Journal of Food Chemistry & Nanotechnology

http://dx.doi.org/10.17756/jfcn.2016-019

Research article

Open Access

Effect of Herbal Antimicrobials on Bacterial Strains of Foods of Vegetable and Animal Origin

Bhoj R. Singh*, Dharmendra K. Sinha and Vinodh Kumar O. R

ICAR-Indian Veterinary Research Institute, Izatnagar-243122, India

***Correspondence to:**
Bhoj R Singh
Act. Head of Division of Epidemiology
Indian Veterinary Research Institute
Izatnagar-243122, India
Tel: +91-8449033222
E-mail: brs1762@ivri.res.in

Received: March 07, 2016
Accepted: July 19, 2016
Published: July 21, 2016

Citation: Singh BR, Sinha DK, Kumar VOR. 2016. Effect of Herbal Antimicrobials on Bacterial Strains of Foods of Vegetable and Animal Origin. *J Food Chem Nanotechnol* 2(3): 115-123.

Published by United Scientific Group

Abstract

At the face of emergence of multiple antibiotic resistant strains, herbal antimicrobials are looked as the future drugs for therapeutics, and also as food preservative. This study was undertaken to understand the antimicrobial activity of some selected herbal preparations on bacteria of food origin. In the study 464 (254 from foods of vegetable origin, 134 from foods of animal origin, 14 from food handlers, 62 reference) strains belonging to more than 104 species of 33 genera of Gram negative (194) bacteria (GNB) and Gram positive (270) bacteria (GPB) were tested for their sensitivity to methanolic extract of *Eupatorium odoratum* (EOME), methanolic extract of *Ageratum conyzoides* (ACME), methanolic extract of *Zanthoxylum rhetsa* seed coat (ZRME), lemon grass (*Cymbopogon citratus*) oil (LGO), sandalwood (*Santalum album*) oil (SWO), patchouli (*Pogostemon cablin*) oil (PO) and agarwood (*Aquilaria crassna*) oil (AO) through disc diffusion method. Besides, minimum inhibitory concentration (MIC) of different herbal preparations was determined for test strains using agar-well dilution method. Sandalwood oil inhibited maximum number of strains (50.5%) followed by LGO (45.9%), EOME (43.8%), ACME (40.4%), PO (38%), ZEME (19.6%) and AO (17.5%). Enterobacteriaceae strains in general showed more resistance to herbal antimicrobials than strains of any other family. The MIC for different herbal preparations varied for different strains from 1 µg mL^{-1} to > 16.384 mg mL^{-1}. The study indicated that herbal antimicrobials being edible and acceptable since ages can be an option for use in foods to control spoilage and choice may depend on type of microbe causing the problem in the specific food unit.

Keywords

Meat, Milk, Food handlers, *Eupatorium odoratum*, *Ageratum conyzoides*, *Zanthoxylum rhetsa*, *Cymbopogon citratus, Santalum album*, *Pogostemon cablin*, *Aquilaria crassna*

Introduction

Use of herbal components in food for food preservation is known since long and many phytochemicals have been used for the purpose [1, 2]. Besides, phytochemicals strongly affect the odour and flavour thus the taste [1]. Phytochemicals are not equally active on different microbes similar to antibiotics thus general recommendations may not be possible for their judicious use [3]. Moreover, different herbal antimicrobials impart different flavours and odours thus are not equally acceptable in different communities [2]. Therefore, many different phytochemicals have been studied for their antimicrobial activities in different regions mainly based on the availability and ethnic use in the society. Similar to emergence of antibiotic drug resistance in strains of clinical and non-clinical origin herbal drug resistance is also now steadily gaining acceptance for

its emergence [3] and it is important to monitor the effect of herbal antimicrobials in environmental, food and clinical strains of potential food spoilage and pathogenic bacteria. This study was undertaken to study the antimicrobial potential of *Eupatorium odoratum*, *Ageratum conyzoides*, *Zanthoxylum rhetsa*, *Cymbopogon citrates, Santalum album, Pogostemon cablin*, and *Aquilaria crassna* ingredients often used for culinary purpose in India and having antimicrobial potential [4, 5]. Therefore,

Table 1: Sensitivity of bacterial strains to different herbal antimicrobials tested using disc (containing 1 mg of active ingredient/ disc) diffusion assay on Mueller Hinton agar (MHA) for non-fastidious bacteria, MHA with lysed sheep blood (5%) for fastidious bacteria (*Bordetella, Brucella, Campylobacter, Listeria, Pasteurella, Streptococcus*).

