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Antibacterial Activity of Selected Plant Aqueous Extracts against Fish Pathogens: An In-Vitro study

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ABSTRACT

Plant derivatives have become a viable alternative to antibiotics in aquaculture. A study was conducted to determine the antibacterial activity of aqueous extracts of *Terminalia chebula, T. bellirica*, *Cynodon dactylon* and the ayurvedic drug Triphala with selected gram-negative bacterial fish pathogens**.** The aqueous extracts of each plant and the product were prepared, and susceptibilities of bacterial isolates to the plant extracts were tested using the agar disc diffusion method. The effect of plant extracts was compared with the commercial antibiotic Ampicillin. The results from the study revealed that the selected extracts of *T. chebula* showed the most pronounced activity with a higher inhibition zone against *Edwardsiella tarda* (18.33 ± 0.52 mm) and *Vibrio alginolyticus* (17.66 ± 0.52 mm). In comparison, the cold extract of *T. bellirica* showed a higher zone of inhibition against *E. tarda* (17.66 ± 0.52 mm). Triphala showed a wide spectrum of antibacterial activity against most of the investigated fish pathogens, with higher action against *E. tarda* (21.66 ± 0.52 mm). Most of the selected extracts showed high activity against *V. alginolyticus.* The current study did not show any effect of *C. dactylon* on any selected pathogens. The study focused on enumerating the simplified use of the above plant extracts in aquaculture.

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1. Introduction

Aquaculture has become the fastest-growing agricultural sector in the world, aiming to enhance food production, poverty alleviation and improve people's health (FAO, 2016). The culture methods have intensified to meet the ever-growing demand for protein-rich food sources, aiming for higher production (Rico *et al.*, 2012). The advent of intensified culture methods paved the way for the rise of pathogens and associated diseases, which later became a major threat to aquaculture (Rasul and Majumdar, 2017). To overcome this, many chemotherapeutic agents like antibiotics have been used to treat diseases and prophylaxis (Aoki *et al.,* 1985; Rasul and Majumdar, 2017). The extensive use of such antibiotics and other synthetic drugs caused many threats to cultured organisms and their consumers on different trophic levels (Jaime *et al*., 2012). Decreased efficacy and resistance of pathogens to antibiotics have necessitated the development of new alternatives (Smith *et al*., 1994). Therefore, it is essential to develop antibacterial drugs made from natural substances. Natural products, especially plants (Phytobiotics), have been investigated for their therapeutic and prophylactic effects on several fish diseases (Pongsak and Parichat, 2009; Olusola *et al.*, 2013; Tania *et al.,* 2018). The presence of various phytochemicals like tannin, terpenoids, alkaloids and flavonoids in plants gives a viable spectrum of pharmaceutical agents to be used against fish pathogens in organic aquaculture (Olusola *et al*., 2013; Shafaque, 2014).

The traditional Indian systems of Ayurveda and Siddha medicines support the importance of medicinal plants in treating diseases (Beusher *et al*., 1994). According to '*Charaka Samhita*', Ayurveda deals with plant-derived products/extracts that can stimulate the host's disease resistance (Kaviratna and Sharma, 1913). *T. bellirica* (Baheda) belongs to the family Combretaceae, and is routinely used as traditional medicine in Ayurveda to get remedies for several ailments such as fever, cough, diarrhoea, skin diseases and oral thrush. Chemical substances of gallic acid, ethyl gallate, galloyl glucose, a new triterpene, belliric acid and chebulagic acid have been isolated from fruits of *T. bellirica* (Rastogi and Mehrotra, 1999). But reports on the antimicrobial activity of *T. bellirica* were scanty, particularly on these strains of microorganisms and their biochemical processes. Elizabeth (2005) studied the antimicrobial activity of *T. bellirica* against human microbial pathogens. Phytochemical analysis of *T. bellirica* extracts showed the presence of secondary metabolites like phenolics, alkaloids, flavonoids and tannins (Nithya *et al.*, 2014).

