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ORIGINAL ARTICLE

Effect of Geographic Disparity on the Antimicrobial Efficacy of Indigenous Plant Extracts Against *Vibrio cholerae*

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ABSTRACT

This study was carried out to investigate the antimicrobial efficacy of methanolic extracts from six medicinal plants including *Amomum subulatum* (Badi Elaichi), *Cinnamomum cassia* (sweet wood), *Eucalyptus camaldulensis* (Murray red gum), *Lagenaria siceraria* (Bottle gourd/Lauki), *Mentha spicata* (Spearmint) and *Zingiber officinale* (Ginger/Adrak) against *Vibrio cholerae*. Different dilutions (15 mg/ml, 12.5 mg/ml, 10 mg/ml, 7.5 mg/ml, and 5mg/ml) of these extracts were prepared and tested against said strains. Considerable antimicrobial activity was noted in the extracts from all tested plants, with various spectrums of activity. *Mentha spicata* showed antimicrobial activity against three clinically isolated *Vibrio cholera* O1 (AMBL1), *V. cholera* O139 (AMBL2), and *V. cholera* Wild type (AMBL3). Quantitatively, *Mentha spicata* exhibited a 3.6 mm zone against the AMBL1 strain, *Zingiber officinale* (3.1 mm) against the AMBL3 strain, *Cinnamomum cassia* (2.9 mm) against the AMBL3 strain, and *Eucalyptus camaldulensis* (2.9 mm) against AMBL2 strain at the concentration of 15mg/ml. Methanolic extract of the minimum inhibitory concentration (MIC) and minimum bacteriostatic concentration (MBC) value of *Mentha spicata* was found to be 1.5 mg/ml against AMBL3 strain, followed by the extracts of *Cinnamomum cassia* against AMBL2 and AMBL1 strain, and extracts of *Lagenaria siceraria* against AMBL2. The extracts of *Cinnamomum cassia* expressed MIC 1.75 and 2.3 mg/ml, and MBC 1.79 and 2.4 mg/ml against AMBL2 and AMBL1 respectively, while extracts of *Lagenaria siceraria* were efficient against the AMBL2 strain of *Vibrio cholerae* with MIC and MBC value of 2.1 mg/ml. There was a significant linear correlation between the efficacy of extracts against *vibrio* spp. and the site of their sampling. Topographically, the higher the altitude of the collection site of the plant, the lower the efficacy. Antimicrobial efficacy was correlated with geo-environmental location and exposure of tested plants. The geographic correlation of samples to the efficacy against vibrio strain is the first of its kind and provides a baseline to extend the trials for other notorious agents like *Mycobacterium tuberculosis,* a communicable pathogen of this region.

Keywords
Antimicrobial Activity, Geographic Variation, Methanol Extracts, Vibrio cholerae.

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INTRODUCTION

Cholera is a clinical-epidemiological disease consisting of acute and secretary diarrhea and is mainly caused by the gram-negative bacterium *Vibrio cholera*[1,2]. More than 200 serogroups of *V. cholerae* have been recognized among which O1 and O139 serogroups are epidemic in nature[3]. Diarrheal diseases including cholera are one of the main causes of morbidity and the second leading cause of death for children under 5 years of age[4,5,6,7]. In adults, cholera is

one of the major causes of severe dehydrating Diarrhea. According to the World Health Organization (WHO), although cholera is immensely under-reported 3-5 million cases occur per year largely in Africa and Asia[8]. Despite all the efforts that have been made, unfortunately, no effective treatment is available to completely cure and prevent the infection caused by *V. cholerae*.