Genus of the bacteria tested (Numbers)	**Species (source, number) of strains tested**	**N**	**Number of strains sensitive to**						
			EO ME	AC ME	LG O	SW O	PO	ZR ME	AO
Acinetobacter (2)	*A. calcoaceticus* (milk, 2)	2	NT	NT	0	0	0	NT	0
Aerococcus (1)	*Aerococcus* spp. (milk 1)	1	NT	NT	0	0	0	NT	0
Aeromonas (12)	*A. eucranophila* (fish 4)	4	NT	NT	4	NT	0	NT	NT
	A. hydrophila (fish 2)	2	NT	NT	2	NT	0	NT	NT
	A. media (fish 4)	4	NT	NT	4	NT	0	NT	NT
	A. salmonicida (fish 1)	1	NT	NT	1	NT	NT	NT	NT
	A. veronii (fish 1)	1	NT	NT	1	NT	0	NT	0
Alcaligenes (1)	*A. faecalis* (milk 1)	1	NT	NT	0	0	0	NT	0
Bacillus (94)	*B. anthracoides* (Axone 3)	3	2	3	3	3	3	1	NT
	B. badius (Axone 7)	7	1	5	7	5	5	4	NT
	B. brevis (Axone 4)	4	2	3	3	3	3	3	NT
	B. circulans (Axone 4)	4	1	4	4	4	4	3	NT
	B. coagulans (Axone 30)	30	9	12	26	27	26	5	2
	B. laterosporus (Axone 1)	1	1	1	1	1	1	0	0
	B. lentus (Axone 8)	8	2	6	8	6	6	4	1
	B. licheniformis (Axone 6)	6	0	0	0	0	0	0	0
	B. marcerans (Axone 4, milk 1)	5	1	4	5	4	2	2	1
	B. mycoides (Axone 2)	2	2	1	2	2	2	0	2
	B. pentothenticus (Axone 16)	16	2	15	15	15	12	15	NT
	B. stearothermophilus (Axone 5)	5	5	3	5	5	5	0	3
	B. subtilis (Axone 3)	0	3	3	1	3	3	0	1
Bordetella (1)	*B. bronchiseptica* (ref 1, MTCC6838)	1	0	0	0	0	0	NT	0
Brucella (3)	*B. abortus* (ref 3, S19, S99, B19)	4	0	2	2	2	2	NT	0
Budvicia (1)	*B. aquatica* (fish 1)	1	Nt	NT	1	NT	0	NT	NT
Burkholderia (4)	*B. cepacia* (ref 1, MTCC438)	1	1	1	1	1	0	NT	1
	B. gladioli (ref ,1 MTCC 1888),	1	0	0	0	0	0	NT	0
	B. pseudomallei (ref 2, MTCC7183, MTCC7259)	2	0	0	0	0	0	NT	0
Campylobacter (4)	*C. jejunii* (ref 4, CJ1-4)	4	0	0	0	0	0	0	0
Citrobacter (7)	*C. diversus* (Axone 1)	1	0	0	0	0	0	1	0
	C. freundii (fish 3, muffen 3)	6	0	0	0	0	0	0	1
Edwardsiella (1)	*E. tarda* (fish 1)	1	Nt	NT	0	NT	0	NT	NT
Enterobacter (23)	*E. agglomerans* (Axone 11, milk 4, ref 1 RAVI)	16	0	4	7	0	4	2	0