T. chebula is routinely used as traditional medicine by tribals of Tamil Nadu to cure several ailments such as fever, cough, diarrhoea, gastroenteritis, skin diseases, candidiasis, urinary tract infection and wound infections (Dash, 1991). The ethanol extract of *T. chebula* fruit showed effective antagonism against *Salmonella typhi* , *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Kannan *et al*., 2009). The antibacterial activity of *T. chebula* extracts against several bacterial, namely, *Helicobactor pylori* (Malckzadeh *et al*., 2001) and *Salmonella typhi* (Rani and Khullar, 2004). *T. chebula* fruit aqueous extract has antifungal antagonism against dermatophytes and yeasts (Dutta *et al.,* 1998). Triphala (Tri = three and phala = fruits), a mixture of dried fruits of *T. bellirica, T. chebula* and *Phyllanthus emblica* is mainly used as anti-microbial, anti-inflammatory, anti-diabetic and anti-hyperlipidemic and immunomodulator (Gaddam *et al*., 2019).

Bermuda grass, *C. dactylon* is also an actively used medicinal plant for gastrointestinal disorders (Nasiri *et al*., 2012). The *C. dactylon* is available throughout the year; domestic animals use the material as food. The plant's juice is astringent and is applied externally to fresh cuts and wounds. It is also useful in the treatment of catarrhal ophthalmic, dropsy, hysteria, epilepsy, insanity, chronic diarrhoea and dysentery. Previous studies showed that butanoic, ethanolic, and methanolic extracts of *C. dactylon* leaves showed a wide range of antibacterial activity. No detailed scientific data is available regarding the efficacy of aqueous extract of the whole plant of *C. dactylon*. The aqueous extract of *C dactylon* was used to determine the antimicrobial activity against *P. aeruginosa*, *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Candida albicans*. (Amita *et al*., 2011)

This study explores the antibacterial potential of aqueous extracts of *T. bellirica, T. chebula,* and *C. dactylon* and the effect of the ayurvedic drug Triphala against selected fish pathogens.

2. Materials and Methods

2.1. Bacterial strains

Pure isolates of *Aeromonas hydrophila* (MCCB-113), *Edwardsiella tarda* (MTCC-2400), *Vibrio alginolyticus* (MRNL-03) and *Vibrio fluvialis* (MRCS-26) were obtained from the Department of Marine Biology, Lakeside Campus, Cochin University of Science and Technology (CUSAT), Kochi and the pure cultures were stored with cryo-protectants for further studies.

2.2. Collection and Maintenance of Plant Materials

Fresh leaves of *C. dactylon* (CL) and dried fruits of *T. bellirica* (TB) and *T. chebula* (TC) devoid of seeds were collected, washed, shade dried (away from the sunlight), ground to a fine powder and stored airtight at room temperature.

2.3. Extract preparation of components of ayurvedic drug

Aqueous extracts of the three plants (CL, TB, TC) were prepared by following the methods of Akueshi *et al*. (2002) modified by Bhattacharjee *et al*. (2006). Each sample was weighed (50 g) and soaked separately into 200 ml distilled water (Hot/Cold) in a conical flask that was stoppered with rubber corks and left undisturbed for 24 h. For the hot extract, the solution was then filtered (sterile filter paper- Whatman No. 1) into a clean conical flask, and the crude extract was prepared by boiling in a water bath (20 min), while the cold extract avoided the heating process. The standard extracts thus obtained were then stored in a refrigerator at 4^{o}C for further use.

2.4. Extracts of Ayurvedic Drugs

The cold extracts of the ayurvedic drug Triphala (TP) were prepared by diluting the powder (W/V) using sterile distilled water and then filtered through sterile filter paper (Whatman No. 1). The solution was stored in a refrigerator at 4^{o}C for further use.