Plant-based therapeutic substances have versatile applications in the agrochemical, nutraceutical, and pharmaceutical industries[9]. Ancient herbal medication practices are the footprints for modern treatment practices. Plant-based medications are formulated based on the ancient testament and are believed to develop modern medicine[10]. The therapeutic value of medicinal plants has been used to cure human diseases for ages. Several natural antiprotozoal, antifungal, and antibacterial molecules have been extracted from natural plants which can be applied parentally or transfused intravenously[11,12,13]. The major advantage of using plant derivatives is to combat the side effects of synthetic counterparts which otherwise are quite effective in case of infectious diseases[10]. Out of the variety of secondary metabolites from medicinal plants, the antimicrobial activities are of most interesting, particularly with a clue that plants bear strong defensive mechanisms against infectious diseases owing to these molecules[14]. The antimicrobial drugs are constituted with a desired property of the substance with selective microbistatic or microbicidal properties against the pathogen only[15], either in combination or alone as a therapeutic agent[16]. Various plant resources are being explored globally to extract antimicrobial activities[17,18]. The target sites for antimicrobials if found different than those antibiotics then there will be a big scope for mitigating the toxicity as well as the same receptor challenges like antibiotic resistance issues and beyond[10]. As an alternative to synthetic antimicrobials, numerous reports are given in medical literature to emphasize the plant-derived value-addable substances still in practice for economical and practical reasons[19,20,21,22]. As a guideline, these studies are pebble marks to seek and validate the antimycotic and antibacterial native molecules particularly to combat the anticholergen.

Pakistan is a South Asian country with a unique steep slope lying between the Arabian sea and China border northward 24-37.5N° and eastward 61.5-75.3E° coordinates. This 2000 km slope facing sunlight provides equally distributed 4 seasons making this region a heaven for contamination of food and water leading to enteric infections. At the same time, plants are a significant resource providing new medicaments for the treatment of diseases[23]. Plant-based anti-enterobacterial activities are distributed across these steep yet varying efficacies. However, most of these claims lack scientific validation and most of the practitioners are Hakeem (herbal doctors) or herbolarios as in the Philippines[10].

This study aimed to determine the comparative anticholeragen efficacy of methanolic extracts of *Amomum subulatum*, *Cinnamomum cassia*, *Eucalyptus camaldulensis*, *Lagenaria siceraria*, *Mentha spicata*, and *Zingiber officinale* against notorious serogroups O1 and O139 of *Vibrio cholerae*. Additionally, the variation of the efficacy of the extracts of these plants concerning their site of origin was to be investigated.

MATERIALS AND METHODS

Six plants with anticholeragen activities i.e., *Amomum subulatum* (BadiElaichi), *Cinnamomum cassia* (Sweetwood), *Eucalyptus camaldulensis* (Murray red gum), *Lagenaria siceraria* (Bottle gourd/Lauki), *Mentha spicata* (Spearmint) and *Zingiber officinale* (Ginger/Adrak) were carried further for study (Figure **2**). All these species were obtained from fairly distant four locations in Pakistan namely Gilgit (N°35. 55 12, E°74.18 28), Lahore (N°31. 55 46, E°74.35 71), Hyderabad (N°17. 38 50, E°78.48 66), and Quetta (N° 30.18 29, E° 66.99 87). The *Amomum subulatum* (fruit), *Cinnamomum cassia* (stem bark), *Lagenaria siceraria* (seeds), *Mentha spicata* (leaves), and *Zingiber officinale* (rhizomes) were obtained from these plants (Figure **1**).

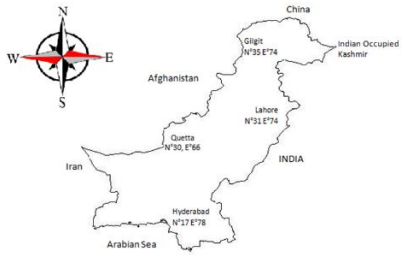

Figure 1. Map of Pakistan with geographically isolated extracts of selected plants with activity against *vibrio cholera* (adapted from www.countryreports.com).

Figure 2. Selected six medicinal plants with anticholeragen activities. Common names and plant parts used for study are given in parentheses.

Preparation of Plant Extracts

The selected dried parts of these plants were macerated into powder form using a grinder machine. About 100 g powder was soaked into 500 mL of absolute aqueous methanol for 7 days at room temperature and then filtered using Whatman filter paper no.1. The filtrate was dried using a rotary evaporator (BÜCHI Rotavapor R-200) under vacuum at 40ºC and freeze-dried for further study.

