Subjecting the leaves to a concentration for stock of the extract

Abstract: There is disclosed a composition for treatment or prevention of bacterial diseases in aquatic animals; more particularly fishes; comprising an extract from betel leaves; wherein the extract comprises hydroxychavicol. The composition further comprises further comprising hydrochavicol, hydroxychavicol acetate, allylpyrocatechol, 3,4-dihydroxybenzaldehyde, pipetone, and tannin. It is anticipated that the composition can prevent and treat bacterial diseases in freshwater and marine fishes. A method is also disclosed herein.
Declarations under Rule 4.17:
— of inventorship (Rule 4.17(iv))

Published:
— with international search report (Art. 21(3))
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
COMPOSITIONS CONTAINING PIPER BETLE EXTRACT FOR PREVENTION AND TREATMENT OF BACTERIAL DISEASES IN AQUATIC ANIMALS

FIELD OF INVENTION

[0001] The present invention is directed to the protection and treatment of aquatic animals, and more particularly, to the protection and treatment of fish and other aquatic animals from bacterial diseases using a composition containing, as the active ingredient, an extract of Piper betle (daun sireh).

BACKGROUND OF INVENTION

[0002] Nowadays, bacterial diseases which are affecting consumable poultry or aquatic based products may not only be detrimental to the physiological well being of the animals, but also can adversely affect the consumers.

[0003] The primary causative factors of infections in aquatic animals and particularly fishes include; contaminated water and failure to maintain a healthy and stress-free environment for the fishes within the confined space; whereby the latter condition occurs in view of aquaculture purposes. These factors thereby result to vulnerability to diseases, especially those of bacterial or fungal origin. It is reported that contaminated water and inconsistent management of confined space for the fishes have been favoring the growth of pathogens that cause fish diseases. For instance, in one part of Asia, several majority known diseases which have been affecting the well-being of fishes and hence fish production and yield; include; Vibriosis, Streptococcusis and Pasteurellosis. The species affected include the seabass, grouper, crimson snapper, tilapia and catfish; causing a total lost of at least USD 648,000 to USD 13.5 mil annually.

[0004] It is reported that many potentially pathogenic bacteria of fish normally exist as commensals or free-living in the environment, that is non-pathogenic to host. Some bacteria cause only surface lesions or gill infections; and in some cases causes bacteremia indicating the presence of bacterial organisms in the bloodstream without clinical signs. These bacteria become pathogenic when the fish is immuno-compromised by the causative factors discussed in an earlier paragraph.
Currently fish disease therapies largely include the use of chemical based compounds such as; antibiotics and vaccines that can lead to adverse side effects. Additionally, most of the drug related therapies are prohibitively expensive and can thereby confine the affordability of fish farmers in their efforts of maintaining a healthy environment for their fishes. For this reason, therapeutic treatments and diseases prevention methods for diseased or injured fish that utilize naturally derived substances or biologically obtainable sources are particularly preferred. These treatments do not adversely affect other aquatic animals as well as plants that also present in the water or hazardous to the environment.

The use of a probiotic bacteria in the treatment of diseased fishes with a spore-forming strain Bacillus having a specific sequence is described by Terhune et. al, in U.S. Pat. App. No. 13/460,238. In another example, there is disclosed the use of a transgenic plant to express of fish disease antigens hence for producing a vaccine is described by Bootland et. al; in U.S. Pat. App. No. US 11/941,032. To prepare a vaccine using this method, a nucleotide sequence which is linked to a regulatory element such that said nucleotide sequence encodes an amino acid sequence which when expressed in the plant and administered to a type of fin-fish or shell-fish.

The present invention is based on the unexpected discovery that extracts of *Piper betle*, commonly or locally called Sireh, are highly efficacious in the therapeutic treatment and prevention of bacterial infections in fish and possibly other aquatic animals.

**SUMMARY OF INVENTION**

The present invention provides a composition which is useful in protecting aquatic animals and more particularly fishes. Also provided is a method of preparation of the extract of *Piper betle*.

In one aspect, there is provided a composition for treatment or prevention of bacterial diseases in aquatic animals comprising an extract from betel leaves; wherein the extract comprising hydroxychavicol.
[0003] In one embodiment, the extract further comprising hydrochavicol, hydroxychavicol, hydroxychavicol acetate, allypyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A, piperol, sterol, chatecol, and tannin.

