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Efficacy of some plant extracts on some *Vibrio* species isolated from some raw vegetable for processing salad

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Abstract

Raw vegetables have been consumed without further processing. Manures applied to soil to enhance its fertility have been major source of contamination of vegetables and fruits. Natural compounds from plants have been used as an alternative antimicrobial against food-borne pathogens. *Vibrio* species were isolated and identified from raw vegetables using standard methods. Preliminary screen of methanolic, ethanolic, and aqueous extracts of four medicinal and edible plants was performed against the *Vibrio* species.*Vibrio cholerae* (100%) and *Vibrio parahaemolyticus* (91%) were the predominant isolates. The potency of commercial oxoid antibiotics was also tested against the *Vibrio* species. Counts between $1.4 \times 10^4 - 8.0 \times 10^4$, $3.3 \times 10^4 - 2.71 \times 10^5$ and $2.5 \times 10^4 - 2.74 \times 10^5$was recorded as the mean from the three locations sampled. High microbial counts from all the sampled markets indicate gross contamination and pose a potential risk to consumers. Minimal bactericidal concentrations (MBCs) were measured for extracts showing high antimicrobial activity. The *Vibrio* species were all sensitive to aqueous plant extracts of *Ocimum gratissimum* (scent leaf), *Xylopia aethiopica* (uda); *Cymbopogon citratus* (lemon grass), *Zingiber officinale* (ginger), *Allium sativum* (garlic) and *Azadirachta indica* (neem) plants. The three *Vibrio* species were sensitive to Tetracycline, Amikacin, gentamycin, Erythromycin. Rifampicin, and Streptomycin, but resistant to Amoxicillin, Ampicillin and Ciprofloxacin.The efficacy of the plant extracts could be as a result of disruption of the cell membranes of *Vibrio. Species* causing increased membrane permeability, a clear decrease in cytoplasmic pH, cell membrane hyperpolarization, and a decrease in cellular ATP concentration.

Keywords: vegetables, plant extracts, *Vibrio* species, antibacterial

Introduction

Vibrio species have long been acknowledged as ubiquitous marine and estuarine organisms capable of causing human illness (Cosa, *et al.*, 2006).*Vibrio cholerae* was first identified as the causative agent of cholera. More recently identified human pathogens, *Vibrio parahaemolyticus* and *Vibrio vulnificus*, are now the predominant etiologies of human seafood borne infections in developed areas (Cosa, *et al.*, 2006).

Fresh fruits and vegetables are perceived by customers tobe healthy and nutritious foods owing to the plethora ofscientifically proven and documented health benefits derivedfrom consuming fresh products (Braide *et al*., 2012; Oguoma *et al*., 2015; Braide *et al*., 2016; Braide *et al*., 2017).

Raw edible vegetables is exposed to microbial contamination from the field to storage (Song *et al*., 2012; McAllister and Toppi, 2012). Animal and human manures had been reported as a vehicle to contamination of fruits and vegetables (Kumar *et al*., 2013; Jiang and Dharmasena, 2015). Some fruits and vegetables are consumed without processing, thereby exposing consumers to infections. Furthermore, fruits and vegetables sold in open markets constitute risk and danger to consumers.

Active components of plant extract has been reported to kill or inhibit bacteria associated with food borne infection (Burt, 2004; Akinyemi *et al*., 2005; Davidson, 1997; Hayek *et al*., 2013; Tajkarimi *et al*., 2010; Tiwari *et al*., 2009; Adeleye *et al*., 2016a; Adeleye *et al*., 2016b; Braide *et al*., 2018; Adeleye *et al*., 2018; Mike-Anosike *et al*., 2018).Plant extracts seem to be a promising solution to the increasing antibiotic resistance, and may also provide better results than synthetic preservatives (Hayek *et al*., 2013)

This paper reports on the antibiotic susceptibility pattern of *Vibrio* species isolated from some raw vegetables.

Materials and Methods

Collection of samples, preparation and inoculation

Raw vegetable salads were collected from three popular markets in Owerri, Imo State, Nigeria.Samples were homogenize in a stomacher blender containing one liter of sterile diluent. One-tenth milliliter was transferred from the stock serially decimally until appropriate dilution of 10^6 was obtained. An aliquot (0.1 ml) the last dilution was inoculated onto freshly prepared surface dried TCBS medium, spread evenly and incubated at ambient temperature for 48 h (Beishir, 1987; Cheesbrough, 2000).