Bacterial Strains

Clinical test strains of *Vibrio cholerae* O1 (AMBL1), and *V. cholerae* O139 (AMBL2) were obtained from the Pakistan Institute of Medical Sciences, Islamabad, while *V. cholerae* Wild type (AMBL3) was isolated at our laboratory (AMBL) and maintained at 4°C using Müeller-Hinton agar.

Antibacterial Studies

The Agar Well Diffusion Method was used to determine the antimicrobial activity of the methanol extract of selected plants against V. cholerae (Bell and Grundy 1968). Briefly, Müeller-Hinton agar medium was prepared by adding 20 gm dehydrated medium in 1 litter distilled water; pH was maintained at 7.0 and autoclaved. The microbial suspensions were prepared equal to McFarland's 0.5 turbidity standard [10^6 colony forming units (CFUs)/mL] and were cultivated (100 µL) on agar medium. Seven wells per plate were punched in agar with a 6 mm sterile cork borer. Plant extracts were used in five different

concentrations (5, 7.5, 10, 12.5, 15 mg/mL). All petri plates were incubated at 37ºC for 24 h. Ampicillin was used as positive and DMSO was used as negative control.

Determination of MIC and MBC

The broth dilution technique as described by NCCLS (1997) was used for the determination of MIC and MBC values[24,25]. Plant extracts were diluted to the highest concentration (100 mg/mL) and then twofold serial dilutions were prepared in the concentration range from 0.001 mg/ml to 100 mg/ml in tubes that contained Müeller-Hinton broth. About 3% conc. of DMSO was added to the tubes, which did not interfere with the antimicrobial efficacy of plant extracts. The density of bacterial cultures in Müeller-Hinton broth was adjusted to McFarland's 0.5 $BaSO_4$ turbidity standard [10^6 colony forming units (CFUs) per mL]. About 1:10 dilution of bacterial suspension was prepared in Müeller-Hinton broth, and 25 µL of diluted bacterial suspension was added to tubes containing plant extracts and incubated for 24 h at 37°C. The MIC was measured by observing the turbidity of incubated tubes. In tubes that did not show the growth of microorganisms, a fraction of 0.1 mL was added to Petri dishes containing Müeller-Hinton agar to measure the possible bactericidal activity of plant extracts.

RESULTS AND DISCUSSION

The plant extracts were prepared with methanol to yield organic extracts as shown in Table **1**. Antibiotic susceptibility (%) of Vibrio cholera strains was determined using disc diffusion method results given in Table **3**. The highest susceptibility was noticed in the case of 1 µg of Oxacillin (OX1) which is 100 percent in the case of all 3 bacteria. It was followed by 10 µg of Methicillin (MET10) with 100% susceptibility against AMBLI and AMBL2 and 96% in the case of AMBL3 strain of bacteria. This was followed by Cefoxitin 30 µg (FOX30), Cephradine 30 µg (CE30) Cephalexin 30 µg (CL30) while Linezolid 30 µg (LZD30) and Quinupristin/Dalfopristin 15 µg (QD15) showed zero percent susceptibility for all three strains.

Table 1. Percentage (%) Yield of the Extract from Respective Part of Plant Using Methanol.

Scientific name	Common name	Family	Part collected	Yield (%)
Amomum subulatum	Badi Elaichi	*Zingiberaceae*	Fruit	5.3 ±0.79
Cinnamomum cassia	Sweetwood	*Lauracease*	Stem bark	47.6 ±1.02
Eucalyptus camaldulensis	Murray red gum	*Myrtaceae*	Leaves	14.8 ± 1.43
Lagenaria siceraria	Bottle gourd	*Cucurbitaceae*	Seeds	8.0 ±0.91
Mentha spicata	Spearmint	*Lamiaceae*	Leaves	35.6 ± 1.23
Zingiber officinale	Ginger/Adrak	*Zingiberaceae*	Rhizomes	27.0 ± 1.07

Table 2. Determination of Zone of Inhibition (mm) of Plant Extracts at Different Concentrations. (Ampicillin = Positive Control; DMSO = Negative Control

