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Original Research Article

ANTI-MICROBIAL POTENTIAL OF CANNABIS PLANT EXTRACT AGAINST BACTERIA ISOLATED FROM GUT AND MUCUS OF OREOCHROMIS MOSSAMBICUS

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Abstract: *Cannabis sativa L. is one of the oldest medicinal plants used by human. The plant has also been used for fiber, oil production and simply as additive for food products. Cannabis plant extract may be used for anti-bacterial activity. Bacteria present naturally on the skin, in the gut and in the slime of live fish although. They are not harmful for the fish. But fish get infected with most micro-organisms due to various causes. The goal of this study was to identify the targeted bacteria from mucus and gut of tilapia fish, to study the antimicrobial resistance of isolated bacteria. Thirty samples of Oreochromis mossambicus were collected from Tounsa Head Barrage, Punjab Pakistan. Mucus was collected through spatula. In the laboratory, fish was dissected; gut was removed with the help of a sterile dissecting box. Bacteria were isolated and grown on nutrient agar. E. coli, Staphylococcus spp, Klebsiella spp, B. cereus were isolated from the gut and the mucus while Enterobacter bugandensis was separated from the gut only of the fish. Anti-microbial activity test was done then measure the zone of inhibition in mm. The cannabis showed higher inhibition in growth of E. coli, and Klebseilla spp., while cannabis plant extract showed less inhibition in growth of Staphylococcus spp, Enterobacter bugandensis and B. cereus. It was concluded from our research that the cannabis may be used to develop antibacterial medicines.*

Keywords: *Oreochromis mossambicus*, antibiotics, cannabis, microbial, purification, microorganisms

Introduction

Cannabis is one of the humanity's oldest cultured plants. It is consideration to have originated in central Asia and was cultivated as early as 8000 BC for food, fiber, oil, medicine and as an intoxicated (Small, 2015). Cannabis plant has been found to be used in a huge amount throughout the world, addressing 65% of all worldwide seizure cases (1.65 million cases) in 2011. 5200 tons of spices and 1000 tons of wax were detained during 2006 (Burgdorf *et al*., 2011). Cannabis is most consistently misused in Pakistan as well as throughout the globe (Cascini *et al*., 2012). It contains various artificially dynamic mixtures, for example cannabinoids, terpenoids, flavonoids, and alkaloids (Hortobagyi *et al*., 2016). *Cannabis sativa* derivates, are similar to hashish and marijuana, and are the most as often as possible over the top unlawful medications around the world (Fischer *et al*., 2011). In fact, the cutting-edge plant assortments of *C. sativa* generally utilized for the developed of fish nets (Guarrera and Savo, 2013). Some of the biochemicals of *C. sativa* have been prominent to influence the movements and physiology of fish (Gyang *et al*., 2004). Aquaculture is an important basis of diet, food, occupation, and the income of millions of people around the world. Fish has been measured as best healthful basis of high-quality proteins, supply of vitamins, minerals that framework the major part of human diet (Harrow *et al*., 2012). Fish farming of tilapia has been carried out in Pakistan since 1951, originally, it produced gently but now it is playing a significant part in the budget of the country by hiring more than 400,000 people (Danelljan *et al*., 2016). Tilapia (*Oreochromis spp.*) is one of the most luxurious species in hydroponics produce believing its capacity to be refined in wide degree of standard condition and to use variable proteins (Ainsworth, 2015). Tilapia are generally freshwater living superficial streams, fishponds, rivers, ponds and less usually found living in salted water. Plant-created food can be used for agricultural, making different to delicate wild stocks and farmed that need animal protein-based feed (Taweel *et al*., 2013). The bacteria can enter in fish through mouth with water, food and travel through the intestinal zone (Olafsen, 2001). The fishes are living in the standard rich in pathogenic