	E. amnigenus (Axone 1, muffen 3)	4	0	0	0	1	0	0	2
	E. gregoviae (Axone 3),	3	0	0	0	0	0	0	NT
Enterococcus (70)	*E. asacchrolyticus* (Axone 1)	1	NT	1	1	1	1	0	NT
	E. avium (Axone 4)	4	0	0	2	1	0	0	NT
	E. caecorum (Axone 28)	28	11	2	9	14	9	2	2
	E. casseliflavus (Axone 3)	3	0	1	2	2	1	0	NT
	E. dispar (Axone 6)	6	4	1	2	3	2	0	0
	E. faecalis (Axone 3)	3	0	1	1	1	0	0	NT
	E. faecium (skin 1)	1	NT	NT	0	1	0	0	NT
	E. gallinarum (Axone 6)	6	0	1	0	0	0	0	NT
	E. hirae (Axone 7)	7	0	1	2	2	1	0	NT
	E. malodoratus (Axone 3)	3	2	0	0	1	1	0	0
	E. mundatii (Axone 3)	3	2	0	2	1	1	0	Nt
	E. raffinosus (Axone 5)	5	2	1	0	1	0	0	0
Erwinia (3)	*E. chrysanthemi* (fish 1, ref 2, E7300, E7300/1)	3	0	0	0	0	0	NT	0
Escherichia (25)	*E. blattae* (fish 3)	3	NT	NT	2	NT	0	NT	NT
	E. coli (Axone 8, milk 9, ref 5, E382, E382/1, YP1, YP2, Eca)	22	1	0	5	0	1	0	0
Hafnia (2)	*H. alvei* (Axone1, milk 1)	2		1	0	0	1	0	0
Klebsiella (15)	*K. oxytoca* (fish 1)	1	NT	NT	1	NT	0	NT	NT
	K. pneumoniae (Axone 8, fish 1, milk 4, muffen 1)	14	0	2	4	1	2	1	1
Lactobacillus (1)	*L. acidophilus* (Axone 1)	1	NT	NT	1	1	1	1	NT
Leminorella (1)	*L. ghrimontii* (fish 1)	1	NT	NT	0	0	0	NT	NT
Listeria (1)	*L. monocytogenes* (ref 1, MTCC839)	1	0	1	1	1	0	NT	1
Micrococcus (2)	*M. agilis* (Axone 2)	2	2	1	1	2	1	0	NT
Pasteurella (3)	*P. canis* (milk 1)	1	NT	NT	1	0	0	0	0
	P. multocida (ref 2, P52, Soron)	2	2	1	2	2	2	0	1
Pediococcus (1)	*Pediococcus* spp. (milk 1)	1	0	0	0	0	0	NT	0
Proteus (20)	*P. mirabilis* (Axone 7, fish 1)	8	NT	0	4	0	0	0	NT
	P. myxofaciens (Axone 1)	1	NT	0	1	0	0	0	NT
	P. penneri (Axone 5, fish 2)	7	NT	0	1	0	0	0	NT
	P. vulgaris (Axone 3, fish 1)	4	NT	0	0	0	0	0	NT
Providencia (5)	*P. rettgeri* (Axone 5)	5		0	0	0	0	0	NT
Pseudomonas (30)	*P. aeruginosa* (Axone 22, milk 2)	24	1	NT	15	9	20	9	1
	P. fluorescens (fish 3, milk 1)	4	NT	NT	0	0	0	NT	0
	P. pseudoalcaligenes (fish 2)	2	NT	NT	2	0	0	NT	NT
Raoultella	*R. terrigena* (milk 1)	1	NT	NT	0	0	0	NT	0
Salmonella	*S. enterica* ssp. enterica serovars (Ref 27 from NSC)	27	0	0	4	2	2	2	1
Serratia	*S. plymuthica* (muffen 1)	1	0	0	0	0	0	0	0
Staphylococcus (80)	*S. aureus (*skin 4, buffen 2, chevon 5, ref 3, ATCC43300, ATCC29213, ATCC700699),	12	NT	NT	4	10	8	2	1
Streptococcus (20)	*S. auricularis (*chevon 2)	2	NT	NT	0	1	1	0	0
	S. caprae (chevon 1)	1	NT	NT	1	1	1	0	0
	S. capitis (buffen 4, milk 1)	5	0	0	3	4	1	NT	1