Quantity (mg/ml)	Test Strain	*Amomum subulatu±o.3m*	*Cinnamomum cassia*	*Eucalyptus camaldulensis*	*Lagenaria siceraria*	*Mentha spicata*	*Zingiber officinale*	*Ampicillin*	*DMSO*
15	AMBL1	1.4±o.03	1.4±o.03	1.4±o.03	2±o.10	3.6±o.2	1.8±o.047	4.7±o.35	0
	AMBL2	2.4±o.06	2.1±o.05	2.4±o.09	2.6±o.10	2.6±o.1	1.6±o.043	3.6±o.2	0
	AMBL3	2.6±o.07	2.9±o.13	2.6±o.10	2.4±o.09	2.4±o.09	3.1±o.17	3.4±o.18	0
12.5	AMBL1	1.7±o.04	1.2±o.01	1.4±o.03	1.6±o.043	1.7±o.045	1.6±o.043	4.7±o.35	0
	AMBL2	1.7±o.03	1.5±o.04	2.2±o.06	2±o.10	1.7±o.45	1.1±o.009	3.6±o.2	0
	AMBL3	1.2±o.01	2.8±o.11	2.1±o.05	1.7±o.045	1.5±o.04	2.1±o.05	3.4±o.18	0
10	AMBL1	1.1±o.009	1.1±o.009	1.1±o.009	1.4±o.03	1.5±o.04	1.6±o.043	4.7±o.35	0
	AMBL2	1.7±o.045	1±o.007	1.9±o.07	1.5±o.04	1.4±o.03	0	3.6±o.2	0
	AMBL3	1.8±o.05	1.9±o.06	2±o.08	1.3±o.025	1±o.007	2.2±o.06	3.4±o.18	0
7.5	AMBL1	0	1.1±o.009	1.2±o.01	1.4±o.03	1.4±o.03	1.6±o.043	4.7±o.35	0
	AMBL2	1.3±o.025	0	1.7±o.045	1.4±o.03	1.3±o.025	0	3.6±o.2	0
	AMBL3	1.5±o.04	1.8±o.047	1.4±o.03	1.2±o.01	1±o.007	1.7±o.045	3.4±o.18	0
5	AMBL1	0	0	0	1.3±o.025	1.4±o.03	1.4±o.03	4.7±o.35	0
	AMBL2	1.2±o.01	0	1.5±o.04	0.9±o.006	1±o.007	0	3.6±o.2	0
	AMBL3	1.4±o.03	1.6±o.043	1.4±o.03	0	0.9±o.006	1±o.007	3.4±o.18	0

Table 3. Antibiotics Susceptibility (%) of *Vibrio cholera* Strains Using Disc Diffusion Method.

Antibiotic discs (abbreviations)	AMBL1	AMBL2	AMBL3
Gentamicin 30 µg (CN30)	72.7±3.6	83.3±4.7	79.3±4.0
Methicillin 10 µg (MET10)	100±5.12	100±5.12	96.6±5.0
Vancomycin 30 µg (VA30)	18.2±1.0	5.6±0.28	79.9±4.1
Levofloxacin 5 µg (LEV5)	45.5±2.2	50±2.4	62.1±2.95
Oxacillin 1 µg (OX1)	100±5.6	100±5.12	100±5.12
Teicoplanin 30 µg (TEC30)	18.2±1.0	5.6±0.28	10.3±0.51
Linezolid 30 µg (LZD30)	0	0	0
Penicillin G 10 IU (P10)	36.4±1.7	55.6±2.65	31±1.6
Co-amoxiclav 30 µg (AMC30)	36.4±1.75	50±2.4	13.8±0.71
Quinupristin/Dalfopristin 15 µg (QD15)	0	0	0
Cephalexin 30 µg (CL30)	90.9±4.5	77.8±3.9	100±5.12
Ciprofloxacin 5 µg (CIP5)	81.8±4.2	66.7±3.1	69±3.1
Ampicillin 25 µg (AMP25)	36.4±1.7	50±2.4	13.8±0.71
Cephalothin 30 µg (KF30)	81.8±4.2	66.7±3.1	75.9±3.8
Erythromycin 15 µg (E15)	90.9±4.5	88.9±4.8	79.3±3.6
Imipenem 10 µg (IPM10)	45.5±2.2	55.6±2.65	24.1±1.41
Cephradine 30 µg (CE30)	81.8±4.2	94.4±4.95	100±5.12
Tetracycline 30 µg (TE30)	36.4±1.7	50±2.4	75.9±3.8
Levofloxacin	45.5±2.2	50±2.4	62.1±2.95

Antibacterial Activity

The antimicrobial activity of selected plant species against selected *Vibrio cholerae* strains was analyzed by the presence and diameter of zones of inhibition (mm) as given in Figure **3a** and **3b**.