Determination of microbial population and characterization

Colony counts obtained on the media were counted and expressed as colony forming units per gram (CFU/g) of the total population (Harrigan and McCance, 1990).

Microbial isolates were characterized based on cultural (colonial), microscopic and biochemical methods with reference to standard manuals. The identities of the isolates were cross-matched with reference to standard manuals for the identification of bacteria (Buchanan and Gibbon, 2000).

Preparation of Plants Extracts

Fresh plant leaves of *Ocimum gratissimum* (scent leaf), *Xylopia aethiopica* (uda); *Cymbopogon citratus* (lemon grass), *Zingiber officinale* (ginger), *Allium sativum* (garlic) and *Azadirachta indica* (neem) plants were rinsed with water and sundried. The leaves were grind with a laboratory blender and soaked in ethanol, methanol and water for 24-48 h in 250 ml conical flask. The filtrate was air dried and stored. Different concentration of the extract was obtained and used for the antibacterial test.

Standardization of Inoculum

Twenty four hour old pure cultures of test isolates (*Vibrio* species) were standardized using McFarland method with cell turbidity equivalent to 1.5 x 10^8 Cfu/ml (Cheesbrough, 2000).

Antibacterial susceptibility Test using Commercial Antibiotics

Standardized pure cultures of test isolates were spread evenly on a freshly prepared and surface dried Mueller Hinton Agar medium and allowed to stand for 20 mins. Commercial antibiotics (oxoid) of known concentrations were placed at equidistance on the medium seeded with the test isolates. Incubated was done at 37^0C for 24-48 h. Zone of inhibition was measured and recorded (Beishir, 1987; Cheesbrough, 2000)

Antibacterial susceptibility Test using Plant Extracts

Different concentrations of the plant extracts was added into a well created on Mueller Hinton medium already seeded pure and standardized cultures of the test organisms. Incubation was done for 24-48 h at 37^0C (Cheesbrough, 2000; McAllister and Toppi, 2012; Oguoma *et al*., 2015; Braide *et al*., 2018).
Plates with positive results (visible zone of inhibition) were further subjected to minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Results

Total counts and colonial characteristics of bacteria isolated from the raw vegetables is shown in Table 1. Colony counts from the three sampling areas ranges between $1.4 \times 10^4 - 8.0 \times 10^4$, $3.3 \times 10^4 - 2.71 \times 10^5$ and $2.5 \times 10^4 - 2.74 \times 10^5$ for Ihiagwa, Relief and Obinze market respectively. Colonial characteristics on the TCBS suggest the presence of *Vibrio* as the dominant species and one unidentified *Vibrio* sp and *Serratia marcesence*.

The microscopic and biochemical characteristics confirms the presence of two dominant vibrio species namely *Vibrio cholerae* and *Vibrio parahaemolyticus* (Table 2)

Table 1: Total counts and colonial characteristics of Bacteria isolated from Raw Vegetables for Salad

Sample code	Colony counts (Cfu/g)	Colony types	Colonial characteristics	Probable identity
SIH1	2.8×10^4	SIH1X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH1Y	Moist and shiny circular orange colonies	*Vibrio* sp
		SIH1Z	Small moist and shiny red colonies	*Serratia* sp
SIH2	2.4×10^4	SIH2X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH2Y	Small moist and shiny circular green colonies	*Vibrio* sp
SIH3	6.1×10^4	SIH3X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH3Y	Small moist and shiny circular green	*Vibrio* sp
SIH4	1.4×10^4	SIH4X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH4Y	Small moist and shiny circular green	*Vibrio* sp
		SIH4Z	Moist and shiny circular orange colonies	*Vibrio* sp
SIH5	1.4×10^4	SIH5X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH5Y	Small moist and shiny circular green	*Vibrio* sp
SIH6	5.4×10^4	SIH6X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH6Y	Small moist and shiny circular green	*Vibrio* sp
SIH7	8.0×10^4	SIH7X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH7Y	Small moist and shiny circular green	*Vibrio* sp
		SIH7Z	Moist and shiny circular orange colonies	*Vibrio* sp
SIH8	2.7×10^4	SIH8X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH8Y	Small moist and shiny circular green	*Vibrio* sp
		SIH8Z	Moist and shiny circular orange colonies	*Vibrio* sp
SIH9	3.9×10^4	SIH9X	Smooth moist and shiny yellow colonies	*Vibrio* sp
		SIH9Y	Small moist and shiny circular green	*Vibrio* sp
		SIH9Z	Moist and shiny circular orange colonies	*Vibrio* sp