	S. caseolyticus (milk 1, chevon 1)	2	NT	NT	1	2	0	1	0
	S. chromogenes (chevon 3, buffen 1, milk 1)	5	NT	NT	2	5	2	1	2
	S. epidermidis (chevon 7, milk 1, skin 3, ref 2, BM, MTCC 449)	13	1	1	4	12	6	4	3
	S. felis (chevon 1)	1	NT	NT	0	0	1	0	0
	S. haemolyticus (chevon 2, milk 1)	3	NT	NT	3	3	3	0	0
	S. hominis (chevon 2)	2	NT	NT	1	2	1	0	0
	S. hyicus (buffen 3, milk 1)	4	0	0	2	3	2	NT	1
	S. intermedius (chevon 6)	6	NT	NT	5	6	5	0	2
	S. lentus (buffen 2)	2	NT	NT	1	2	1	NT	1
	S. scheferi (chevon 1)	1	NT	NT	1	1	1	1	0
	S. sciuri (Axone 12, skin 3)	15	7	4	3	9	6	0	0
	S. warneri (milk 1)	1	NT	NT	1	1	1	NT	NT
	S. xylosus (Axone 2, chevon 1)	3	0	2	0	3	1	0	0
	S. agalactiae (milk 3, ref 1, CJ1)	4	1	1	1	2	1	1	1
	S. bovis (milk 3)	3	NT	NT	3	NT	3	NT	0
	S. dysgalactiae (ref 2, CB, CJ3)	2	0	0	NT	1	0	0	0
	S. equi (milk 1, ref 1 MTCC3522)	2	1	1	1	2	2	NT	1
	S. gallinarum (skin 1)	1	NT	NT	1	1	1	1	NT
	S. intestinalis (milk 5)	5	2	2	2	3	0	NT	1
	S. pyogenes (milk 1, skin 1, ref 1, CJ4)	3	1	1	2	0	0	0	1
Yersinia	*Y. enterocolitica* (ref 1 MTCC3100)	1	0	0	0	0	0	NT	0
Number of total strains tested			162	282	460	404	460	331	206
Number of strains sensitive			71	114	211	204	175	65	36
Percent (%) of strains sensitive			43.8	40.4	45.9	50.5	38.0	19.6	17.5

N, number of strains tested; NT, not tested; EOME, methanolic extract of *Eupatorium odoratum*; ACME, methanolic extract of *Ageratum conyzoides*; LGO, lemon grass (*Cymbopogon citrartus*) oil; SWO, sandalwood (*Santalum album*) oil; PO, patchouli (*Pogostemon cablin*) essential oil; ZRME, methanolic extract of seed carps of *Zanthoxylus rhetsa,* AO, agarwood (*Aquilaria crassna*) oil; NSC, National *Salmonella* centre (Vet) at Indian Veterinary Research Institute, Izatnagar; MTCC, microbial type culture collection, Chandigarh; ATCC, American type culture collection; ref, reference strains procured from VTCC (Veterinary type culture collection, Indian Veterinary Research Institute) or MTCC and other reference labs, the culture number of reference labs are given for their identity in the repository.

antimicrobial potential testing of the herbal preparations was not only restricted to reference bacterial strains but strains isolated from food handlers and foods of animal and vegetable origin were tested.

Materials and Methods

Bacterial strains

A total of 464 strains belonging to more than 104 species of 33 genera (Table 1) available in the laboratory repository in glycerol stocks were revived as per standard protocol and tested for their identity and purity using morphological, growth and biochemical characteristics [6] and stored on brain heart infusion agar slants for the period of the study [6]. Of the 464 strains tested 254 were from fermented soybean product (Axone) consumed in North East India, 12 were isolated from buffen (buffalo meat), 32 from chevon (goat meat), 49 from buffalo milk, 33 from fish, 8 from muffen (mithun, *Bos frontalis*, meat), 14 from hand swabs of milk and meat vendors, and 62 reference strains of either American type culture collection (ATCC), Microbial type culture collection centre (MTCC), National *Salmonella* centre (NSC), *Brucella* Referral centre, or from Veterinary type culture collection (VTCC) of the Institute (Table 1).