2.5. Antibacterial activity Test

The bacterial cultures were inoculated (100 ml nutrient broth- Himedia) and incubated for 24 hrs at 37^{o}C. The susceptibility of isolates to the plant extracts was tested using the agar disc diffusion method (Bauer *et al*., 1966; NCCLS, 1993). The test was performed by inoculating the surface of agar plates with respective bacterial strains (using a single dip of a swab from culture broth). Each plant extract was coated onto the paper disc (20 µl) and placed in the plate. Ampicillin disc 10 mcg (Himedia) of 5 mm was used as the positive control in all plates inoculated with bacteria, while autoclaved distilled water (20 µl) was used as a vector. Triplicate plates were incubated at 37^{o}C for 24 hrs after which the plates were examined and the diameter of the inhibition zones (IZD) was measured to the nearest millimeter. Relative Inhibition Zone Diameter (RIZD) was calculated following Rojas *et al*. (2006).

RIZD (%) = (IZD of sample / IZD of antibiotic) × 100

One-way analysis of variance (ANOVA) and post hoc Duncan test were used to evaluate the differences in the inhibition zones (mean ± SD) among plant extracts and pathogens. All the statistical analyses were performed using the SPSS Statistic 20 software. Statistical significance was designated as P value < 0.05.

3. Results and Discussion

Bacteria is the most common pathogen in both culture and wild environments that cause major losses to the aquaculture industry worldwide (Gerba, 2015). The extensive use of synthetic antibiotics to treat these infectious pathogens leads to side effects both in fishes as well as humans (Malini *et al*., 2013). To overcome this, the development of an alternative was required (Sridevi *et al*., 2011). Among these, herbal plants in aquaculture emerged as a viable source of pharmaceuticals against fish pathogens (Alviano and Alviano, 2009).

The screening for antibacterial activity of aqueous plant extracts against selected pathogens was examined by the presence and absence of a zone of inhibition (Table 1; Figs. 2-5). The hot extracts of TC showed a higher inhibition zone against *E. tarda* (18.33 ± 0.52 mm), while a lower inhibition zone (12.66 ± 0.52 mm) was shown by the cold extract against *V. fluvialis*.

Many investigations reported that the use of compounds extracted from medicinal plants was beneficial for the development of antiobiotics (Abouzeed *et al*., 2013). The present study investigated the antagonistic activity of some phytobiotic aqueous extracts, such as *C. dactylon, T. bellirica* and *T. chebula* and the ayurvedic drug Triphala against selected fish pathogens. The current study revealed that Triphala could be used as a broad-spectrum antibiotic for treating various fish diseases. And also, hot and cold extracts of *T. chebula* and cold extracts of *T. bellirica* exhibited a similar effect. Extracts of *C. dactylon* had no activity towards any of the selected fish pathogens. The cold extract of TB showed a higher zone of inhibition against *E. tarda* (17.66 ± 0.52 mm), whereas both the cold and hot extracts showed lower activity against *A. hydrophila*

Table 1. Zone of Inhibition (mm) of Extracts of *Terminalia chebula, T. bellirica*, Thriphala and Ampicillin against *Aermonas hydrophila, Edwardsiella tarda, Vibrio alginolyticus* and *V. fluvialis*

Treatment	*A. hydrophila*		*E. tarda*		*V. alginolyticus*		*V. fluvialis*		F value
	Mean	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**	
Ampicillin	35.00^{f}	0	21.00^{e}	0.89	39.00^{d}	0.89	14.00^{d}	0	2063.750***
TC Hot	15.67^{d}	0.52	18.33^{d}	0.52	17.67^{c}	0.52	15.67^{e}	0.52	42.500***
TC Cold	13.67^{c}	0.52	14.33^{b}	0.52	16.33^{bc}	1.03	12.67^{c}	0.52	30.833***
TB Hot	11.00^{b}	0	13.00^{a}	0	15.67^{b}	0.52	11.33^{b}	0.52	204.583***
TB Cold	10.00^{a}	0	17.67^{c}	0.52	13.67^{a}	1.03	14.00^{d}	0.89	110.417***
TP	16.33^{e}	1.03	21.67^{f}	0.52	16.67^{bc}	2.58	9.67^{a}	0.52	70.403***
F value (*Comparing treatment*)	1900.00***		233.143***		319.539***		88.857***		

*** P < 0.005; a, b, c, d, e, f: Means with the same superscript within each bacterial strain do not differ from each other (DMR Test)

(10 mm and 11 mm). Triphala showed a wide spectrum of antibacterial activity against most of the investigated fish pathogens, with the highest zone of inhibition against *E. tarda* (21.66 ± 0.52 mm) and lowest against *V. fluvialis* (9.66 ± 0.52 mm). Extracts of *C. dactylon* had no activity towards any of the selected fish pathogens.