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microorganisms. The mucus concealed by the skin of fish displayed more antimicrobial properties (Austin *et al*., 2006). The mucus emitted from fishes additionally fills in as a divider between the inside and outside climate which is worried as a fat having protecting framework (Kuppulakshmi *et al*., 2008). It is now commonly accepted that the intestinal zone of fish principally the intestine holds a large number of bacteria. Bacteria arrive into the fish with food and drinking water and assemble in the intestine (Friedlander *et al*., 1996). The assortment of new microorganisms was effectively done from bacterial vegetation of fish GI bundles that contain a huge, separated, and beneficial microbial people (Dimitroglou *et al*., 2011). Isolate and assess various strains from the gut of Oreochromis niloticus for the conceivable use as probiotics in aquaculture (Wu *et al*., 2001). *Streptococcus spp*., is related with deadly outcomes in tilapia and is the most important fish pathogen (Laith *et al*., 2017). Isolate and biochemically identifies *E. coli* from gills, muscles intestine, surface of tilapia fish and water from fish (Garcia and Servais, 2004). *Klebsiella pneumoniae* (*K. pneumoniae*) is a capsulated Gram-negative bacteria that is a well-described risky pathogen for both community-acquired (Wyres and Holt, 2016). *B. cereus* is a connective mediator of abdominal and non-abdominal diseases. *Enterobacter* is documented as a major pathogen in nosocomial pollutions and is connected with food adulteration (Moradigaravand *et al*., 2016). Recognizable proof of the microorganisms all through reenacted contamination tests, biochemical tests, 16s RNA examination and sequencing of the GBS-express quality CAMP-factor unequivocal quality (Henderson *et al*., 2017).

Materials and methods

The research was conducted in Microbiology laboratory (Institute of Molecular Biology and Biotechnology) of the University of Lahore, Pakistan. The Cannabis plants were collected from the Gujranwala.

Preparation of plant extract

The plants were cleaned and dried at room temperature. Then, these plants were grind with the help of electrical grinder. A total of 50g fine powder was mix with 100 ml of Ethanol. Then the Rotary Shaker, adjusted at 120 rpm was used to shake all the ingredients for 24 hours. After that period, the initial filtrate was obtained through using filter paper. Plant extract were collected and dried. The plant extract was used in concentration of 50µl, 100µl, 150µl, 200µl, 250µl for antimicrobial activity test.

Collection of fish sample

Oreochromis mossambicus was obtained from the Head Tounsa Barrage on the River Indus. Twenty samples of fish were collected. Mucus was collected in the sterile vials before dissection. Sterile spatula was used to scrap mucus from the dorsal body of fish cautiously. Later, fish sample were shifted into an icebox and it was transported into the laboratory of The University of Lahore. Fish was weighted before and after the collection of mucus though using electric weight balance. After that, fish was dissected, and gut was removed from each sample with the help of sterile instruments.

Identification of bacteria

Nutrient media was prepared for growth and testing of bacterial strains. Different colonies were seen on nutrient agar then these isolated colonies were separated and streaked on fresh nutrient agar. These plates were placed in an incubator for 24 hrs. Next day growth of bacteria was observed. Pure colonies of bacteria were isolated and purified on agar and colonies were preserved after that.

Gram staining techniques

This technique was performed to determine gram characteristics of bacteria. After fixing the bacterial smear, crystal violet was first applied for 30 seconds and rinsed with water. It was followed by the addition of mordant iodine for one min which fixed the stain. After rinsing with water, the slide was washed with alcohol for 30 seconds. The bacterial slides were subsequently stained with the safranin dye counterstain for 45 seconds and rinsed again. The slide was blot dried and was observed under a light microscope for negative and positive staining. In microbe identification used deferential media to distinguish the colonies of different organisms. MacConkey Agar, Mannitol Salt Agar, Eosin Methylene Blue, Blood Agar, Biochemical Test

Characterization of isolated bacteria

The isolated bacterial strains were identified through Urease Test, Methyl Red Test, Indole Test, Catalase Test, Simon Citrate Test, Triple Sugar Iron Test and Coagulase Test.

Antibiotics sensitivity test procedure

A Sterile Muller-Hinter Agar (MHA) plate was taken. Bacterial culture was streaked by a cotton swab. After completing streaking, the plates were dried for 10 min. Later, Cannabis plant extract different with different concentrations was prepared applied on the solidified agar through sterilized micropipette tip. Used sterilized forceps, gently filter paper discs into the agar valves. Inoculated the plates cautiously and incubated at the room temperature for 24 hrs. Then, diameter of zone of inhibition of every

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used cannabis extract was measured by comparing the obtained measurements from each valve.

Results

The sample of fish *Oreochromis mossambicus* also known as a Tilapia and common name in Pakistan is Chira fish. *Oreochromis mossambicus* has been collected from the Head Tounsa Barrage, Pakistan.