[0004] In another embodiment, the composition is used for treatment and prevention of bacterial diseases in freshwater and marine fishes.

[0005] In yet another embodiment, the composition provides antibacterial properties against Vibriosis; Streptococcosis; Aeromonasis and Nocardiosis.

[0006] In a further aspect of the present invention, there is provided a method for preparing a composition useful for treatment or prevention of bacterial diseases in aquatic animals; comprising: drying the betel leaves for a predetermined period of time; reducing the leaves into powdered form; immersing the powder form in alcohol solution for a predetermined period thus forming a mixture, and the mixture shaken for a predetermined period of time; filtering the mixture; and subjecting the filtered mixture to a concentration process to obtain a dried form or obtain a constant weight and providing the mixture in a form suitable for oral administration to the aquatic animal.

[0007] In yet another embodiment, the composition provides antibacterial properties against Vibriosis; Streptococcosis; Aeromonasis and Nocardiosis.

[0008] In a further embodiment, the powder is immersed in ethanol for a duration of three days.

[0009] In yet another embodiment, the shaking is performed continuously at 200 rpm.

[0010] In a further embodiment, the concentration process is performed at a temperature of 60°C.

[0011] In another embodiment, the method further includes preparing the mixture in stock form.

[0012] In yet a further embodiment, providing the mixture in a form suitable for oral administration includes: diluting 100 mg of the mixture with 0.5 ethanol; diluting the mixture with 40 ml of water and dispersing on 1 kg of fish pellet to obtain dose of 100 mg/kg treatment; and drying the pellet.
In yet another aspect, there is provided a use of a therapeutically effective amount of betel leaves extract in the manufacture of a medicament for the treatment of bacterial diseases in aquatic animals.

In a further aspect, there is provided an aquatic animal feed supplement comprising an extract of betel leaves; the supplement is capable for treatment of bacterial diseases in said aquatic animal.

**BRIEF DESCRIPTION OF DRAWINGS**

The present invention features, objects and advantages thereof may be best understood by reference to the following detailed description when read with accompanying drawings in which:

- **FIG. 1** shows a flowchart of the method in preparing the composition in accordance with an embodiment of the present invention;

- **FIG. 2** shows a bar graph as an example depicting the inhibition zone plotted against concentration (mg/ml) in accordance with an embodiment of the present invention;

- **FIG. 3** shows another bar graph as an example depicting the efficacy of the extract plotted against a variety of Bacteria Strains; in accordance with an embodiment of the present invention;

- **FIG. 4** shows the percentage of fish survival; in particular for fishes which were fed with *Piper betle* extract before being infected with a type of bacteria in accordance with an embodiment of the present invention;

- **FIG. 5** shows the results obtained in the bacterial challenge; depicting the effects of the composition of the present invention and standard antibiotic (Oxytetracycline); in accordance with an embodiment of the present invention;

- **FIG. 6** shows the results of the fish blood toxicology study in accordance with an embodiment of the present invention.
[0022] In line with the above summary, the disclosed description and examples relates to a composition comprising extracts of *Piper betle* or betel leaves and method of producing a composition containing said extracts for the treatment and prevention of bacterial diseases in fishes. It shall be apparent however to one skilled in the art that the exemplifications are provided to better elucidate the embodiments of the present invention and therefore should not be construed as limiting the scope of protection. Some of the details may not be described at length so as not to obscure the invention.

[0023] For the purpose of clarity; an "extract" suitable for use in the various embodiments of the disclosure can be obtained from any part of a plant. The extract may comprise of active compounds, derivatives, and synthetic or semi-synthetic additional components. The extract can be in liquid or dry form; or a combination/mixture of both forms.

[0024] In accordance with an embodiment, the composition provides one or more of the following benefits in an oral care composition: antibacterial, antioxidant, anti-inflammatory, and/or healing properties. The composition of the present invention arresting a micro-organism, such as bacteria or by eliminating the said micro-organism(s) present on contact with the composition of the present invention.