The majority of the studies in Triphala were concentrated on human pathogens. The current study evaluated only the aqueous extract of Triphala, being positive to antagonistic studies, and exploring the possibilities of other extracts like chloroform, ethanol, methanol, etc. Triphala showed strong antibacterial activity against gram positive bacteria like *Staphylococcus epidermis* and *S. aureus* and moderate activity towards gram negative bacteria such as *Proteus vulgaris, Salmonella typhi,* etc. (Tambekar and Dahikar, 2011). In the current study, Triphala showed a strong activity against gram negative bacteria *E. tarda*, a fish pathogen; it shows a path forward for exploring various extract preparatory procedures and their possible incorporation through feed (at different concentrations) and other means to be explored.

Earlier studies compared aqueous and methanolic extracts of *T. bellirica* against *Staphylococcus aureus*, *Streptococcus sp.*, *Escherichia coli*, *Pseudomonas aeruginosa* and proved that the aqueous extract has a higher potential than methanolic extract (Thenmalar *et al*., 2017). In the studies by Elizabeth (2005), the aqueous extracts of T. *bellirica* showed higher activity against *S. aureus* at a concentration of 4mg, and the ethanolic extracts also showed similar effects against *Streptococcus pneumoniae*, *Salmonella typhi*, *S. typhimurium*, *E. coli* and *C. albicans*. The Aqueous extract of dry fruit showed the highest zone of inhibition against *S. aureus*, *E. coli* and *P. aeruginosa*. In a study by Nithya *et al*. (2014), it was observed that the aqueous extract of *T. bellirica* exhibited significant activity against the tested bacterial and fungal isolates, compared with chloroform and petroleum ether extract, respectively. The ethanolic extract had less effect over the aqueous extract against *Pseudomonas aeruginosa*, which goes hand in hand with our results on assessing the effectiveness of aqueous extracts than using expensive other modes of extraction with different solvents. Another advantage of using aqueous extract is the easier application procedure for the farmer, rather than going for expensive and laborious multiple solvent extractions.

Mostafa *et al.* (2011) studied the relative effectiveness of an aqueous extract of *T. chebula* against *V. cholera* and concluded that it showed only 34% activity when compared to antibiotics (Ampicillin- 40%). In the current investigation, the hot extract of *T. chebula* displayed a more pronounced activity of 110.71% (RIZD), and the cold extract displayed 89.29% against *V. alginolyticus* compared to antibiotic (Amp- 10 mcg). The aqueous and ethanolic extracts of *T. chebulla* show activity against *S. epiodermidis* and *Bacillus subtilils* and are considered as an important candidate in elucidating its antimicrobial activity (Kannan *et al*., 2009).

Ali *et al.* (2014) conducted an in-vitro antibiotic and herbal sensitivity test against *E. tarda* isolates by disc diffusion method, and reported that the majority of the isolates were resistant to antibiotic Ampicillin while the herbal extracts of *T. bellirica* and *T. chebula* exhibited strong antibacterial activity against all of the selected *E. tarda* isolates. The present study is also in line with the findings of the former one, with a hot extract of *T. chebula* and a cold extract of *T. bellirica* exhibiting highest activity to *E. tarda* than any other bacterial strain in the study, and the antibiotics Ampicillin also showed a similar range of activity when compared to herbal extracts, which is contrary to the above findings.