Isolated colonies of bacteria

The results from figure 1 are displaying isolated colonies of bacteria on nutrient agar. The bacterial strains in figures Fm 3, Fm 4, Fm 22 showing the bacterial colonies on nutrient agar that were isolated from the fish mucus, the Fg 1, Fg 3, Fg 26 are showing bacterial colonies on nutrient agar which were separated from the gut and streak on nutrient agar. The figure 2 indicated the gram positive and negative test results.

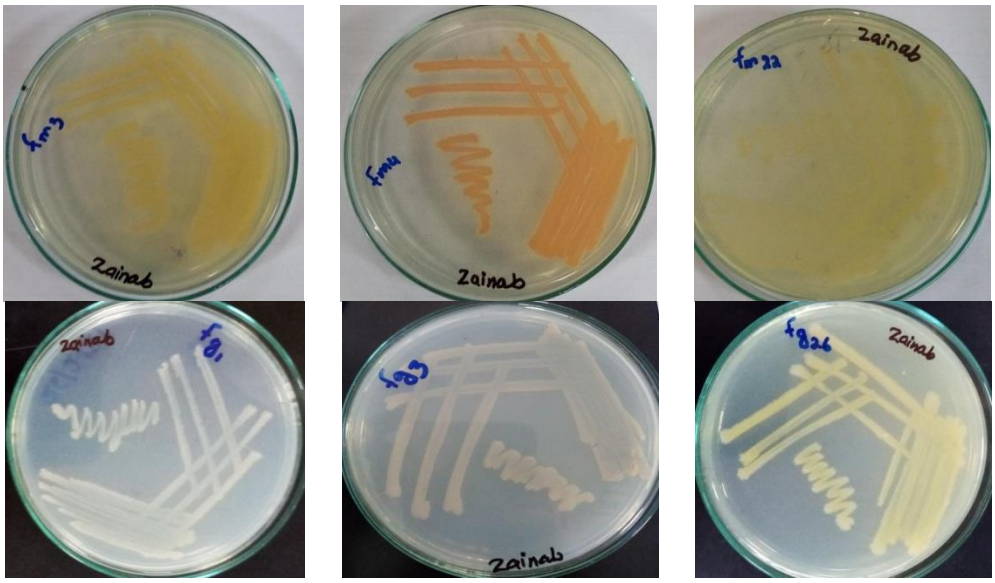

Fig 1: Bacterial growth on Nutrient agar (Fm 3, Fm 4, Fm 22) growth of mucus sample on nutrient agar and (**Fg 1, Fg 3, Fg 26**) growth of gut sample on nutrient agar.

Microscopy gram staining

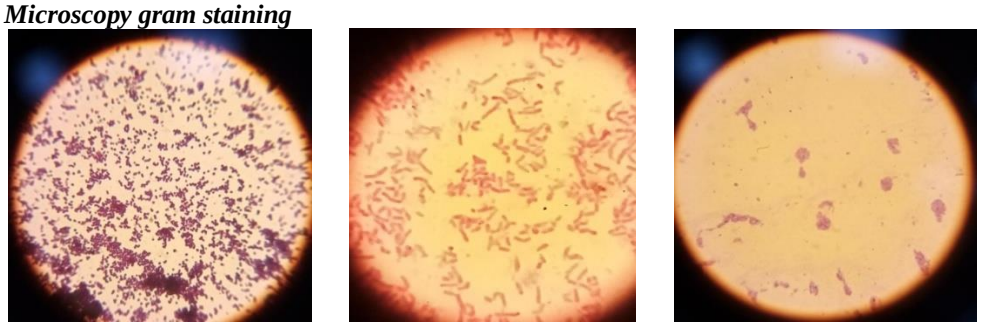

Fig 2: **(A)** is showing cocci shaped in purple-colored clusters form Gram-positive bacteria **(B)** is representing Rod shaped pink colored of gram-negative bacteria **(C)** Is showing cocci shaped in pink colored clusters and colonies form gram-negative bacteria.

Bacterial colonies on differential media macconkey agar

According to fig 3 A, B and C the *E. coli* has given pink colonies on MacConkey agar, *Klebsiella ssp.* has given mucoid colonies and *Enterobacter bugandensis* show mucoid colonies.