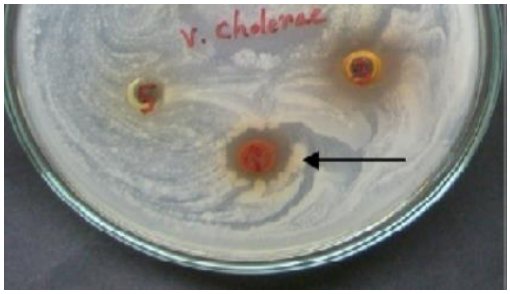

Figure 3a. Arrow showing antimicrobial activity of methanolic extract of *Amomum subulatum* on pathogen *Vibrio cholerae*.

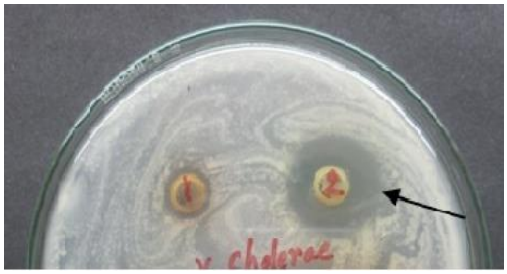

Figure 3b. Arrow showing antimicrobial activity of methanolic extract of *Amomum subulatum* against pathogen *Vibrio cholerae*.

All plants had an antimicrobial effect on all the selected bacterial strains but their activity differed considerably. The most active plants were *Mentha spicata* (3.6 mm) against AMBL1 strain, *Zingiber officinale* (3.1 mm) against AMBL3 strain, *Cinnamomum cassia* (2.9 mm) against AMBL3 strain and *Eucalyptus camaldulensis* (2.9 mm) against AMBL2 strain at the concentration of 15 mg/ml. *Mentha spicata* showed antimicrobial activity on all 5 concentrations of methanolic extracts against three strains of *Vibrio cholera* (AMBL1, AMBL2, and AMBL3). The methanolic preparations of plant extracts have been a common practice as reported previously[25]. Further, the yield of extract using methanolic formulation is quite significant e.g *Cinnamomum cassia* stem bark showed 47.6% yield which is predominantly due to their essential oils and seems miscible with the methanol[26].

The concentrations used in this study include 15, 12.5, 10, 7.5, and 5 mg/mL. Anticholergenic activity against tested bacterial strains was shown by all plants but their activity rate differed considerably. The most active plants were *Menthaspicata* (3.6 mm) against AMBL1 strain, *Zingiberofficinale*(3.1 mm) against AMBL3 strain, *Cinnamomum cassia* (2.9 mm) against AMBL3 strain and *Eucalyptus camaldulensis* (2.9 mm) against AMBL2 strain at the concentration of 15mg/mL. *Menthaspicata* showed antimicrobial activity in all the 5 concentrations used against three strains of *Vibrio cholerae*. These plants are

mostly used to cure different diseases, so their use as antimicrobial agents is quite possible. Especially *Mentha spicata* is the most used treatment of diarrhea. So its use as an antimicrobial agent is justified. It showed good inhibition zones and a remarkable effect has been found against the AMBL1 strain. It was demonstrated that the methanolic extracts of selected plant species exhibited the highest antibacterial effect towards the AMBL3 strain than other bacterial strains. These results are similar to Nazia and Tariq (2006) who reported that *Cinnamomum cassia* showed an antibacterial effect against pathogenic bacteria[26].

