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In vitro Screening of Herbal Extracts and Antibiotics against Bacteria Isolated from Fish Products at Retail Outlets

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Authors' contributions

This work was carried out in collaboration between all authors. Authors UK and SS isolated bacterial pathogens and performed all experiments. Author NS prepared all the extracts of medicinal plants. Author TG helped in performing literature search and in writing the manuscript. Author SA designed and supervised the study and revised the manuscript for critically important contents. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Many studies have been conducted on the antibacterial activity of medicinal plants against human pathogens. However, a little has been done on fish pathogens. The aim of this research work was to isolate bacterial pathogens from spoiled fish leading to human diseases and compare the efficacies of selected antibiotics and medicinal herbal extracts against these infectious pathogens.
Study Design: An experimental study.
Place and Duration of Study: Biotechnology Lab, Department of Zoology, University of AJ&K, Muzaffarabad, Pakistan, between Feb 2011 and August 2012.
Methodology: Bacterial pathogens *Enterobacter amnigenus, Serratia odorifera, Salmonella* Typhimurium and *Shigella flexneri* were isolated from spoiled fishes. Various extracts of seed and stem parts of medicinal plants including *Cinnnamomum zylanicum*, *Cuminum cyminum*, *Syzygium aromaticum*, *Curcuma long Linn*, *Trachyspermum ammi* and *Momordica charantia* (both seeds and green parts of Bitter gourd) against common fish

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associated bacterial pathogens by filter disc diffusion method.
Results: The highest zone of inhibition was observed by Ciprofloxacin against *S.* Typhimurium (61 mm), whereas 55 mm by Gentamicin and 51 mm by Streptomycin against *S. flexneri*. However, Penicillin G, Ampicillin, and Amoxicillin had no effect on *S. flexneri* and *E. amnigenus.* The extracts of green part of *M. charantia* showed better results as compared to the seed extracts. Phytochemical screening of medicinal plants indicated that individual compounds *viz*., thyme from ajwain, ar-turmerone from turmeric, eugenol, taninns and flavonoids from clove have antimicrobial activities.
Conclusion: Current study supports the traditional use of medicinal plants as antibacterial agents.

Keywords: Antibacterial activity; antibiotics; filter disk diffusion method; medicinal plants; phytochemical analysis.

1. INTRODUCTION

Bacteria, a major group of pathogens, and fishes are susceptible to several pathogenic diseases which lead to elevate the mortality rates, diminish productivity efficiency, and causing high economic losses to the fish farmers [1,2]. Some bacterial fish pathogens are tied with human's diseases called zoonotic or food-borne diseases [3], and transmitted through various ways *viz*., after getting injured while cleaning aquarium with exposed hands [4-6], injuries from fish, e.g. by thorns [7], and through fish bite [8].

Several antibiotics are used to treat both human and fish bacterial infections [2,9]. The wide use of antibiotics produces antibiotic resistant bacterial strains (ARB) and could have negative impact on the therapy of fish and human's diseases [10]. Regarding the problem of bacterial resistance, there is a vital requirement to discover and establish new drugs and alternative therapies to control these diseases. Owing the ability to synthesize many different substances, the herbal plants are one of the richest sources that researchers used as potential and promising pharmaceutical agents against fish pathogens [11]. Previous literatures indicated the inhibitory action of herbs against fish bacterial pathogens, as an antibacterial agent [12-27].

However, there is insufficient awareness about antimicrobial activity of herbs from Pakistan as a natural source of treatment for fish associated bacterial pathogens. Therefore, the objective of the current study was to evaluate the antibacterial activity of various solvent extracts obtained from medicinal plants in Biotechnology lab, Muzaffarabad, Pakistan on most frequently isolated bacteria from fish products at retail outlets.