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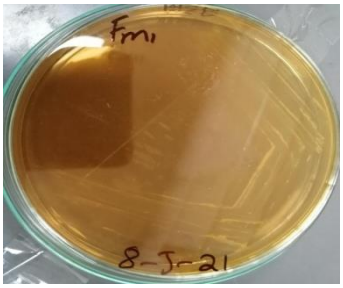

Fig 3: **(A)** Growth of *Klebsiella spp*. on MacConkey Agar **(B)** Growth of E. *coli* on MacConkey Agar **(C)** Growth of *Enterobacter bugandensis* on MacConkey agar.

Blood agar

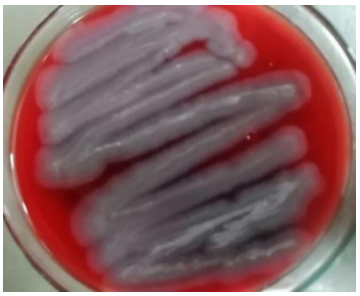

Fig 4: **(A)** Is showing *Klebsiella* no hemolysis on blood agar **(B)** is showing *B. cereus* beta hemolysis on blood agar

Mannitol salt agar: MSA is selective media that is used for the isolation of *Staphylococcus ssp.* produce yellow colonies on MSA (Figure 5).

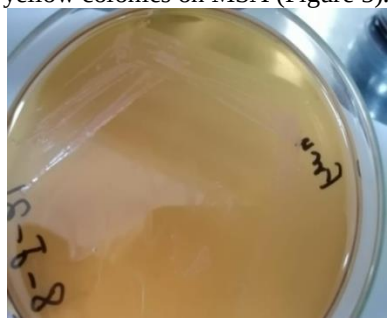

Fig 5: Growth of *Staphylococcus spp.* on MSA media.

Eosin methylene blue: It is show that *E. coli* gave green metallic sheen colonies on EMB media (Figure 6).

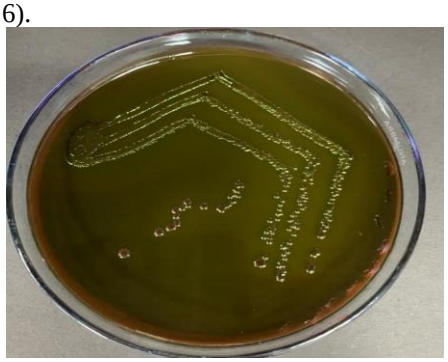

Fig 6: Growth of *E. coli* on EMB

Biochemical characterization There is a different test that was used for the identification of bacteria (Figure 7). The results from figures 7-12 indicated the biochemical test for gram positive and negative bacterial strains.

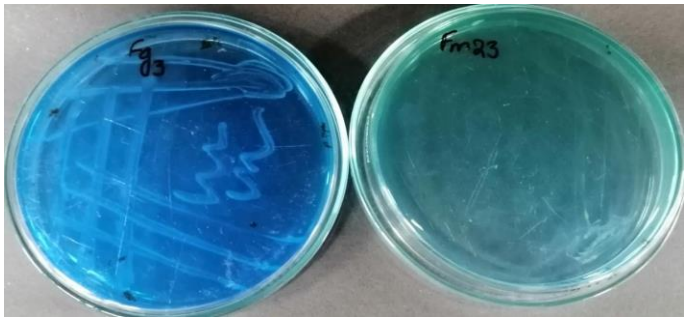

Fig 7: Simon Citrate shows Blue color that it is positive for bacteria and Green is negative bacteria.

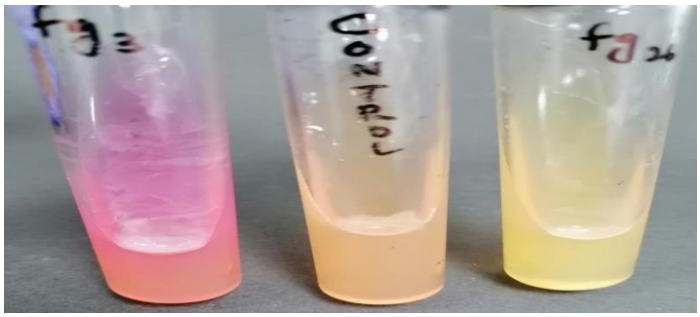

Fig 8: Urease test showed Pink color after inoculation of bacteria and Yellow color show negative result in Urease test.