This study did not show any effect of *C. dactylon* on the selected pathogens. The absence of antibacterial activity does not mean that the bioactive compound is not present in the plant or the plant has no activity against the selected pathogen. In previous studies, aqueous extract of *C. dactylon* showed antimicrobial activity against *P. aeruginosa*, *E.coli*, *S. aureus, and K. pneumoniae* (Amita *et al*., 2011). Boobalan *et al*. (2014) studied the effect of

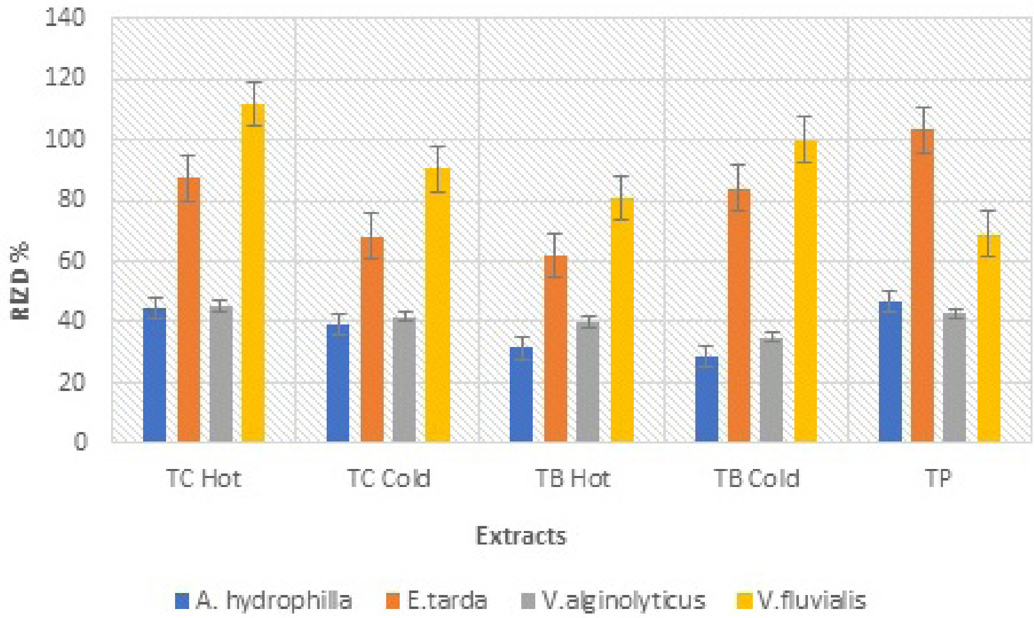

Fig. 1. RIZD (%) of Extracts *Terminalia chebula, T. bellirica* and Thriphala against *Aermonas hydrophila, Edwardsiella tarda, Vibrio alginolyticus* and *V. fluvialis*

different extracts of *C. dactylon* against *Salomnella sp*. and found that the aqueous extracts showed less effective when compared with ethanolic and chloroform extracts. The presence of adequate quantities of active constituents in the extract to exhibit antagonistic activity can be the reason for the negative result. Thus, more in-depth studies on different preparatory procedures (duration of soaking, duration of heating, etc.) extracts (different solvents) of these herbs also need to be examined for fish pathogens, and its in-vivo assay is to be attempted.

The percentage relative inhibition zone diameter of selected plant extracts, when compared with the antibiotic (Ampicillin 10 mcg), revealed that the hot extract of TC and Triphala showed a greater inhibition than the other extracts (Fig. 2). TC hot extract showed an inhibition zone greater than the antibiotic against *V. fluvialis* with a RIZD of 111.85%. Triphala also showed a RIZD of 103.14% against *E. tarda* with an inhibition zone equal to the zone produced by antibiotic (21 ± 0.89 mm).