2. MATERIALS AND METHODS

2.1 Isolation and Screening of Fish Pathogens

Spoiled fish samples *viz*., Rita Rita and *Schizothorax plagiostomus* were collected from the fish market, Muzaffarabad, Azad Jammu and Kashmir, Pakistan. Collected samples were aseptically taken to the Biotechnology lab for bacteriological examination. The chosen fish were cut down into small pieces, sterilized/washed with 70% absolute ethanol and later d_3H_2O (double distilled deionized water) for 5 min to remove excess ethanol. Washed sample products were placed on Nutrient agar (NA) medium supplemented with methyl red

and crystal violet. After the incubation of 24 hrs at 37ºC, the small portion of growth area were picked with sterilized loop and again streaked on the different selected medium such as Nutrient gar (NA; supplemented with crystal violet and methyl red), MacConky agar (MA), Xylose lysine deoxycholate agar (XLD), and Thiosulphate citrate-bile salts- sucrose agar (TCBS), respectively for the screening of single pathogen. Stock cultures were grown in Nutrient Broth at 37ºC and stored at -20ºC as 60% glycerol stock before used for antibacterial analysis. All growth mediums were purchased from Oxoid Company.

2.2 Biochemical Tests of Isolated Pathogens

The bacterial pathogens were identified and confirmed by using conventional microbiological and biochemical procedures from microbiology lab of Combined Military Hospital (CMH), Muzaffarabad, Pakistan [20,21]. The gram staining was aimed at differentiating gram reactions, sizes, shapes and arrangement of cells of the isolates. Various biochemical tests such as oxidase, catalase, urease, H_2S, citrate utilization, API 10 and Voges proskeur test were used for the confirmation of test pathogen.

2.3 Plant Materials and Extraction

Medicinal plants *Cinnnamomum zylanicum* (Cinnamon; sample C), *Cuminum cyminum* (Cumin; sample B), *Syzygium aromaticum* (Clove; sample A), *Curcuma long Linn* (sample E), *Trachiyspirum ammi* (Carom seeds; sample D), and *M. charantia* were purchased from the super market of Muzafarrabad, Azad Jammu and Kashmir, Pakistan. The collected plant material was air dried at room temperature for 10 days and then powdered with the help of a grinder. The powdered material was weighed (5.80, 5.73, 9.38, 7.90, and 5.25 mg) and soaked in 250 ml of different solvents *viz.*, chloroform (Chl) and isoamylalcohol (ISO) for 48 hrs at room temperature except *M. charantia* [22]. After soaking, the extracts were filtered using Whatman 41 filter paper. The filtrates were collected and stored in refrigerator at 4ºC before use. Whereas the extracts of *M. charantia* (both seeds and green parts) were prepared consecutively with, chloroform (N2, N7), methanol (N5, N10), ethyl acetate (N3, N8), n-hexane (N1, N6) and ethanol (N4, N9) using a Soxhlet extractor for 48 hrs. All the extracts were concentrated using rotary flash evaporator and preserved at 4ºC in airtight bottle until further use [17]. All the extracts were subjected to antibacterial activity assay. The extracts of seeds were indicated as N1, N2, N3, N4, N5 whereas green parts of *M. charantia* as N6, N7, N8, N9, and N10, respectively.

2.4 Phytochemical Screening of Extracts

Phytochemical analysis of chloroform, isoamylalcohol, ethanol, ethyl acetate, n-Hexane and methanol extract of the screened plants were performed to check the presence or absence of active secondary metabolites or different constituents such as tannins, alkaloids, flavanoids, and phenols are given below [18,23,26].

2.4.1 Flavonoids test

1 g powdered + 10 ml ethyl acetate, heated over a steam bath (40–50°C) for 5 min, filtrate was treated + 1 ml dilute ammonia. A yellow coloration indicated positive test.

2.4.2 Tannin test

0.5 g powdered was boiled in 20 ml distilled water for few min, + 3 drops of 5% $FeCl_3$. Development of brownish-green or blue black coloration was taken as positive.