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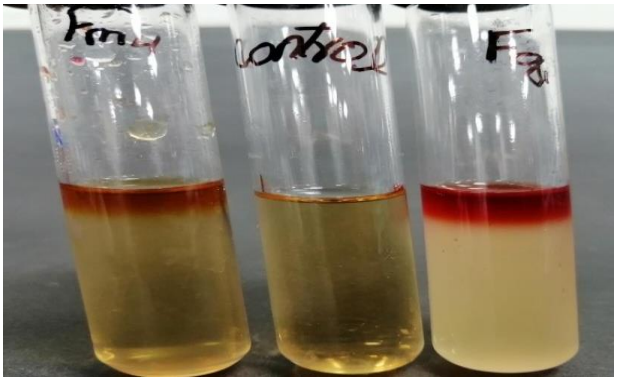

Fig 9: The Red color of Methylene blue has demonstrated that is a positive result.

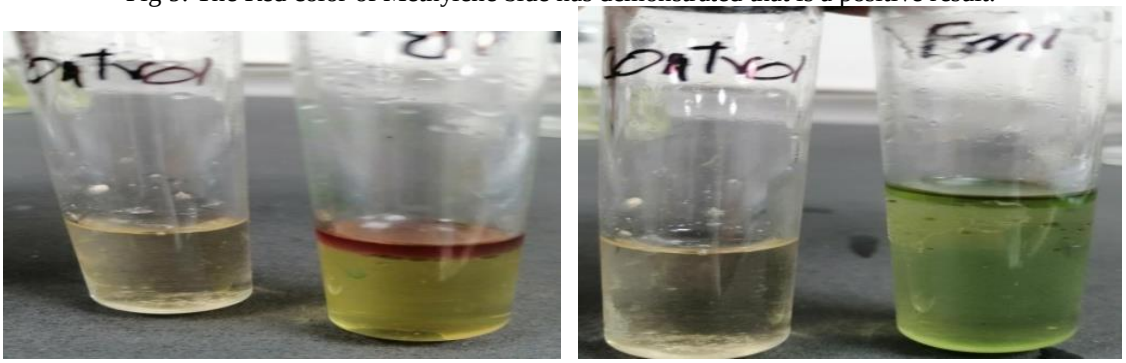

Figure 10: A positive reaction is denoted by the appearance of a color change on the bacterial smear within 2-3 minutes. Indole positive shown bright pinkcolor. Indole negative Green / absence of color showed Indole negative.

Fig 11: **(A)** Acidic slant, acidic butt and gas production show positivity of TSI test.

(B) Yellow slant, yellow butt and gas production of H_2 S of TSI test.

(C) Yellow slant, yellow butt and no gas production H_2S of TSI test.

(D) Yellow slant, red butt and gas reproduction of H_2S of TSI test.

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Fig 12: Catalase test produced bubble after inoculation of bacteria that show positive result.

Catalase test was not produced bubble after inoculation of bacteria show that shows negative result.

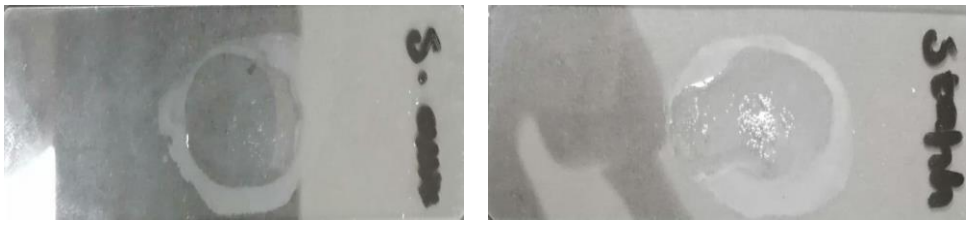

Fig 13: Coagulase showed that is a positive for both **(A)** and **(B)**

The results indicated the following isolated bacterial strains. The E. coli, Staphylococcus spp, Klebsiella spp, B. cereus were isolated from the gut and the mucus while Enterobacter bugandensis was separated from the gut of the fish.

Table 1 Isolated bacteria

Isolated Bacteria from mucus	**Isolated Bacteria from gut**
E. coli	*E. coli*
Staphylococcus spp.	*Staphylococcus spp.*
Klebsiella spp.	*Klebsiella spp.*
B. cereus	*B. cereus*
	Enterobacter bugandensis

Morphological and biochemical tests

Table 2: *Klebsiella* gave Pink to Purple colonies on EMB. It showed these results after biochemical characterization. It is Simmon Citrate test positive, TSI positive, Urease negative, MR negative Catalase test positive, Indole negative.