SIH10	4.9 x 10^4	SIH10X SIH10Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SRL1	6.1 x 10^4	SRL1X SRL1Y SRL21Z	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies Small moist and shiny red colonies	*Vibrio* sp *Vibrio* sp *Serratia* sp
SRL2	1.28 x 10^5	SRL2X	Moist and shiny light lemon green colonies	*Vibrio* sp
SRL3	1.36 x 10^5	SRL3X SRL3Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SRL4	2.71 x 10^5	SRL4X SRL4Y SRL4Z	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies Mucoid raised rough cream colonies	*Vibrio* sp *Vibrio* sp *Vibrio* sp
SRL5	1.06 x 10^5	SRL5X SRL5Y SRL5Z	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies Mucoid raised rough cream colonies	*Vibrio* sp *Vibrio* sp *Vibrio* sp
SRL6	2.19 x 10^5	SRL6X SRL6Y SRL6Z	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies Small moist and shiny red colonies	*Vibrio* sp *Vibrio* sp *Serratia* sp
SRL7	1.96 x 10^5	SRL7X SRL7Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SRL8	2.49 x 10^5	SRL8X SRL8Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SRL9	9.6 x 10^4	SRL9X SRL9Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SRL10	3.3 x 10^4	SRL10X SRL10Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SOBZ1	2.5 x 10^4	SOBZ1X SOBZ1Y SOBZIZ	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies Small moist and shiny red colonies	*Vibrio* sp *Vibrio* sp *Serratia* sp

SOBZ2	1.27 x 10^4	SOBZ2X SOBZ2Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SOBZ3	2.8 x 10^4	SOBZ3X SOBZ3Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SOBZ4	2.9 x 10^4	SOBZ4X	Yellow colonies	*Vibrio* sp
SOBZ5	2.74 x 10^5	SOBZ5X SOBZ5Y SOBZ5Z	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies Moist and shiny bright red colonies	*Vibrio* sp *Vibrio* sp *Vibrio* sp
SOBZ6	1.16 x 10^5	SOBZ6X SOBZ6Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SOBZ7	5.4 x 10^4	SOBZ7X SOBZ7Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SOBZ8	1.16 x 10^5	SOBZ8X SOBZ8Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SOBZ9	3.6 x 10^4	SOBZ9X SOBZ9Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp
SOBZ10	5.4 x 10^4	SOBZ10X SOBZ10Y	Smooth moist and shiny yellow colonies Small moist and shiny circular green colonies	*Vibrio* sp *Vibrio* sp

SIH, Salad Ihiagwa Market; SRL, Salad Relief Market; SOBZ, Salad Obinze Market

Table 2: Microscopic and Biochemical Characteristics of Bacterial isolates

Microscopic morphology	cat	MR	Mn	Ar	Suc	Lac	Na+	LOC	AD	KIA	EH	Identity of isolates
Small slender Gram negative rods in comma or sickle shape	+	+	+	+	+	-	+	+	+	+	+	*Vibrio cholerae*
Small slender Gram negative rods in comma or sickle shape	+	-	+	-	+	-	+	+	+	+	+	*Vibrio parahaemolyticus*
Small gram negative rods in singles and short chains	+	-	-	+	+	-	-	-	-	-	-	*Serratia* sp
Small slender Gram negative rods in comma or sickle shape	+	-	-	-	+	-	+	+	+	-	-	*Vibrio* sp

Cat, catalase; MR, methyl Red Reduction Test; Mn, mannitol; Ar, Arabinose; Suc, Sucrose; Lac, Lactose; Na+, Sodium; LOC, Lysine and Ornithine decarboxylase assay; AD, Arginine dihydrolase Test; KIA, Kligler Iron Agar, Production of Hydrogen Sulphate; EH, Esculin Hydrolysis Test

Table 3 shows the percentage occurrence of bacteria isolated from the raw vegetables. The percentage occurrence is in the order *Vibrio cholerae* > *Vibrio parahaemolyticus* > unidentified *Vibrio* sp > *Serratia marcescence*.The three *Vibrio* specie and *Serratia marcescence* were isolatedfrom the raw vegetable in the all the markets sampled (Table 4).