2.4.3 Phenols test

Extract was mixed with few drops of diluted Folin Ciocalteu reagent and aqueous sodium carbonate solution. The mixture was allowed to stand for 10 min and formation of gray colour indicates the presence of Phenolic groups.

2.4.4 Alkaloids test

Few drops of dilute HCL and 0.5 ml Wagner's reagent has added. A brown flocculent precipitate indicates the presence of alkaloid.

2.5 Sensitivity Test of Antibiotics

Sensitivity of antibiotics against test strains was determined by filter disc diffusion method [25]. Sensitivity was predictable with clear zone surrounding the disc. The potency of antibiotics (5 mm in diameter) per disc are as follows; Amoxicillin (10 µg), Streptomycin (10 µg), Tobramycin (10 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Sulfomethoxyzol (25 µg), Tetracycline (10 µg), Penicillin G (10 µg), Trimethoprim (5 µg), Ampicillin (10 µg).

2.6 Antibacterial Activity through Filter Disk Diffusion Method

Filter disc diffusion method was used for testing of medicinal plant extracts against four bacterial fish pathogens viz., *Shigella flexneri, Enterobacter amnigenus, Salmonella* Typhimurium*,* and *Serratia odorifera* [24,25,28,29]. Whatman No. 1 filter paper disc (5 mm diameter) was impregnated with crude (10 µl) plant extracts was placed on Nutrient agar (NA) which was previously swabbed with bacterial fish pathogens. The sterile disc impregnated with only solvents used as a negative control. All the plates were incubated at 37ºC for 24 hrs under static conditions. After 24 hrs the zone of inhibition appearing around the discs were measured and recorded in millimetre (mm) diameter. Each experiment was conducted thrice, and the mean of the results were calculated for both the test and control.

3. RESULTS

3.1 Identification of Pathogens

Various selective, enriched and differential media have been used for the screening of bacterial pathogens. Crystal violet and methyl red indicators were used for the screening of Gram negative bacteria as compared to Gram positive bacteria (Fig. 1A and 1B). It was observed that all isolated pathogens indicated growth on types of medium used except TCBS (Fig. 1A and 1B). A series of biochemical tests were carried out for the identification of *S. flexneri, E. amnigenus, S.* Typhimurium*,* and *S. odorifera* pathogenic bacterial strains (Table 1 and 2). *Enterobacter*, *Salmonella, Shigella,* and *Serratia* are the members of the *Enterobacteriaceae* family (enteric bacteria). These are Gram negative short rods that do not form spores and are facultative anaerobes. They are oxidase negative, and catalase positive

except some *Shigella* strains (Table 2). All isolated bacterial pathogens fermented glucose (Table 2).

3.2 Inhibitory Effect of Medicinal Plants

The antibacterial activity of solvent extracts of medicinal plants *viz*., *S. aromaticum* (sample A), *C. cyminum* (sample B), *C. zylanicum* (sample C), *T. ammi* (sample D), *C. long Linn* (sample E), and *M. charantia* were studied against pathogenic bacterial strains through filter disc diffusion method (Fig. 2A-2C). Antibacterial potential of solvent extracts and several standard antibiotics were assessed in terms of zone of inhibition of bacterial growth. The results of the antibacterial activities are presented in Fig. 2A-2C. Growth inhibition (GI) was recorded as very high (++++), high (+++), medium (++) and low (+), which indicated zones of inhibition between 45-65, 30-45, 12-25, and below 12 mm, respectively.

