapia treated with methylen bleu that had a low eosinophil counts.

Table 2 — Hematology analysis of Tilapia fish blood samples.

	Control Tilapia	Control Tilapia + Methylen blue	<i>C. atlantica</i> + <i>P. aeruginosa</i>	<i>J. oxycedrus</i> + <i>P. aeruginosa</i>	<i>P. aeruginosa</i> + <i>J. oxycedrus</i>	<i>P. aeruginosa</i> + <i>R. tournefortii</i>
White blood cells / mm ³	41700	44800	102300	28600	51300	42500
Neutrophils	/	4749	5831	5 720	6 618	300
Lymphocyte	/	38707	58616	9 352	6 310	41 600
Monocytes	/	493	409	143	257	400
Basophils	/	134	307	114	51	100
Eosinophils	/	717	37237	13 270	38 065	/

Discussion

Aquaculture is an important economic activity in the Mediterranean basin. It contributes greatly to human nutrition throughout the world. However, it involves the use of antibiotics for treatment and prophylaxis resulting in antibiotic resistance that rapidly spreads in the aquatic microbial communities leading to important productivity losses. In addition, the antibiotic resistance can reach human pathogens making vain the use of antibiotics for human health (Pepi and Focardi, 2021). Scientists are now exploring new products from nature specially plants that have the ability to limit infections and microbial resistance in humans as well as in animals.

Tilapia culture constitutes a significant source of income and nutrition. However, intensive farming led to the global tilapia disease outbreaks, with bacterial infections causing important mortalities and morbidities, threatening sustainable production (Haenen et al., 2023).

Plants are important source of bioactive components, they are relatively safe, available and cheap and are not limited to the pharmaceutical sectors but also found in the aquaculture sectors (Rahimi et al., 2022).

The present work aimed to evaluate the anti-microbial activity of the ethanolic and aqueous extract of 5 medicinal plants (*A. sativum*, *O. basilicum*, *R. tournefortii*, *C. atlantica* and *J. oxycedrus*) selected based on previous studies against 13 pathogenic microbial species. The best plants showing high antimicrobial activity (*R. tournefortii*, *C. atlantica* and *J. oxycedrus*) were assessed in vivo against *P. aeruginosa* infected Red Tilapia fish.

After the preparation of the ethanolic and aqueous extracts, we noticed that generally the ethanolic extracts of all tested plants had higher yields compared to the aqueous ones. According to Patra et al. (2006) ethanol has an effect on the cytoplasmic membrane which increases the yield. The extraction yield of the plant extracts strongly depends on the polarity of the solvent, which determines quantitatively and qualitatively the extracted compounds. In addition, the choice of extraction solvent depends on their safety to the living organisms (Franco et al. 2008; Plaskova and Mlcek, 2023).

Moreover, the choice of solvent depends also on the studied activity and on the chemical attributes of the secondary metabolites being extracted. A water-alcohol solvent mixture is commonly utilized, resulting in extra efficient extraction because water hydrates plant walls as the alcohol is chemically similar to most active components extracts from the plant material. Also, the alcohol has the advantage of preserving the extract (Bitwell et al., 2023). The highest yields are usually obtained with ethanol and methanol and their mixtures with water. However, water and ethanol are the most used solvents due to their low toxicity (Franco et al. 2008; Naili et al. 2010).

In this study, the antimicrobial activity of the ethanolic extract of *R. tournefortii* demonstrated the best inhibitory action against the 13 tested microbial species compared to aqueous extract as well as to both extracts of all tested plants. The plants of the genus *Rosmarinus*, native of the Mediterranean area, have been used traditionally in food preservation because they prevent microbial contamination (Nieto et al., 2018). Two species are found in Algeria *R. officinalis* L. and *R. tournefortii* de Noé, the last one is an endemic species in the north of Africa and Spain (Outaleb et al., 2020). According to Tahri et al. (2013) the major compounds found in *R. tournefortii* and *R. officinalis* plants are α -pinene, camphene, β -pinene, 1,8-cineole and camphor, the polyphenolic profile of this plant is characterized by the presence of carnosic acid, carnosol, rosmarinic acid and hesperidin, as major components and its extract contain also epirosmanol, rosmanol, methylcarnosate and isorosmanol. These compounds are known for their antioxidant, antimicrobial and more biological activities.