The antimicrobial activity of the phytobiotics against investigated pathogens was in the order:

T. chebula (Hot) > Triphala > *T. bellirica* (cold) > *T. chebula* (cold) > *T. bellirica* (hot)

4. Conclusion

This study investigated the antibacterial activity of plant extracts, namely, *T. chebula, T. bellirica, C. dactylon* and Triphala, against the selected pathogens, among which hot extract of *T. chebula* and Triphala showed the most promising result. *T. chebula* and Triphala could be promising sources

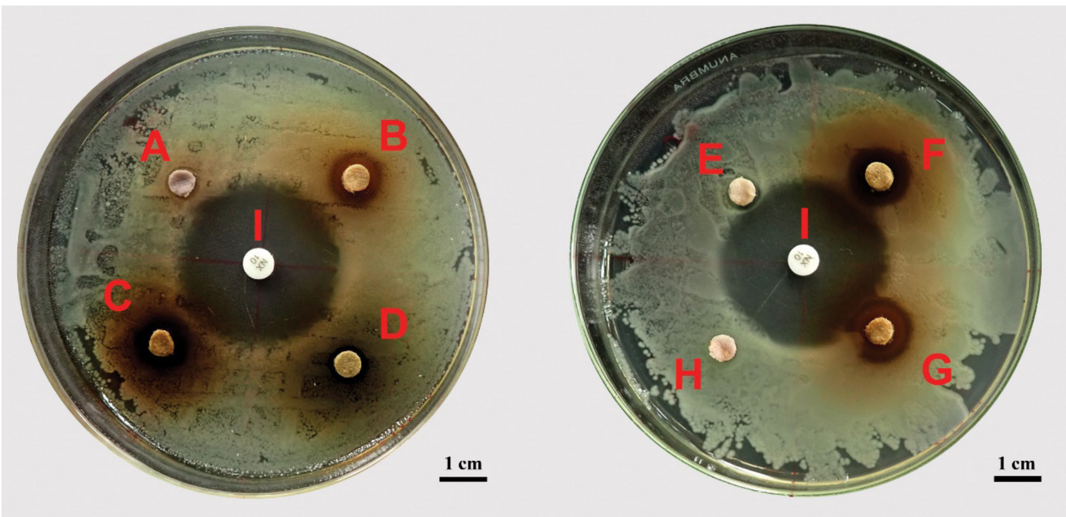

Fig. 2. Activity of extracts against *Aermonas hydrophila*

A – Vector, B – TP, C - TB Hot, D – TB Cold, E – CL Cold, F – CL Hot, G - TC Cold, H – TC Hot, I - Ampicillin

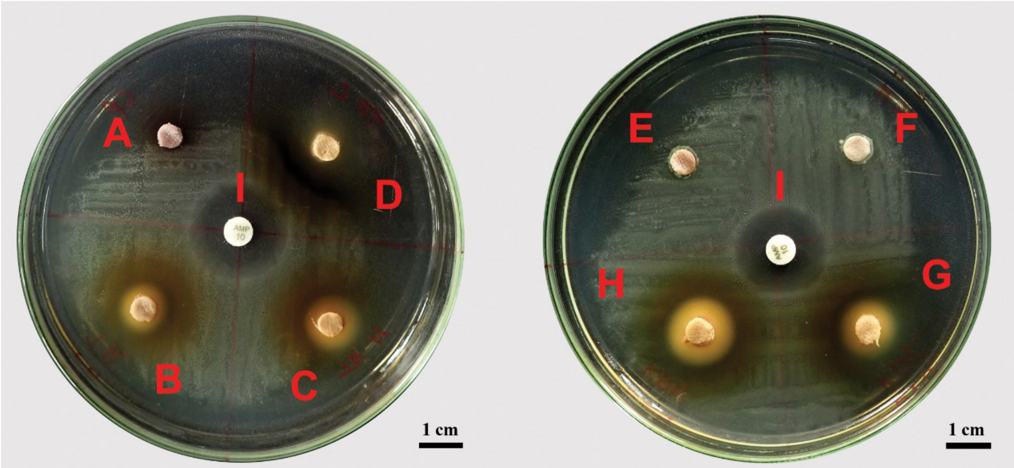

Fig. 3. Activity of extracts against *Edwardsiella tarda*
A – Vector, B – TP, C - TB Hot, D – TB Cold, E – CL Cold, F – CL Hot, G - TC Cold, H – TC Hot, I – Ampicillin