The screening results of our study confirm the possible use of medicinal plants as a source of antimicrobial agent. The inhibitory effects of chloroform extract of sample A showed zone of inhibition (21, 21, 23, 25 mm) for *S. flexneri, E. amnigenus, S.* Typhimurium*,* and *S. odorifera*, respectively (Fig. 1A). On the other hand no significant result was observed in case of isoamylalcohol extract of *S. aromaticum* (Fig. 1A). The inhibitory effects of (ISO) extract of sample B were observed against *S.* Typhimurium and *S. flexneri* (11, 25 mm) whereas sample B had no effect against *E. amnigenus* and *S. odorifera* (Fig. 1A). On the other hand the significant and moderate zone of inhibition of (Chl) extract of sample B was observed (35, 17 mm) against *S.* Typhimurium and *S. flexneri* while the less inhibition was measured against *E. amnigenus* and *S. odorifera* (9, 8 mm), respectively (Fig. 2A). The inhibitory effects of (ISO) and (Chl) extracts of sample C (45, 15, 31, 17 mm) and (15, 33, 13, 14 mm) were measured against all four tested bacterial pathogens (Fig. 2A). Chloroform extracts of sample D showed significant zone of inhibition (35, 35, 31, 22 mm) against four pathogens as compared to the (ISO) extracts (29, 15, 13, 17 mm), respectively. The chloroform extract of sample E had no effect on the growth of *S. flexneri* whereas 20, 13, 18 mm zone of inhibition was measured against *S.* Typhimurium*, E. amnigenus* and *S. odorifera.* In the same way 21, 35, 23, 15 mm zone of inhibition was measured by using (ISO) extracts of sample E against all tested pathogens (Fig. 2A). The solvents *viz*., chloroform (Chl) and isoamylalcohol (ISO) had no effect and used as negative control. The tested medicinal plants contain more or less same phytochemicals like saponin, flavonoids whereas indicated that these plants are rich in tannin and phenolic compounds.

3.3 Inhibitory Effect of *M. charantia*

The extracts of green parts showed strong antibacterial activity as compared to the extracts of seed parts (Fig. 2B). In the present study, the antibacterial efficacy of various solvent extracts namely chloroform, ethyl acetate, ethanol, n-Hexane and methanol of *M. charantia* against the fish pathogenic bacteria showed varied level of inhibition. N7, N9, and N10 extracts of *M. charantia* indicated 10-32 mm zone of inhibition against *S. flexneri, E. amnigenus, S.* Typhimurium*,* and *S. odorifera*, respectively (Fig. 2B). NI, N2, and N4 had no effect on all tested bacterial pathogens whereas N5 and N6 had no effect on *E. amnigenus* and *S. odorifera*. The highest zone of inhibition was shown by N10 and N9 against *S. odorifera* (32 mm), *E. amnigenus* (30, 30 mm), *S.* Typhimurium (30 mm), respectively (Fig. 2B). The extracts of N6 and N8 had less activity against *E. amnigenus* (6 mm), *S.* Typhimurium (4 mm). On the other hand n-Hexane, chloroform, methanol, ethanol and ethyl acetate had no effect on the growth of all tested pathogens.

3.4 Inhibitory Effect of Standard Antibiotic Discs

The effect of different antibiotics provided useful information for the treatment of bacterial fish and human diseases under laboratory condition. All isolated pathogens *viz*., *S. flexneri, E. amnigenus, S.* Typhimurium, and *S. odorifera* were significantly inhibited by Streptomycin (25, 53, 35, 45 mm), Tobramycin (25, 25, 27, 20 mm), Gentamicin (25, 55, 45, 35 mm), Ciprofloxacin (62, 45, 30, 42 mm), and Tetracycline (25, 25, 20, 15 mm), respectively (Figure 2C). On the other hand Ampicillin and Amoxicillin had no effect on all tested pathogens. Penicillin G, Trimethoprim and Ampicillin had low effect on the growth of *S. flexneri, E. amnigenus, S.* Typhimurium, and *S. odorifera*, respectively (Fig. 2C).