TEST	***Klebsiella spp.***
Growth on nutrient agar	Light Yellow color
Microscopy	Gram negative and Rods
Growth on Blood agar	White colonies
Growth on MacConkey agar	Pink colonies
Simmon Citrate	+
TSI test	+
Urease test	-
MR test	+
Catalase	+
Indole test	-
Coagulase	+

Table 3: *E. coli* gave Green metallic sheen colour on EMB as well as dark pink colonies in macConkey test. After biochemical characterization it was recognize *E. coli* is Simmon citrate positive, TSI positive, urease negative, MR positive, catalase and indole are positive.

Test	***E. coli***
Growth on nutrient agar	Light yellow colour
Microscopy	Gram Negative and Rods
Growth on EMB agar	Green metallic sheen colour
Growth on MacConkey agar	Dark pink colonies
Simmon Citrate	+
TSI test	+
Urease test	-
MR test	+
Catalase test	+
Indole test	+
Coagulase	+

Table 4: *Bacillus spp.* given beta hemolysis on blood agar. Simmon Citrate positive, TSI positive, Urease positive, MR positive, Catalase positive and Indole Negative.

Test	***B. cereus***
Growth on nutrient agar	Light yellow color
Microscopy	Gram positive and Rods

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Growth on Blood agar	Beta hemolysis
Growth on MacConkey agar	Mucoid colonies
Simmon Citrate	+
TSI test	+
Urease test	+
MR test	+
Catalase test	+
Indole test	-
Coagulase	+

Table 5: Bacteria *staphylococcus spp* demonstrated yellow colony on MSA agar. It showed Simmon citrate positive, Urease positive, MR positive, Catalase positive and Indole negative, TSI positive.

Test	***Staphylococcus spp.***
Growth on nutrient agar	Orange color
Microscopy	Gram positive and cocci
Growth on MSA agar	Yellow colonies
Simmon Citrate	+
TSI test	+
Urease test	+
MR test	+
Catalase test	+
Indole test	-
Coagulase	+

Table 6: *Enterobacter bugandensis* showed gamma hemolysis on blood agar and pink to purple colonies on EMB agar**.** This table is demonstrating that the *Enterobacter bugandensis* shown positivity on Simmon citrate, TSI and Catalase.

Test	***Enterobacter bugandensis***
Growth on nutrient agar	Light yellow color
Microscopy	Gram negative and Rods
Growth on Blood agar	Gamma hemolysis
Growth on MacConkey agar	Light yellow mucoid colonies
Growth on EMB agar	Pink to purple colonies
Simmon Citrate	+
TSI test	+
Urease test	+
MR test	-
Catalase test	+
Indole test	+
Coagulase	+

Antimicrobial activity

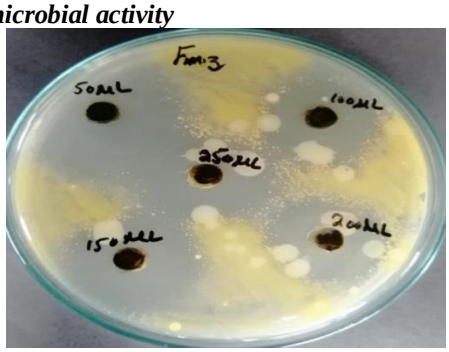

Fig 13: Zone of inhabitation of cannabis plant extract against *Klebsiella spp.*

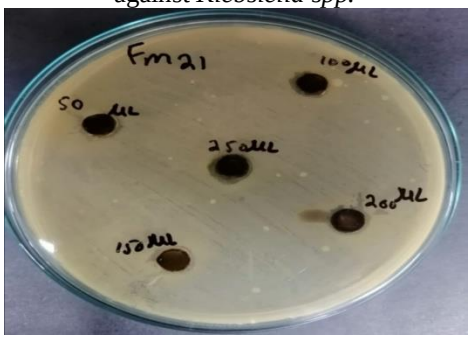

Fig 14: Zone of inhabitation of cannabis plant extract against *E. coli.*

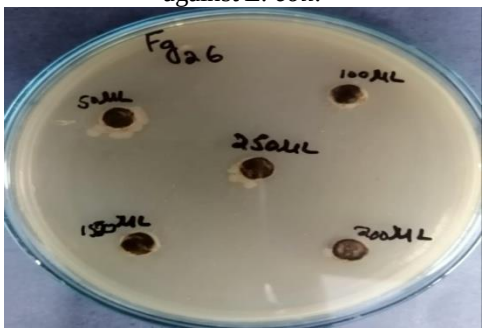

Fig 15: Anti-microbial activity of cannabis extract against *B. cereus* was absent