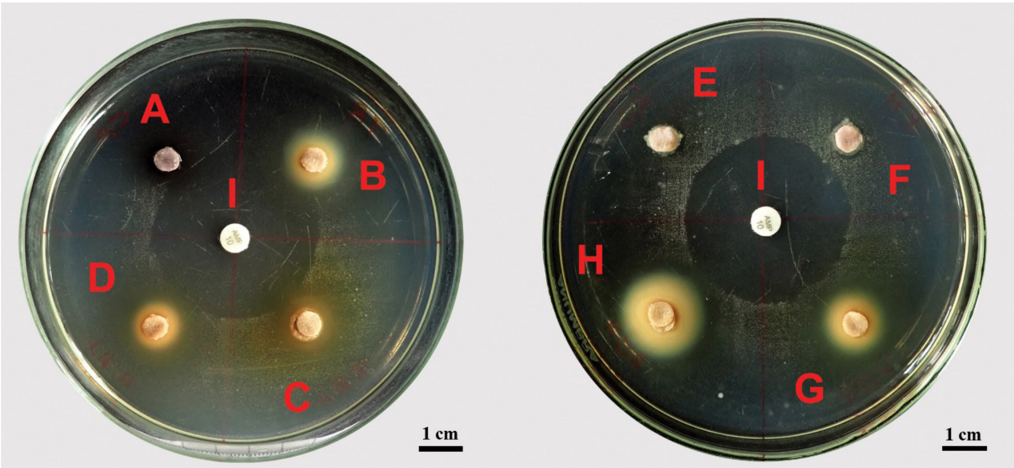

Fig. 4. Activity of extracts against *Vibrio alginolyticus*
A – Vector, B – TP, C - TB Hot, D – TB Cold, E – CL Cold, F – CL Hot, G - TC Cold, H – TC Hot, I – Ampicillin

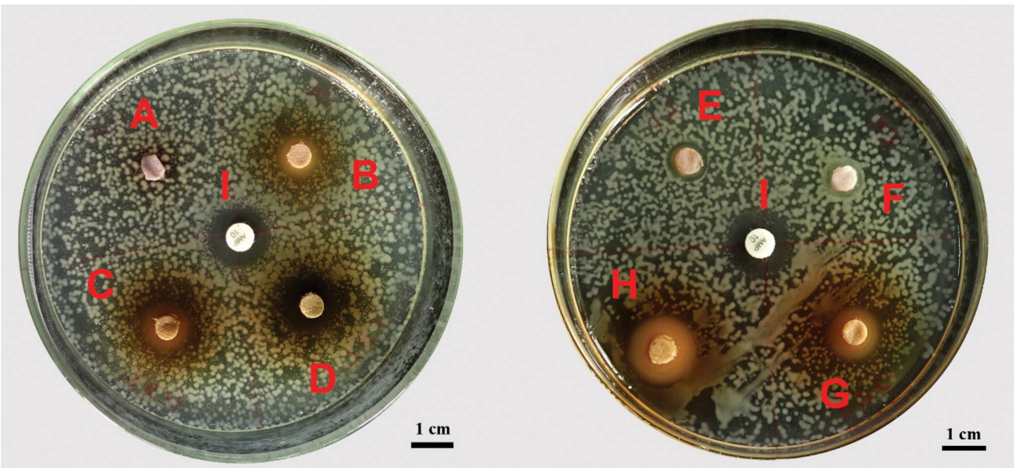

Fig. 5. Activity of extracts against *Vibrio fluvialis*

A – Vector, B – TP, C - TB Hot, D – TB Cold, E – CL Cold, F – CL Hot, G - TC Cold, H – TC Hot, I – Ampicillin of new drug candidates in the aquaculture industry. Being cost-effective and eco-friendly, it is especially beneficial for small-scale farmers to pave a new way of treatment against fish pathogens without debasing the culture environment. The results of this experiment will be of immense use in the future in treatment and prevention. Further research needs to focus on elucidating the bioactive components of such plant products and investigate the effectiveness of various herbs and ayurvedic herbal preparations in fish pathogens and in-vivo assay.

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