[0025] In one embodiment, the composition comprises extracts from *Piper betle*; also known as Betel leaves in some countries. It is largely found central and eastern Malaysia; and throughout tropical Asia, Madagascar and East Africa. The leaves of the plant is the edible part; whereby it is known to be pungent, bitter in taste but aromatic.

[0026] It is discovered through experimental methodologies to be provided herein that the extracts of *Piper betle* exhibited significant antibacterial activities against gram positive and gram negative bacteria.

[0027] In accordance with an embodiment; the composition of the present invention comprises hydrochavicol, hydroxychavicol, hydroxychavicol acetate, allpyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A, piperol, sterol, chatecol and tannin. One of the main compounds of the composition of the present invention which
contributes to the antibacterial properties and inhibitory properties is hydrochavicol. It is further anticipated that the composition may provide antimicrobial properties due to the presence of hydrochavicol.

[0028] The present invention is also related to a method of protecting aquatic animals and in particular fishes against bacterial infections; the method comprises applying to the fishes an effective amount of the composition comprising Piper betle extracts. In one embodiment, for oral administration to the diseased fish; the composition is provided in pellets or solution form; whereby in which ever form; the composition is administered to the diseased fish via oral routes; such that the active ingredient of the extract is able to inhibit toxic production and initiate expression of enzymes for pathogenesis through disruption of the permeability barrier of the respective microbial membrane structure. In addition, the extract exhibits antioxidant or free radical scavenging activity hence promoting healing capabilities to the diseased fish.

[0029] In an embodiment, the composition of the present invention can be used against Vibriosis; Streptococcus; Aeromonasis and Nocardiosis.

[0030] In one embodiment, and with reference to a flowchart generalizing the method of the present invention; in FIG. 1; preparing the composition comprises the steps of: drying the Piper betle leaves for a predetermined period of time 101; and reducing the leaves into powdered form 102; immersing the powder in alcohol solution for a predetermined period thus forming mixture, and the mixture shaken for a predetermined period of time 103; filtering the mixture 104; subjecting the filtered mixture b a concentration process to obtain a dried form or obtain a constant weight 105; and re-suspended in alcohol to obtain stock of the extract 108. Finally, upon obtained the stock, it is provided in a form suitable for oral administration to the diseased fish, in is anticipated that the stock may be provided as feed supplements ; in the form of pellets or any suitable form that can be consumed by fishes.

[0001] The preparation of the composition in accordance with an embodiment of the present invention will now be described herein with reference to the experimental examples. However, it should be understood that the scope of the present invention is not limited to these examples. Also from the following examples, it will be seen that the invention can be applicable for various types of fishes.
EXAMPLE 1

i. Preparation of Extract

[0002] In the first step; an amount of matured betel leaves were collected; washed and dried under a shaded area for 5 days or in another option; dried in an oven for 3 days at 40°C. The leaves are then grounded to reduce the size into powdered form using an electric grinder and followed by filtering process using a 1 mm mesh cloth. 50 grams of the powder were then immersed in 750 ml absolute ethanol (99.6%) and kept on shaker for 3 days. The resulting solution were filtered with filter paper and thereby concentrated until completely dry or until a constant weight was obtained; using a rotary evaporator at a temperature of 60°C. The extract obtained was then re-suspended in absolute ethanol to obtain stock of 100 mg/ml; by using the formula:

\[
\text{Needed ethanol in (h) = weight of obtained product in (g) x 1ml} \\
\frac{100mg}{1}\]

[0003] For oral administration to the fish, 100 mg stock were first diluted with 0.5 ml absolute ethanol and followed by 40ml water. The solution is sprayed on 1 kg of fish pellet to obtain dose of 100mg/kg treatment. The pellets were then dried under the sunshade before being administered to fish.

EXAMPLE 2

ii. Inhibition of Bacteria Growth - In Vitro Test

[0004] The crude extract of Piper betle was diluted to obtain concentration of 100, 90,80,70,60,50,40,30,20 and 10 mg/ml. Thirty microlites (30 ul) of each diluted extract was incorporated with the antibiotic assay disc of 6 mm. The discs were then diffused on the Mueller-Hinton media which was previously swabbed with bacteria. The discs were incubated for 24 hours and inhibition zone were measured. Inhibition zones above 15mm were considered as optimum result indicating potential use as treatment. The results obtained has shown that the Piper betle extract can be used as treatment for Vibrio sp., Aeromonas hydrophilia, Streptococcus sp., Flexibacter sp. and Norcardia sp. FIG. 2 shows a bar graph as an example depicting the inhibition zone plotted against concentration (mg/ml). In this graph it is shown that the highest bacterial growth inhibition activity occurred when the concentration was at 19 mg/ml. Accordingly, the inhibition activity increases with higher concentration of the extract. FIG. 3 shows
another bar graph as an example depicting the efficacy of the extract plotted against a variety of Bacteria Strains.