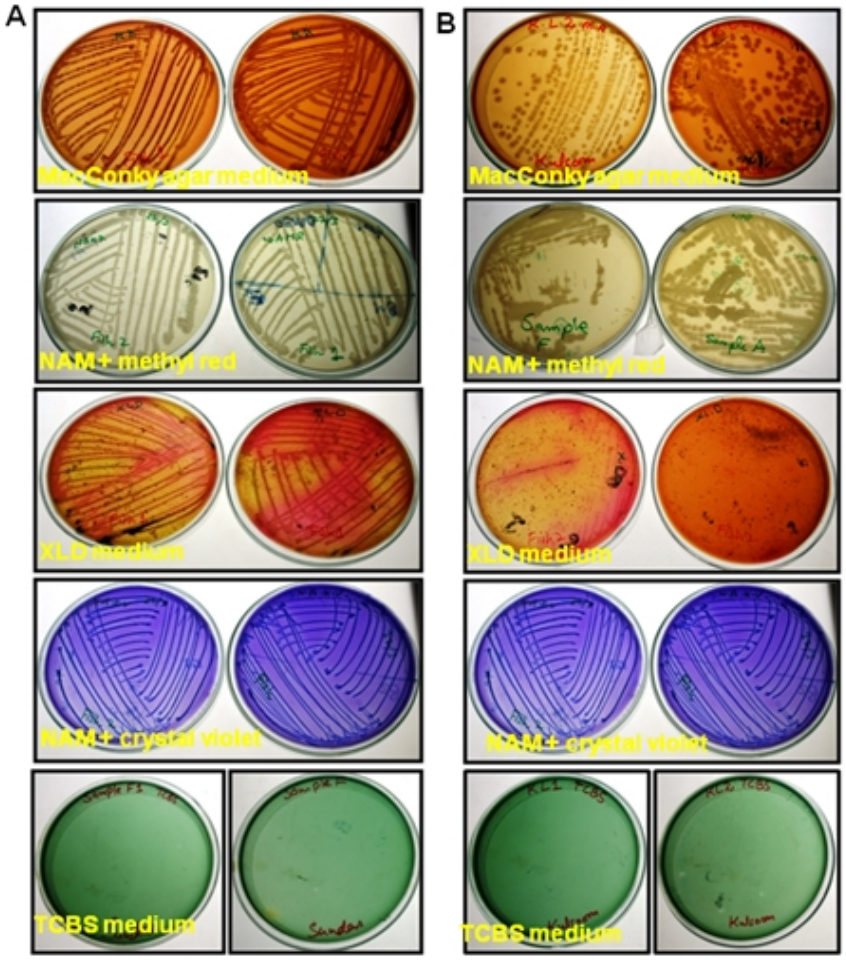

Fig. 1. Screening and identification of isolated bacteria from fish products by using various growth media. (A) Identification of *Enterobacter amnigenus* (left), and *Serratia odorifera* (right). (B) Identification of *Shigella flexneri* (left) and *Salmonella typhimurium* (right)

Table 1. Screening of biochemical tests for the identification of fish associated pathogens

Biochemical techniques used for bacterial identification	Name of bacterial pathogens			
	Shigella flexneri	***Enterobacter amnigenus***	***Salmonella* typhimurium**	***Serratia odorifera***
Gram staining	-VE rod	-VE rod	-VE rod	-VE rod
Citrate test	-VE	+VE	+VE	+VE
Catalase test	+VE	+VE	+VE	+VE
Coagulase test	NA	NA	NA	NA
Indole test	-VE	-VE	-VE	-VE
Methyl red test	+VE	NA	+VE	NA
Oxidase test	-VE	-VE	-VE	-VE
Urease test	-VE	-VE	-VE	+VE
Motility test	+VE	+VE	+VE	NA
Carbohydrate test	+VE	+VE	+VE	+VE
Voges proskeur test	-VE	+VE	-VE	+VE
API 10	+VE	+VE	+VE	+VE
Ziehl Nelson staining	NA	NA	NA	NA