Listeria monocytogene and *Enterobacter cloacae* were the most sensitive to the ethanolic extract followed by the two strains of *S. aureus*, *B. subtilis* and *S. enteritidis*, these results are in accordance with those of Manilal et al., (2021). Regarding *C. albicans* we noticed that the aqueous extract of rosemary had the best inhibitory action against one strain compared to the other microbial strains but no effect on the other one. In another study, Saeidi et al., (2019) showed that *Rosmarinus officinalis* methanolic extract has a good activity against *candida albicans* and inhibited their growth on the concentration of 0.1 mg/ml. According to Fazeli-Nasab et al. (2021) the ethanolic extract has a great antimicrobial activity against *E. coli* and *P. aeruginosa*. Nieto, et al. (2018) the antimicrobial effect of rosemary extract is due mainly to carnosic acid, the compound interact with the cell membrane of the microorganism, causes changes in genetic material and the loss of membrane functionality and its structure.

J. oxycedrus is one of herbal tree that is found in the Mediterranean region (Karaman et al., 2003). According to Mrid et al. (2019) the aqueous extract of juniper contains different hydroxycinnamic acids including caffeic acid, p-coumaric acid, salicylic acid, p-hydroxybenzoic acid and some flavonoids compounds hesperidin, naringenin and rutin with the absence of terpenes compounds. The ethanolic extract contains alkaloids, tannins, saponins, phenolics, flavonoids, steroids, terpenoids, cardiac glycosides, coumarins (Živić et al., 2019). The antimicrobial effect of juniper is due to the high presence of α -pinene, α -terpinolene, limonene, β -myrcene and β -terpinene (Isik et al., 2020). In our study, we noticed a better antimicrobial action of the extracts on Gram positive bacteria compared to Gram negative bacteria, this result was also observed by Pepeljnjak et al. (2005).

Atlas cedar is one of the endemic plants that is found in Algeria, it belongs to the family of Pinaceae (Belkacem et al., 2021). α -pinène, α -phellandrène and limonène are the major compounds found in the essential oil of atlas cedar (Ez-Zriouli et al., 2023).

Regarding Atlas cedar, the aqueous extract demonstrated the best inhibitory action compared to the aqueous extracts of the other plants tested. *E. coli* demonstrated the higher sensitivity with a MIC of 8 mg/ml. Ez-Zriouli et al. (2023), showed that *P. aeruginosa*, *Salmonella* and *S. aureus* are resistant to the essential oil of *C. atlantica* while *E. coli* is more sensitive.

A. sativum is an aromatic herb belonging to the family of Amaryllidaceae (Lu et al., 2024). Garlic is native to the central of Asia, southern Europe, the Mediterranean basin, India, and China (Shemesh-Mayer et al., 2023). The compounds found in garlic are saponins, phenols and polysaccharides. The major bioactive compounds of garlic are organosulfur compounds, such as diallyl thiosulfonate (allicin), diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), E/Z-ajoene, S-allyl-cysteine (SAC), and S-allyl-cysteine sulfoxide (alliin) (Shang et al., 2019).

Even though, several studies showed the efficacy of garlic as antimicrobial agent, in this study no inhibition was observed with both aqueous and ethanolic extracts of garlic against all the tested microbial species. This is probably due to the use of an already dried and grounded garlic bought from a market. The storage condition may have altered the properties of the plant components which led to this result. Indeed, according to Abidullah et al. (2021) fresh garlic crude extract has a high range of antibacterial activity compared to the stored one. According to Tahri et al. (2013), dried herbs lose their quality slowly. A previous study demonstrated that the fresh garlic anti-microbial activity is more efficient than the freeze-dried garlic (Rahman et al., 2006). Drying can also cause changes in the physical properties such as color and structure, as well as the deterioration of aroma compounds or degradation of nutritional substance reducing the product quality (Ratti et al., 2007).

In a previous study, we demonstrated that the aqueous extract of *O. basilicum* had no inhibitory effect against all tested microbial species. In this study we tested the ethanolic extracts of leaves and stems of basil.

O. basilicum is an aromatic herb belonging to the Lamiaceae family (Mohammed et al., 2020). *O. basilicum* is native to tropical and subtropical regions and is found in Africa, India and other Asian regions. Nowadays, it is cultivated all over the world (Ahmed et al., 2019). It is known locally as Reihan.

In this study, we could notice that the ethanolic extracts of both plant parts have relatively the same effect on the tested microbes. Exception is made for the two strains of *C. albicans* that showed resistance to the ethanolic extract and *E. coli* that showed resistance to the aqueous extract. According to Romano et al. (2022), the major bioactive compounds that are found in *O. basilicum* are rosmarinic acid that have antimicrobial activity, caffeic, chicoric, and ferulic acids. The major bioactive compounds of basil leaves essential oil are linalool, linalyl acetate, elemol, and geranyl acetate (Ahmed et al., 2019).

To confirm our in vitro result, we make a in vivo and a challenge test using a prepared food based with the plants that gives the best result rosemary, juniper and atlas cedar.