Table 3: Percentage occurrence of Bacterial isolates in samples

Bacterial isolates>	Percentage occurrence (%)
Vibrio cholera	100
Vibrio parahaemolyticus	91
Serratia sp	8
Vibrio sp	32

Table 4: Distribution of Bacterial isolates across sample locations

Sample locations	Distribution of Bacterial isolates (%)
SIH	*Vibrio cholerae; Vibrio parahaemolyticus; Vibrio* sp; *Serratia* sp
SRL	*Vibrio cholerae; Vibrio parahaemolyticus; Vibrio* sp, *Serratia* sp
SOBZ	*Vibrio cholerae; Vibrio parahaemolyticus; Vibrio* sp, *Serratia* sp

The level of contamination in the three markets as shown in Table 5 is in the order SIH (96%), SRL (85%) and SOBZ (72%). Table 6 shows high level of resistance of the *Vibrio* species to Amoxicillin, Ampicillin and Ciprofloxacin. The three *Vibrio* species were sensitive to Tetracycline, Amikacin, gentamycin, Erythromycin.Rifampicin, and Streptomycin. The *Vibrio* species were all sensitive to aqueous plant extracts of *Ocimum gratissimum* (scent leaf), *Xylopia aethiopica* (uda); *Cymbopogon citratus* (lemon grass), *Zingiber officinale* (ginger), *Allium sativum* (garlic) and *Azadirachta indica* (neem) plants

Table 5: Level of contamination across the sample locations (market)

Bacterial isolates	Level of contamination (%)
SIH	96
SRL	85
SOBZ	72

Table 6: Antibiotic susceptibility Test

Bacterial isolates	TE	AK	CN	E	AMC	RD	S	CIP	AMP
Vibrio cholerae	10	12	12	10	0	12	14	0	0
Vibrio parahaemolyticis	12	14	12	10	0	14	12	0	0
Serratia sp	10	0	0	0	12	16	12	10	12
Vibrio sp	10	14	12	16	0	10	10	0	0

TE, tetracycline;AK, amikacin;CN, gentamycin; E, erythromycin; AMC, amoxicillin; RD, rifampicin; S, streptomycin; CIP, ciprofloxacin; AMP, ampicillin

Table 7: Effects of Plant Extracts on Bacterial Isolates

Bacterial isolates	Ginger	Garlic	Lemon grass	*Xylopia aethiopica*	Scent leaf	Neem plant
Vibrio cholerae	10	12	12	8	0	12
Vibrio parahaemolyticus	12	12	16	14	0	10
Serratia sp	10	0	8	0	0	8
Vibrio sp	12	14	12	10	10	16

Discussion

Vibrios are ubiquitous and abundant in the aquatic environment. A high abundance of vibrios is also detected in tissues and/or organs of various marine algae, animals and zooplankton.

Raw vegetable are subjected to contact with several foodborne pathogens that may be found in the surrounding soil, air and water sources and even faecal material that may be present in the field conditions**(**Song *et al*., 2012; McAllister and Toppi, 2012; Kumar *et al*., 2013; Jiang and Dharmasena, 2015**)**. This was evident from the results as all samples showed the presence of *Vibrio* species irrespective of the sampling location. The high microbial population (1.4×10^4 - 2.71×10^5 Cfu/ml)in the raw vegetables shows gross contamination from source. The level of contamination from the markets sampled is equally worrisome giving the high rate of consumption of salads processed from vegetables. Improper processing and handling, temperature irregularities during transportation and storage, and inadequate sanitation options can also lead to foodborne outbreaks associated with frequently consumed foods(Harrigan and McCance, 1990). The ability of these pathogens to survive the processing and supply chain and reach consumers, causing outbreaks, has made interventions important in terms of sanitation measures. The *Vibrio* species were resistant to three of the nine commercial antibiotics tested against. All the plants extract tested against the organisms proved effective. Natural antimicrobials such as plant essential oils and extracts are gaining popularity as an alternative to commercially used chemicals such as chlorine and hydrogen peroxide(Nwaoguikpe *et al*., 2011; Braide *et al*., 2012; Braide *et al*., 2013; Braide *et al*., 2015; Oguoma *et al*., 2015; Oranusi *et al*., 2015; Okwuwe *et al*., 2015; Adeleye *et al*., 2016a; Adeleye *etal*., 2016b; Braide *et al*., 2018; Adeleye *et al*., 2018; Mike-Anosike *et al*.,2018). Consumers are more aware of the harmful effects of these chemicals and prefer natural alternatives. Even though the exact mechanism of action may be unknown, the efficacy of the compounds present in the plants in reducing the survival of pathogens makes them prime candidates for alternate sanitizer.

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