Herbal antimicrobials

Methanolic extract of *Eupatorium odoratum* fresh leaves (EOME) collected during February and March, methanolic extract of *Ageratum conyzoides* fresh leaves (ACME) collected during October and November, methanolic extract of *Zanthoxylum rhetsa* seed coat (ZRME) purchased from Medziphhema market (Nagaland) in December were prepared in the laboratory as described earlier [7]. Briefly, the

herbs were cleaned to remove any dirt and dried in shade for 5-7 days and pounding to powder. A 250 g of powder was mixed with 500 ml of methanol (99.9%, Merck India Ltd.) in a 2 L conical flask and allowed to stand overnight on a rotary shaker (50 rpm) at 25 °C. Next morning, the flask contents were filtered through glass wool and filtrate was allowed to dry in crystallization bowls at 45 °C for 4-6 h or till the entire methanol got evaporated. The dry contents were weighed and again dissolved in methanol to contain 50 mg in one mL of solution (EOME). 20 µL of solution containing 1 mg of dry weight of methanolic extract was poured on to individual 6 mm sterile discs and discs were dried in air and stored at 4 °C [8].

Lemon grass (*Cymbopogon citratus*) oil (LGO), sandalwood (*Santalum album*) oil (SWO), patchouli (*Pogostemon cablin*) oil (PO) and agarwood (*Aquilaria crassna*) oil (AO) were purchased from Nagaland Fragrance Ltd., Dimapur and discs were prepared as described earlier to contain 1 mg of oil per disc [8]. All discs were stored at 4-8 °C throughout the period of study.

The amount of herbal antimicrobial in each disc (1 mg) was decided on the basis of previous reports indicating that most of the antimicrobial phytochemicals have MIC in range of 0.05-0.1% i.e., 0.5 to 1 mg mL^{-1} [2, 3].

Antimicrobial sensitivity assay

Antimicrobial sensitivity of all bacterial strains for different herbal drugs and ampicillin was determined as described earlier using disc diffusion and agar well diffusion (for minimum inhibitory concentration, MIC) methods [8]. Before testing, strains were grown overnight in Mueller Hinton broth, MHB (BBL, Diffco), for non-fastidious organism and MHB with 5% lysed sheep blood (LSB) for fastidious (*Bordetella, Brucella, Campylobacter, Listeria, Pasteurella, Streptococcus*) bacteria. The growth was adjusted with sterile phosphate buffer saline (PBS) to optical density (590 nm) of 0.1 before inoculating Mueller Hinton agar (MHA) or MHA-LSB plates through swabbing. Appropriate antibiotic discs were applied at 15-20 mm distance and ampicillin (10 mg) discs (BBL, Diffco) were used as positive antibiotic control for disc diffusion method. For determining MIC, 140 mm plates containing 16 wells already sealed with 1% sterile agarose were swab inoculated. The central well received 50 µL of sterile dimethyl sulphoxide (DMSO) and 1 to 15 number wells had suitably diluted herbal preparation in 50 µL DMSO to dispense 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192 and 16384 µg of the test extract or oil. For MIC plates were not inverted for first 4 hours of incubation. Plates were incubated aerobically for all but *Brucella* and *Campylobacter* strains which were incubated under 5% CO_2 pressure at 37 °C for 24 h. After incubation diameter of zone of inhibition around discs was measured in mm. Any zone of growth inhibition around herbal discs was indicative of sensitivity of bacteria to the material in the disc while the well containing the least amount of extract or oil with visible zone of growth inhibition was taken as MIC of the drug. All the experiments were repeated to conformity. Interpretations for ampicillin sensitivity were made in accordance of CLSI guidelines [9].

Table 2: Source of bacteria and their sensitivity to different herbal antimicrobials tested using disc (containing 1 mg of active ingredient/disc) diffusion assay on Mueller Hinton agar (MHA) for non-fastidious bacteria, MHA with lysed sheep blood (5%) for fastidious bacteria (*Bordetella, Brucella, Campylobacter, Listeria, Pasteurella, Streptococcus*).