The result confirm that the rosemary aqueous extract don't have a therapeutic or an immune effect on *Pseudomonas aeruginosa* because the fish fed with the rosemary food then infected with the bacteria were died after 5 days .

Regarding the mortality rate the infected fish and fed with juniper is better the fish fed with atlas cedar, but regarding the morphologic characteristics the fish fed with cedar keeps their red color while the juniper fish had a darkness .

The eyes of the negative control were corroded which is one of *Pseudomonas aeruginosa* clinical sign in tilapia and we observed a darkness in some infected fish specially for the fish infected and fed with the rosemary diet, and some dark point that appears in the *Juniperus* fed fish which is *P. aeruginosa* infection symptoms (Haenen et al., 2023). According to Eissa et al. (2010) injecting tilapia with *Pseudomonas aeruginosa* showed mortality rates of 93.34 %.

We observe that only the kidney of positive control fish treated with methylen bleu was damaged, according to Olufayo, M. (2016) methylene bleu can be toxic and cause a damage in the internal organs of the fish and a high mortality on a concentration higher than 100mg/l .

For the hematological analysis , the number of white blood cells (WBCs) is higher in fish fed with *C. atlantica* before infection with *P. aeruginosa* compared to the other groups. Whereas, the group fed with *J. oxycedrus* before infection showed the lower number of WBCs compared to the healthy control , and we observed total leukocyte counts that were significantly greater than those in healthy tilapia , this was also observed by Sebastião et al. (2011) , the hematological changes can occur due to the physiological and immunological responses to infection and stress caused by bacteria (Martins et al., 2008).

According to Rauta, Nayak and Das (2012) immune activation is a normal, protective mechanism that the fish uses to fight off the pathogenic bacteria. The increased WBC levels are a sign that the immune system is functioning properly to combat the infection.

Neutrophils are defined as soldiers of our innate immune system (Tahir & Zahra, 2023) , they are the first line of defense during an inflammatory response (Havixbeck et al., 2015) , neutrophils phagocytose bacteria and destroy them by releasing digestive enzymes from their cytoplasmic granules that target the cell walls of bacteria (Witko-Sarsat et al., 2000) , lymphocyte involved in the acquired or antigen-specific immune response given that they are the only cells in the organism able to recognize and respond specifically to each antigenic epitope (Cano & Lopera, 2013) , Monocytes are white blood cells that derive from the bone marrow (Espinoza & Emmady, 2023) they play important immunoregulatory and tissue-repairing roles to decrease immune reactions and promote tissue regeneration (Ma et al., 2019) , basophils are frequently used for allergy testing to assess the existence of allergen-specific IgE on their cell surface (Miyake et al., 2020) High Basophil Count May indicate chronic inflammation in the body, Low Basophil Count (Basopenia) During severe allergic reactions, basophils are activated and degranulated, temporarily reducing the count(Tchen et al., 2024) , Eosinophils play a crucial role in immune defense against microbial infections and allergic reactions, it can also release toxic proteins and free radicals that can help eliminate microorganisms.(Lombardi et al., 2022) , those all are a type of WBC , the high count of those cells indicate a severe immune response (Mank et al., 2024)

In our study the fish fed with *C. atlantica* and then challenged with *P. aeruginosa* shows the highest number of the WBCs which indicates a strong immune activation what significate the effect of this plant on the immune system while the fish fed with *J. oxycedrus* that shows a lower WBCs even compared with the healthy control significate suppress the immune response.

CONCLUSION

Tilapia has become one of the most cultivated fish contributing significantly to food security and economic growth, particularly in developing nations. However, Tilapia culture is challenged by various infectious diseases leading to severe economic losses. The current treatment to those infection is using antibiotics and chemicals, nevertheless the overuse of them can lead to antibiotics resistance becoming ineffective.

Through this study we could demonstrate the effectiveness of ethanolic and aqueous extracts of four plant species i.e. *Cedrus atlantica*, *Juniperus oxycedrus*, *Rosmarinus tournefortii*, and *Ocimum basilicum* as antimicrobial agents against 13 pathogenic microbial strains. Whereas, both extracts of *A. sativum* did not show antimicrobial action.

Moreover, *C. atlantica*, *J. oxycedrus*, and *R. tournefortii* were tested in vivo and showed reduction of the impact of infection caused by *P. aeruginosa* in Tilapia fish suggesting an anti-infectious and immunostimulant effect.

As a follow-up to this work, it would be interesting to:

- Explore different extraction techniques, to isolate and enhance the bioactive components more effectively.

- Evaluate the biological activity of combinations of the tested plants.

- Explore other natural product such as mineral products and their combinations with plants as therapeutic agents to treat tilapia infections.

- Produce tilapia food supplements or medication based on plant ethanolic extracts.

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