The plants obtained from heightened regions (Gilgit) showed lesser activity and hence efficacy than the ones from plains (Hyderabad). The physiological adaptation of extracts and hence molecules counteracting with the peizophilic adaptation of serotypes at the varying height of 5000 feet provides a significant clue to relate the mode of action of metabolites and pathogenicity correlated with the pressure, temperature, and humidity[27]. Further, the extracts from plants of the same species are adapted or tend to act differently under varied geographical distributions and hence the anticipated therapeutic mechanism[28].

Topographically, the higher the altitude of the collection site of the plant, the lower the efficacy. A linear reciprocal correlation was found concerning the efficacy of antimicrobials. The plants at altitude are less exposed to environmental perturbation, population density in their surrounding environment, and hence exposure. This acquired efficacy of antimicrobials is consistent amongst all tested plants[29]. Antimicrobial efficacy was correlated with geo-environmental location and exposure of tested plants. A detailed quantitative investigation is proposed. There was a significant linear correlation between the efficacy of extracts against *vibrio* spp. and the site of their sampling (Table **4**). Topographically, the higher the altitude of the collection site of the plant, the lower the efficacy. Antimicrobial efficacy was correlated with geo-environmental location and exposure of tested plants. The geographic correlation of samples to the efficacy against vibrio strain is first of its kind and provides a baseline to extend the trials for other notorious agents like *Mycobacterium tuberculosis,* a communicable pathogen of this region[30].

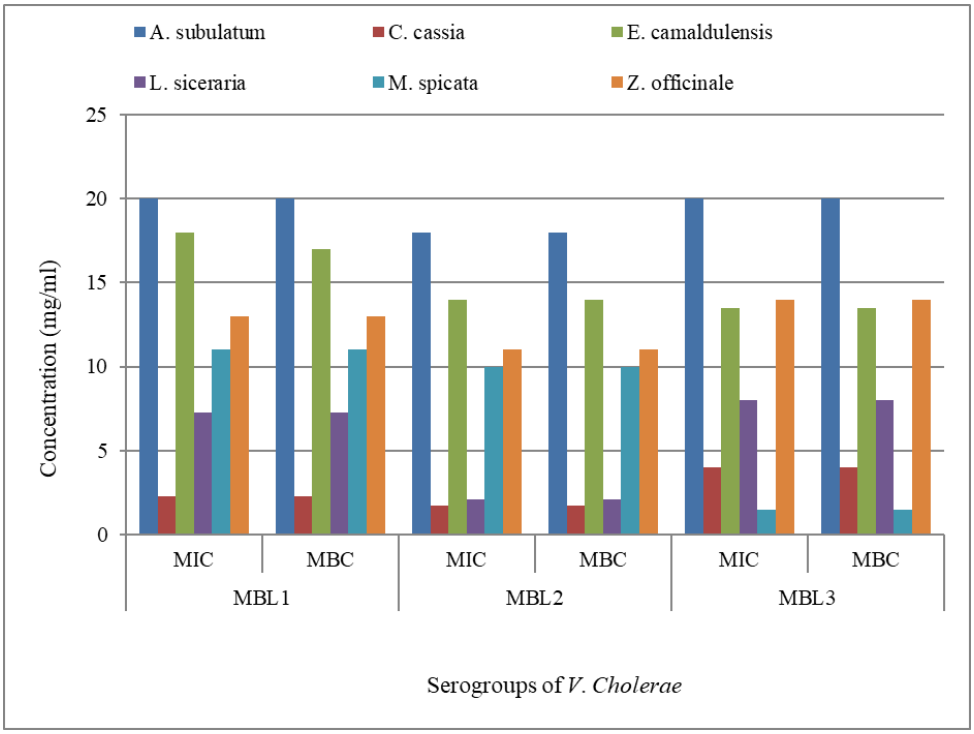

Figure 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) exhibited in mm of plant extracts on three strains (AMBL1, AMBL2, and AMBL3) of *Vibrio cholerae*.