Fig 16: Anti-microbial activity of cannabis extract against *Staphylococcus spp.*, was absent

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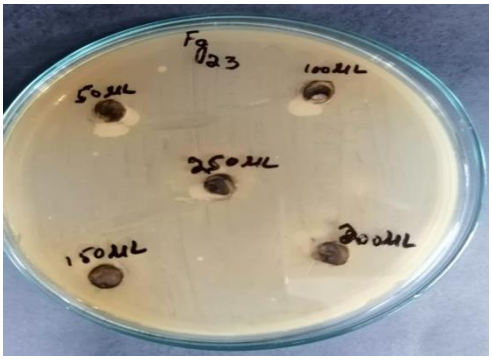

Fig 17: Anti-microbial activity of cannabis extract against *Enterobacter bugandensis* was absent

Table 7 Antimicrobial activity of cannabis plant extract from tilapia fish bacteria (gut & mucus)

Bacterial strains	**Dose 50 µL/mm**	**Dose 100 µL/mm**	**Dose 150 µL/mm**	**Dose 200 µL/mm**	**Dose 250 µL/mm**	**Control**
E. coli	9	10	10	11.1	13	00
Klebsiella spp.	28	24	26	26	27	00

The results from table 7 indicated that the *Klebsiella* spp. showed sensitive as compared with the *E. coli* against the applications of cannabis plant extract.

Discussion

During the study, bacteria were isolated from the gut and mucus of the fish *E. coli*, *Klebsiella spp., B. cereus, staphylococcus spp.*, were isolated. *B. cereus* showed prevalence in the mucus and *Enterobacter bugandensis* showed prevalence in the gut. *E. coli, Klebsiella spp*., and *staphylococcus spp*., were identify by bio-chemical characterization. *Cannabis* extract were used against bacteria and it was checked that which extract is most effective against bacteria. It was observed that *Cannabis* extract were resistant to bacteria and sensitive to bacteria. *Cannabis* extract was effective against *E. coli* and *Klebsiella spp.*,

In this study, E. *coli, Klebsiella spp., B. cereus, S. aureus* and *Enterobacter bugandensis* were isolated from the fish gut and the mucus samples. Study on the bacterial microphlora of some fresh water fish in tropical water (Nachimuthu *et al.,* 2015). The most predominant organisms isolated from the skin the gills of fish belong to the *Enterobacteriaceae* family. *S. aureus, E. coli, Klebsiella spp.*, from skin samples (Tilahun et al., 2019). Borkar separated *Klebsiella spp., E. coli spp*., from the fish (Borkar Pranjali *et al.*, 2017).

E. coli was isolated from the gut and the mucus. Shimeles isolated *E. coli* and identify from the skin samples (Shimeles *et al.,* 2019). *E. coli* isolated from gills, skin, muscles and intestine of the fish. The isolation of the *E. coli* indicates fecal and environmental pollution (Yagoub, 2009). *E. coli* was confirmed after the characterization of biochemical test and morphology. After microscopy it was analyzed that *E. coli* is rod-shaped bacilli and pink color is demonstrated in fig 3(B). Displays the pink colonies on macConkey agar fig 3(B) and green metallic sheen colonies on EMB agar are revealed in fig 6. There is different test was performed for the identification of *E. coli*. These results agreed with Thilahun who showed that *E. coli* is a rod-shaped bacillus. *E. coli* isolated which revealed characteristics colony morphology such as circular, smooth, pink colony in macConkey agar. The bacterial colony appeared green metallic sheen on EMB was regarded as *E. coli* (Tilahun *et al.,* 2019).