Source (no. of strains)		EOME	ACME	LGO	SWO	PO	ZRME	AO
Axone, fermented soy-beans (254)	Tested	107	226	254	225	254	248	50
	Sensitive	60	101	132	125	123	53	11
Buffen, buffalo meat (12)	Tested	NT	NT	12	12	12	NT	12
	Sensitive	NT	NT	7	12	6	NT	4
Chevon, goat meat (32)	Tested	NT	NT	32	32	32	32	32
	Sensitive	NT	NT	15	29	22	6	5
Buffalo milk (49)	Tested	15	14	49	40	45	5	44
	Sensitive	6	6	16	13	9	1	6
Hands of food handlers (14)	Tested	NT	NT	14	14	14	14	NT
	Sensitive	NT	NT	6	11	2	3	NT
Muffen, Mithun meat (8)	Tested	8	8	8	8	8	8	8
	Sensitive	0	0	0	0	0	0	4
Fish, swabs from gills (33)	Tested	NT	NT	33	11	33	NT	NT
	Sensitive	NT	NT	21	0	0	NT	NT
Reference strains (62)	Tested	32	32	58	62	62	24	60
	Sensitive	5	7	14	10	10	2	6

NT, not tested; EOME, methanolic extract of *Eupatorium odoratum*; ACME, methanolic extract of *Ageratum conyzoides*; LGO, lemon grass (*Cymbopogon citrartus*) oil; SWO, sandalwood (*Santalum album*) oil; PO, patchouli (*Pogostemon cablin*) essential oil; ZRME, methanolic extract of seed carps of *Zanthoxylus rhetsa,* AO, agarwood (*Aquilaria crassna*) oil.

Statistical analysis

Correlation coefficient was calculated (MS Office Excel-7) to find out correlation between sensitivity (zone of inhibition in mm) of test strains to different herbal antimicrobials and ampicillin discs. To estimate association between proportions of sensitive and resistant strains of microbes of different species, different genera and of different sources to herbal antimicrobials χ^2 test was performed (MS Office Excel-2007). The statistical comparison was done for only those genera or sources where number (n) of strains tested was ≥ 10.

Results

On testing of 388 bacteria isolated from foods, 14 from food handlers and 62 reference strains belonging to more than 104 species of 33 genera (Tables 1 and 2) of Gram negative (194) bacteria (GNB) and Gram positive (270) bacteria (GPB) for their sensitivity to methanolic extract of *Eupatorium odoratum* (EOME), methanolic extract of *Ageratum conyzoides* (ACME), methanolic extract of *Zanthoxylum rhetsa* seed coat (ZRME), lemon grass (*Cymbopogon citratus*) oil (LGO), sandalwood (*Santalum album*) oil (SWO), patchouli (*Pogostemon cablin*) oil (PO) and agarwood (*Aquilaria crassna*) oil (AO) wide variation in antimicrobial activity of different herbs was evident. On the basis of oxidase reaction, test strains could be grouped in to four classes (Table 3) including oxidase +ve GNB (59), oxidase -ve GNB (135), oxidase +ve GPB (96), and oxidase -ve GPB (174).

The study revealed (Table 1) that sandalwood oil inhibited maximum number of strains (50.5%) followed by LGO (45.9%), EOME (43.8%), ACME (40.4%), PO (38%), ZEME (19.6%) and AO (17.5%). However, 54.6% bacteria were detected sensitive to ampicillin discs. In general, more of the ampicillin sensitive strains were also more often sensitive to EOME (p, 0.05), ACME (p, 0.04), AO and SWO (p, 0.03) than ampicillin resistant strains. However, difference was insignificant among the two groups for their sensitivity to LGO (p, 0.1), PO (p, 0.16) and ZRME (p, 0.64).

Although none of the herbal preparation tested had antimicrobial activity parallel to ampicillin, zone of inhibition induced by herbal antimicrobial discs was positively correlated with zone of inhibition induced by ampicillin discs. The correlation in growth inhibition with ampicillin could be defined as strong, positive and highly significant (p, 0.01) for SWO, EOME and AME, moderately significant (p, 0.05) for AO and LGO, and insignificant for PO and ZEME. Sandalwood oil (SWO) being the most effective antimicrobial photochemical (Table 1) inhibited growth of the maximum number of strains (50.5%) followed by LGO (45.9%), EOME (43.8%), ACME (40.4%), PO (38%), ZEME (19.6%) and AO (17.5%).