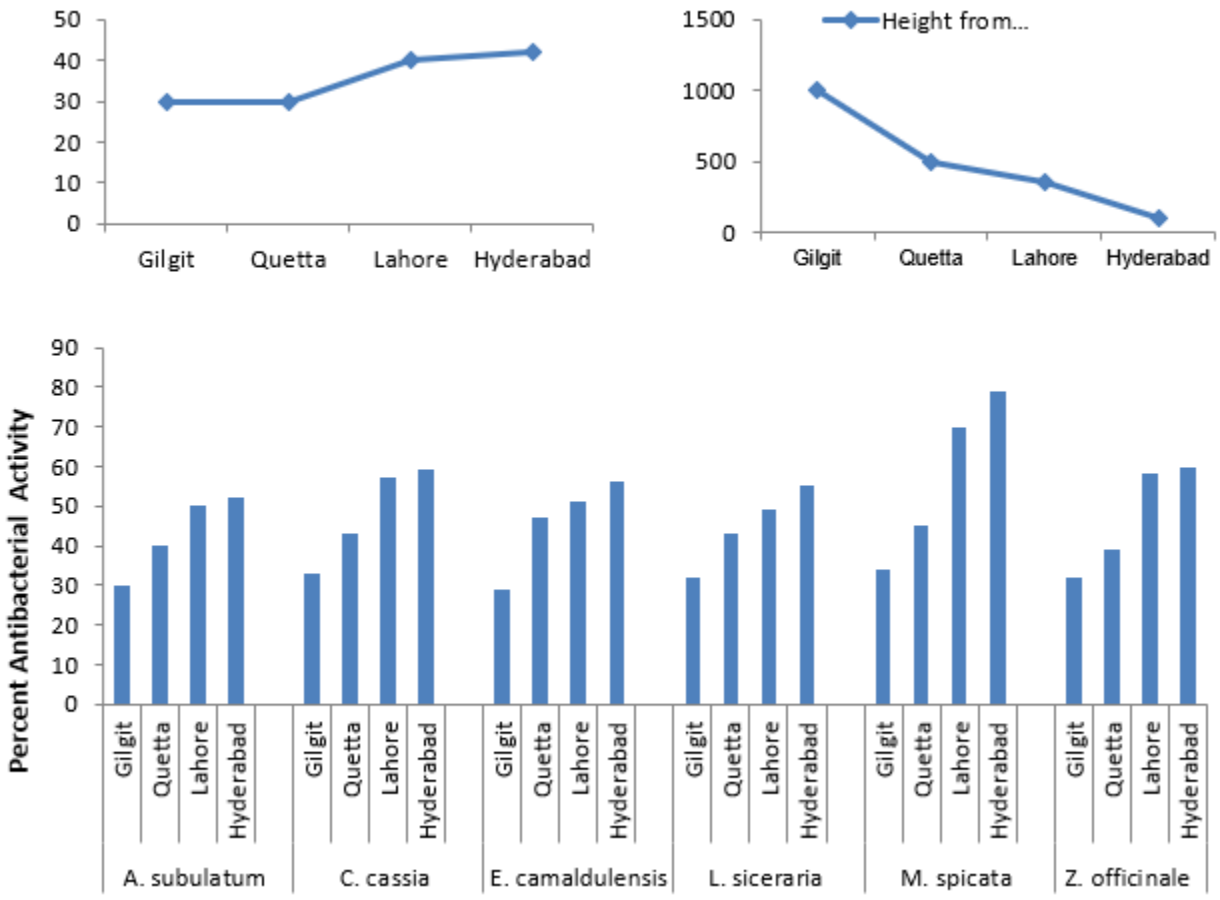

Figure 5. Correlation of antimicrobial activities located at a distance geographically. Locations include Gilgit: N°35. 55 12, E°74.18 28; Lahore: N°31. 55 46, E°74.35 71; Hyderabad: N°17. 38 50, E°78.48 66; Quetta: N° 30.18 29, E° 66.99 87

CONCLUSION

Though these findings are insufficient to proclaim a crucial treatment for *Vibrio* infection, however, this is suggested that the formulations of extracts from *Mentha spicata* obtained from plains would be atop considering the efficacy of the plant. Further, medicinal plants acquire anticholergenic activities on the basis of exposure to wild-type infectious strains of vibrio and the temperature of the region.

CONFLICT OF INTEREST

None.

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