EXAMPLE 3

II. Treatment And Prevention Test

[0005] The treatment and prevention test was conducted on Sea Bass (Lates calcarifer) for marine fish and tilapia (Oreochromis nilotica) for fresh water fish. The fish were divided into two groups and fed with pellet coated with Piper betle crude extract at a dose of 100 mg/kg feed; while control group were fed with commercial pellet.

[0006] For the prevention test, the fish were fed with pellet for one week before being challenged with bacteria at LD50 via immersion. For the treatment test, the fish were first infected through immersion with the respective bacterium at LD50 for 15 min, wash off and treatment was given upon infection. The treatment lasted for 7 consecutive days, whereby observation was made on mortality and clinical signs. Bacterial isolation was performed form the liver and kidney of dead fish and bacteria obtained were identified.

Results 1 - Treatment and Prevention

[0007] The results from the prevention test showed that 65% treated fish survived the challenge compared to that of control as shown in FIG. 4. The treatment test has shown positive results against Vibriosis hence 100% survival upon treatment. The same bacterial species were re-isolated from the kidney of moribund fish to be used in the bacterial challenge.

[0008] FIG. 5 shows the results obtained in the bacterial challenge; depicting the effects of the composition of the present invention and standard antibiotics. In the bacterial challenge, the clinical signs became apparent within 24 hours post challenge. Ulceration started at site of bacterial administration (Vibrio alginolyticus) initially with sloughing off scales, followed by occurrence of hemorrhagic spots all over the body. These spots then progressed to form epidermal lesions that further enlarged within the muscles causing ulcerations. The treated fish however, started to heal after 48-h and were completely recovered after 120-h in treated group. While in the control group, fish
started to die after 48-h and 100% mortality was observed after more than 6 days. It is further observed that the healing process was found faster in the group treated with *Piper betle* crude extract compared to group treated with commercial antibiotics.

**Results 2 - Therapeutic Effects**

[0009] The *Piper betle* extract prepared in accordance with the method of the present invention was used to treat a case of nocardiosis outbreak involving fingerlings of Red Snapper, *Lutjanus erythropterus* cultured in re-circulating tank systems. The infected fish was observed to have the following characteristics:

- lethargic;
- gulping near water surface;
- inappetance;
- skin discoloration;
- emaciated;
- occasional swollen muscle at caudal peduncle area and surrounding the eyes

[0010] Additionally, post-mortem examinations on the affected fish showed whitish nodules in almost all organs including the digestive tracts. Histological examinations of the kidneys and spleen stained with Grocotts revealed hyphae-like organisms, suggesting *Nocardia sp.* Infection.

[0011] Four different treatments were given, these are betel extract, potassium permanganate, oxytetracycline and acriflavine. Absolute (100%) mortality was observed in those treated with potassium permanganate while mortality following oxytetracycline and acriflavine treatments were 78%. It is further observed that mortality in fish treated with *Piper betle* extract in feed at 100 ppm for a period of 4 weeks were eventually reduced to 15%. Accordingly, the survived fish showed discovery and was harvested at 7 months at an average weight of 700g. It is therefore observed that the extracts of *Piper betle* can be used against *Nocardia sp.* infections in fishes.
**Results 3 - Blood Toxicology Study**

[0012] For toxicity determination of *Piper betle* crude extract on fish was conducted through haematology study. Fishes were divided into groups and were fed with betle crude extract at 100 mg/kg for a period of 1, 2, 3, and 4 weeks before blood were sampled for haematology study. It is observed that; with reference to FIG. 6; which shows the results of the toxicology study. Accordingly, there was no significant difference on the haematocrit, red blood, white blood, and hemoglobin values except for thrombocytes count which was higher in fishes treated with *Piper betle* extract for prolonged period. It is believed that such results were obtained owing to the presence of hydroxychavicol in the extract which were found to potentiate platelet counts. However, all altered haematology values were restored to normal after 1 week post treatment.