+VE= Indicated; -VE= Not indicated; NA= Not applicable

Table 2. Identification of fish associated pathogens through API10 test

	Active Ingredients		Reactions/Enzymes	Name of bacterial pathogens			
				Shigella flexneri	***Salmonella* Typhimurium**	***Serratia odorifera***	***Enterobacter amnigenus***
API 10 strip	ONPG	2-nitrophenyl-ßDgalactopyranoside)	ß-galactosidase	+VE	-VE	+VE	+VE
	GLU	D-glucose	fermentation / oxidation (Glucose)	+VE	+VE	+VE	+VE
	ARA	L-arabinose	fermentation / oxidation (Arabinose)	+VE	+VE	+VE	+VE
	LDC	L-lysine	Lysine Decarboxylase	NU	+VE	+VE	-VE
	ODC	L-ornithine	Ornithine Decarboxylase	NU	+VE	+VE	+VE
	CIT	trisodium citrate	Citrate utilization	-VE	+VE	+VE	+VE
	H_2S	sodium thiosulfate	H_2S production	-VE	+VE	-VE	-VE
	URE	Urea	Urease	-VE	-VE	-VE	-VE
	TDA	L-tryptophane	Tryptophane Deaminase	NU	-VE	-VE	+VE
	IND	L-tryptophane	Indole production	-VE	-VE	+VE	-VE
	OX	Dryslide oxidase	cytochrome-Oxidase	+VE	NU	NU	NU
	NO_2	Nitrogen Dioxide	Ntrite reductase activity	NU	NU	NU	NU