Bigger proportion of bacteria of foods of vegetable food (Axone) origin (Table 2) was sensitive to EOME (p, 0.009) and PO (p, 0.0002) than bacteria from foods of animal origin or from food handlers. Bacteria on hands of food handlers were more often sensitive to LGO than those from muffen (p, 0.03); to SWO than those from reference, milk, fish and muffen (p, 0.01); but less sensitive to PO than the strains from buffen (p, 0.05), chevon (p, 0.01) and Axone (p, 0.01). Though agar wood oil was the least effective antimicrobial in the study, its activity was significantly more evident on isolates of muffen origin than strains of buffen ((p, 0.03) and chevon (p, 0.04) origin. However, more of the *Staphylococcus* strains of buffen (p, ≤ 0.02) and chevon (p, ≤ 0.03) origin was sensitive to SWO

Table 3: Gram staining and oxidase reaction in relation to sensitivity of bacteria of food origin to different herbal antimicrobials tested using disc (containing 1 mg of active ingredient/disc) diffusion assay on Mueller Hinton agar (MHA) for non-fastidious bacteria, MHA with lysed sheep blood (5%) for fastidious bacteria (*Bordetella, Brucella, Campylobacter, Listeria, Pasteurella, Streptococcus*).

Oxidase production and Gram reaction		EOME	ACME	LGO	SWO	PO	ZRME	AO
Oxidase +ve G +ve (96)	Tested	39	87	96	90	96	89	22
	Sensitive	33	59	83	80	75	37	10
	% sensitive	84.6	67.8	86.5	88.9	78.1	41.6	45.5
Oxidase +ve G -ve (59)	Tested	22	38	59	34	59	27	20
	Sensitive	4	26	35	14	24	9	3
	% sensitive	18.2	68.4	59.3	41.2	40.7	33.3	15
Oxidase -ve G +ve (174)	Tested	72	83	170	171	172	139	102
	Sensitive	33	22	65	106	66	13	18
	% sensitive	45.8	26.5	38.2	62	38.4	9.4	17.6
Oxidase -ve G -ve (135)	Tested	29	74	135	109	133	76	62
	Sensitive	1	7	28	4	10	6	5
	% sensitive	3.4	9.5	20.7	3.7	7.5	7.9	8.1

EOME, methanolic extract of *Eupatorium odoratum*; ACME, methanolic extract of *Ageratum conyzoides*; LGO, lemon grass (*Cymbopogon citrartus*) oil; SWO, sandalwood (*Santalum album*) oil; PO, patchouli (*Pogostemon cablin*) essential oil; ZRME, methanolic extract of seed carps of *Zanthoxylus rhetsa,* AO, agarwood (*Aquilaria crassna*) oil; G +ve, Gram positive; G −ve, Gram negative.

than strains isolated from Axone and milk. Chevon origin *Staphylococcus* strains were also more often sensitive to PO than those isolates from milk (p, 0.05) and from food handlers (p, 0.01). For sensitivity of bacteria to other herbal antimicrobials, source of microbe was a significant determinant (Table 2).

Instead of source, genus of bacteria was more associated with sensitivity of bacteria to herbal antimicrobials (Tables 1 and 2). More of the *Bacillus* spp. strains were sensitive to all of the herbal preparations than strains of other bacteria (p, < 0.01). *Aeromonas* spp. strains were more often sensitive to LGO (*p*, < 0.001) while *Pseudomonas* spp. strains to ACME (*p*, < 0.01) than strains of other bacteria. Enterobacteriaceae strains in general showed more resistance to herbal antimicrobials than strains of any other families including Bacillaceae, Micrococaceae, Vibrionaceae and Pseudomonadaceae. More of the *Pseudomonas* spp. strains were sensitive to all (except EOME) herbal preparations tested than *E. coli, Klebsiella, Proteus* and *Salmonella* genus strains (p, ≤ 0.05).