[0013] As discussed in an earlier paragraph, the composition in accordance with an embodiment of the present invention, the composition in accordance with the present invention is used for providing antibacterial properties against bacterial diseases affecting aquatic animals and in particular fishes; whereby the composition has shown significant effects against; but not limiting to; vibriosis, streptococosis, aeromonasis, and nocardiosis.

[0014] In one aspect it is understood by a person skill in the art that the composition of the present invention may be used in pure form or in combination with additional components which preferably do not materially affect the properties of the composition. In another aspect, the composition may be incorporated in pharmaceutical formulations for treatment of one or more diseases or infections in fishes.

[0015] From the foregoing, it would be appreciated that the present invention may be modified in light of the above teachings. It is therefore understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.
WHAT IS CLAIMED IS:

1. A composition for treatment or prevention of bacterial diseases in aquatic animals comprising an extract from betel leaves; wherein the extract comprising hydroxychavicol.

2. The composition as claimed in Claim 1 wherein the extract further comprising hydrochavicol, hydroxychavicol, hydroxychavicol acetate, allypyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A, piperol, sterol, catechol, and tannin.

3. The composition as claimed in Claim 1 wherein the composition is used for treatment and prevention of bacterial diseases in freshwater and marine fishes.

4. The composition as claimed in Claim 1, wherein the composition provides antibacterial properties against Vibriosis; Streptococcosis; Aeromonasis and Nocardiosis.

5. A method of preparing a composition useful for treatment or prevention of bacterial diseases in aquatic animals; comprising:

   drying the betel leaves for a predetermined period of time (101);
   reducing the leaves into powdered form (102);
   immersing the powder form in alcohol solution for a predetermined period thus forming a mixture, and the mixture shaken for a predetermined period of time (103);
   filtering the mixture (104); and
   subjecting the filtered mixture to a concentration process to obtain a dried form or obtain a constant weight (105) and providing the mixture in a form suitable for oral administration to the aquatic animal.

6. The method as claimed in Claim 5, wherein the powder is immersed in ethanol for a duration of three days.

7. The method as claimed in Claim 5, wherein the shaking is performed continuously at 200 rpm.
8. The method as claimed in Claim 5, wherein the concentration process is performed at a temperature of 60°C.

9. The method as claimed in Claim 1, wherein the method further includes preparing the mixture in stock form.

10. The method as claimed in Claim 9, wherein providing the mixture in a form suitable for oral administration includes: diluting 100 mg of the mixture with 0.5 ethanol; diluting the mixture with 40 ml of water and drying the pellet.

11. Use of a therapeutically effective amount of betel leaves extract in the manufacture of a medicament for the treatment of bacterial diseases in aquatic animals.

12. An aquatic animal feed supplement comprising an extract of betel leaves; the supplement is capable for treatment of bacterial diseases in said aquatic animal.
Collecting and drying the leaves for a predetermined period of time

Reducing the dried leaves into powder form

Immersing powder in alcohol solution to form a mixture and shaken for a predetermined period of time

Filtering the mixture

Subjecting the mixture to a concentration process for obtaining a dried form or constant weight

Preparing a stock of the extract

FIG. 1
Inhibition zone (mm)

Concentration (mg/ml)

FIG. 2

Efficacy of betel extract on common bacterial pathogens of fish (disc diffusion method)

FIG. 3
FIG. 6
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
A61K 36/67(2006.01)i, A61K 31/05(2006.01)i, A61K 9/00(2006.01)i, A61K 9/16(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 36/67; A61K 31/05; A61K 9/00; A61K 9/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic database consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: piper betle, extract, bacterial disease, aquatic animal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>KR 10-2013-0080667 A (NATUROBIOTECH CO., LTD.) 15 July 2013 See the whole document.</td>
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See patent family annex.

Further documents are listed in the continuation of Box C.

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"K" document member of the same patent family

Date of the actual completion of the international search
29 November 2016 (29.11.2016)

Date of mailing of the international search report
29 November 2016 (29.11.2016)

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