Staphylococcus spp. was identifying from the Tilapia fish (Sivanathan *et al.,* 2012). And *S. aureus* was detected during the study (Hurwitz *et al.,* 2004). *Staphylococcus spp*. also isolated from fish sample. It is gram positive and cocci were analyzed by microscopy displayed in the fig 3(A). *Staphylococcus spp*. had given the yellow colonies on MSA agar as it has been showed in the fig 5. Moreover, it has been displayed in the table 4 that *S. aureus* had given Simmon citrate positive, urease positive, catalase positive, MR positive are also used same method for isolation of *Staphylococcus spp* (Figures 7-13). Which take samples from and part of muscles intestine a loop full from incubated growth was streaked on blood agar, mannitol agar incubated plates at 37°C for 24 hrs after that, observed streaking results and selected colonies were submitted to gram staining, morphological characteristics bio-chemical test for *staphylococcus spp*., (Maddah-Ali and Niesen, 2014). These species are possible pathogens, the presence of bacteria is a cause for concern and any mishandling of the fish will contribute to the transfer of the pathogens to human (Apun *et al.,* 1999).

Klebsiella spp. separated from the gut and the mucus. Borkar isolated *Klebsiella spp.* from the gut and mucus of tilapia fish (Borkar and Mahajan, 2017). *Klebsiella spp.* had shown microscopy results that *Klebsiella spp*. showed gram-negative bacteria, pink color, and rod shaped. *Klebsiella spp.* was streaked on macConkey, as shown in fig 7(A).On macConkey *Klebsiella spp.* had given pink to purple colonies. Also, table revealed that different test was positive such as Simon citrate, TSI test, catalase test was positive for *Klebsiella spp*. while negative for indole test and urease test. These results are same Adeshina who shown that all 66 and isolated were gram-negative and ferment lactose, sucrose and mannitol

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sugars (Adeshina *et al.*,2016). Fishery products are important not only nutritional perspective, as well as for some countries around the world for international trade and foreign exchange earners. Also act as a carrier of many infectious and other health risks. Quality management is a vital importance in the processing and trading of fishery product. Most current method of quality management are time consuming and inconvenient (Borkar et al., 2017; Calder and Yaqoob, 2009).

B. cereus was isolated from the gut and mucus of the fish. These finding are similar to Rasool who also isolated *Bacillus spp*. from different part of the fish. On gram-staining, all these isolated were Gram-positive rods and sometime spores were also seen (Rasool and Hemalatha, 2017) as shown in the table 4. The fig 3(B) shows the growth of *B. cereus* on the blood agar was separated from the gut and the mucus of fish. It gave Beta hemolysis on blood agar. *B. cereus* is gram- positive, and rod and these results are like Rasool and Hemalatha (2017) result. Similarly, it had been examined that *B. cereus* is Simon citrate positive, TSI positive; catalase positive, urease positive, and indole are negative. Natarajan and Rajikkannu also reported that morphological characteristics of *B. cereus* revealed that it is a Gram positive, rod shaped and motile. Biochemical results were positive citrate utilization, catalase it showed negative results for indole (Huritz et al., 2004; Natarajan and Rajikkannu, 2014).

Isolated bacteria like *Klebsiella spp.*, *Bacillus spp.*, *Enterobacter spp*. from the gut and mucus (Apun *et al*., 2000). Sekar and Chandramohan also studied the *Enterobacter spp*. from the Mugil capito and reported it as the causative agent of sea (Sekar and Chandramohan, 2008). Similarly, in this study *Enterobacter bugandensis* was also isolated from the gut of fish. The microscopy result had been identifying in the fig 2(C) that *Enterobacter* is gram-negative and rod. *Enterobacter* was streaked on blood agar and MacConkey is shown Gamma hemolysis on the blood agar and mucoid colony in fig 3(C). It has been shown in table 5 that is Simmon citrate positive, TSI positive, catalase positive, indole positive and MR are negative. Antibiotics are importance in the treatment, control, prevention of illness and death caused by infectious diseases in both animals and humans (Aarestrup *et al*., 2008). As a result of release of antibiotics into water sources through the excretion of the numerous metabolites of presents compounds founds in the aquatic environments of human and animals (Kümmerer, 2009). The risk of aquaculture fishing for bacterial infection is high due to the weak hygienic condition inherent in the water environments. Large quantities of various grades of antimicrobial are used in aquaculture facilities worldwide in fish feed for monitoring and treatment purposes (Nachimuthu et al., 2015; Novosalvskij *et al.*, 2016).

Conclusion

The presence of bacteria in the fish indicates pollution level in the environments. All isolated stains from the fish collections have been tasted for antibiotics sensitivity test which indicated that the cannabis extract were effective against *E. coil* and *klebseila spp.*

Conflict of interest

The authors declared absence of conflict interest.

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