+VE= Positive; -VE= Negative; NU= Not used

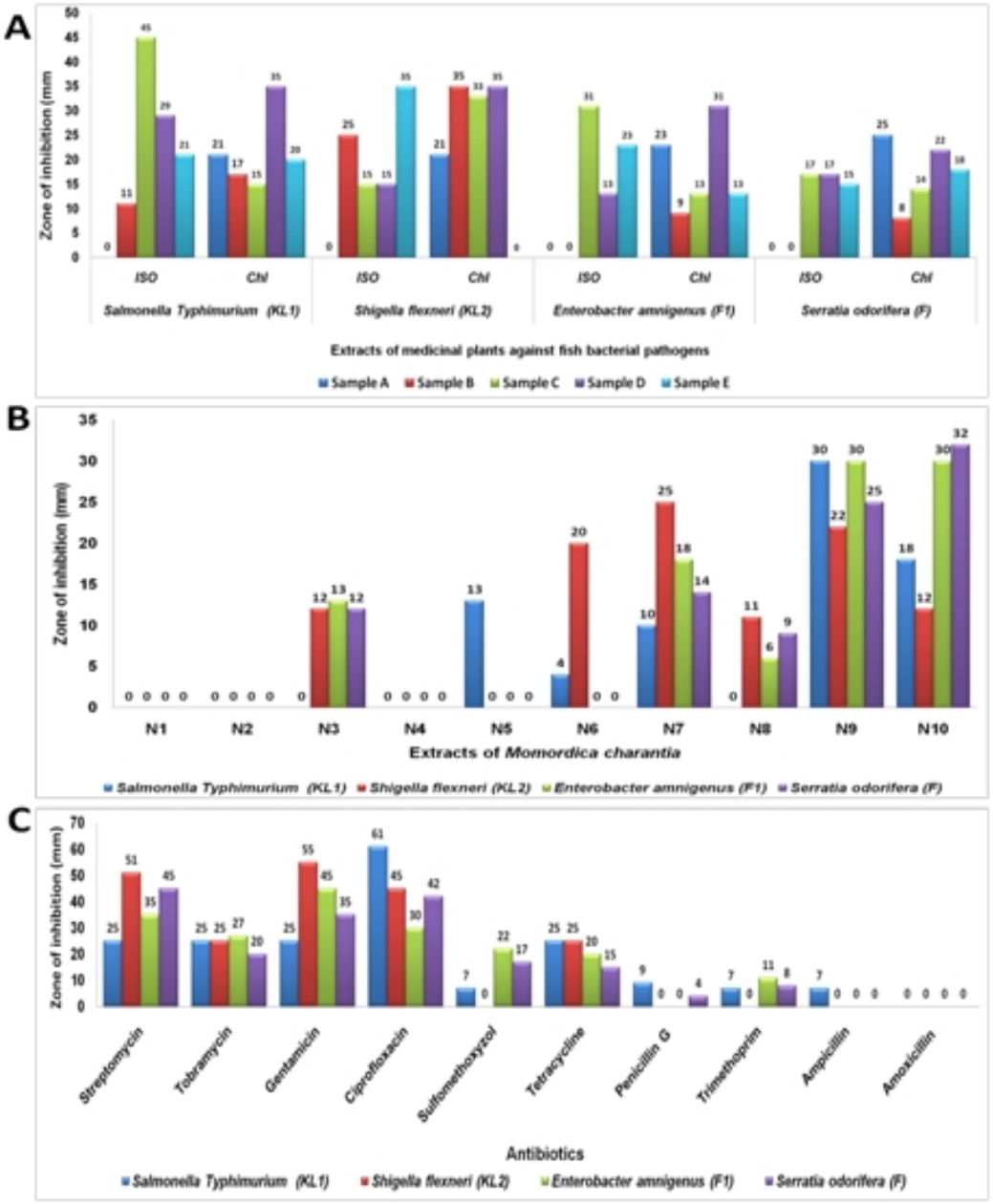

Fig. 2. Antibacterial activity against fish associated pathogens through filter disc diffusion method. (A) Antibacterial activity of medicinal plants against pathogens; (B) Antibacterial activity of *M. charantia* against pathogens; (C) Sensitivity tests of antibiotics against pathogens

4. DISCUSSION

In vitro antibacterial activity of the chloroform, ethanol, ethyl acetate extracts of different coastal medicinal plants were screened against different Gram positive and Gram negative fish bacterial pathogens [30,31]. It has been demonstrated that green extracts of *M. charantia* were found to be more effective against all tested *S. flexneri, E. amnigenus, S.* Typhimurium*,* and *S. odorifera*. Parekh and Chanda [32], and Alam et al. [33], reported that methanol is highly effective solvent for extracting the phytochemicals from the plant material. Similarly, the current scenario indicated the significant activity of methanol extract, which is equal or slightly lesser than the standard antibiotics, tends to show that the active compounds of the plants are better extracted with methanol as compared to the other solvents like n-Hexane (Fig. 2B).
However, the antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world [34, 35]. Several reports indicated the pharmacological activities of turmeric such as antioxidant, anti-protozoal, anti-microbial, antivenom, anti-tumor, anti-inflammatory, hepatoprotective, anti-allergic, anti-ulcer, antidyspeptic and antidepressant [36-49]. Similarly, our results also confirmed that *Curcuma long Linn* (sample E) have therapeutic activities against bacterial infections and may be used as antibacterial agent (Fig. 2A).

The results suggested the presence of high concentration of active compounds/phytochemicals with biological activity in all the extracts of the analyzed plants that can be a helpful therapeutic key. The tested medicinal plants contain more or less same phytochemicals like saponin, triterpenoids, steroids, glycosides, anthraquinone, flavonoids, proteins and amino acids [30, 32]. These plants are also rich in tannin and phenolic compounds, and may possess antimicrobial activities against a number of microorganisms.

5. CONCLUSION

The present study justifies the traditional use of medicinal plants to treat various infectious diseases caused by the microbes. Therefore, it has been concluded that the crude extracts of medicinal plants may be used enough as a drug to treat diseases caused by those bacteria, which are sensitive to the above mentioned samples. The toxicological and clinical trials of pure compounds should be carried out in model animals before use in human being. However, further studies are needed to better evaluate the potential effectiveness of the crude extracts as the antimicrobial agents.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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