In general, more of the oxidase +ve strains were sensitive to herbal preparations tested than oxidase -ve strains (p, < 0.01). Similarly, more of the G +ve strains were sensitive to herbal preparations than G -ve strains (p, < 0.02), except insignificant difference in sensitivity of the two type of the strains to ZRME (p, 0.12). Among all four groups of bacteria on the basis of Gram's staining and oxidase production, O +ve G +ve bacteria were the most sensitive group (p, < 0.05) for all the preparations tested in the study barring a few exceptions (Table 3). Exception included almost equal sensitivity of O +ve G +ve and O +ve G -ve strains to ZRME and ACME (p, > 0.44). Strains showing O +ve G -ve reaction were often more sensitive than O -ve G -ve strains (p, < 0.01) for all the preparations but almost equally sensitive to EOME and AO. Similarly, more of O -ve G +ve strains were sensitive to herbal antimicrobial preparations tested than O -ve G -ve strains (p <, 0.01) but equally sensitive to AO and ZRME.

The MIC for different herbal preparations varied for different strains (Table 4). For PO and SWO, it ranged from 1 µg mL^{-1} to > 16.384 mg mL^{-1}, for LGO it ranged from 4 µg mL^{-1} to > 16.384 mg mL^{-1}, for AO and ZRME it ranged from 8 µg mL^{-1} to > 16.384 mg mL^{-1}, while for EOME and ACME it ranged from 16 µg mL^{-1} to > 16.384 mg mL^{-1}. In terms of MIC, *Salmonella* strains were the most resistant strains (MIC never less than 640 µg mL^{-1}) closely followed by *Citrobacter, E. coli*, and *Klebsiella* strains (MIC was always ≥ 340 µg mL^{-1}). *Bacillus* and *Aeromonas* spp. strains were the most sensitive ones, never had MIC ≤ 4.096 mg mL^{-1} for any of the herbal antimicrobial and *Streptococcus* was next sensitive genus (MIC ≤ 8.192 mg mL^{-1}).

Table 4: Minimum inhibitory concentration (MIC) of the herbal antimicrobials against bacteria isolated from foods and food handlers.

Name of herbal antimicrobial Drug	Range of MIC in µg mL^{-1} for different bacterial strains tested	Five bacterial strains with the least MIC (MIC in µg mL^{-1})
Sandalwood oil	1 to more than 16384	*Streptococcus pyogenese* (1) *Pasteurella canis* (1) *Pasteurella multocida* (2) *Brucella abortus* (8) *Salmonella enterica* ser Gallinarum (640)
Patchouli oil	1 to more than 16384	*Streptococcus pyogenese* (1) *Bacillus polymyxa* (1) *Brucella abortus* (2) *Bordetella bronchiseptica* (2) *Staphylococcus aureus* (4)
Agarwood oil	8 to more than 16384	*Acinetobacter calcoaceticus* (8) *Alcaligenes faecalis* (8) *Bacillus subtilis* (64) *Brucella abortus* (128) *Streptococcus pyogenese* (128)
Zanthoxylum rhetsa oil	8 to more than 16384	*Bacillus pantothenticus* (8) *Streptococcus aglactiae* (8) *Streptococcus equi* ssp. *equisimilis* (8) *Proteus mirabilis* (64) *Acinetibacter calcoaceticus* (128)
Lemon grass oil	4 to more than 16384	*Brucella abortus* (4) *Bacillus pantothenticus* (8) *Bacillus mycoides* (8) *Aeromonas hydrophila* (8) *Acinetibacter calcoaceticus* (8)
Eupatorium odoratum methanolic extract	16 to more than 16384	*Bacillus subtilis* (16) *Burkholderia cepacia* (64) *Streptococcus equi* ssp. *equisimilis* (64) *Bacillus mycoides* (128) *Bacillus pantothenticus* (256)
Ageratum conyzoides methanolic extract	16 to more than 16384	*Streptococcus pyogenes* (16) *Bacillus subtilis* (32) *Burkholderia cepacia* (64) *Streptococcus equi* ssp. *equisimilis* (64) *Staphylococcus aureus* (64)

All resistant strains detected sensitive with disc diffusion assay had MIC < 1024 µg mL^{-1} while for resistant strains it varied from 1.024 mg mL^{-1} to 16.384 mg/ mL^{-1}. *Salmonella* strains were the most resistant strains (in terms of MIC) in the study for most the preparations tested closely followed by *Citrobacter, E. coli*, and *Klebsiella* strains. *Bacillus* strains were the most sensitive ones, never had MIC > 4.096 mg mL^{-1} and *Streptococcus* was next sensitive genus.

Discussion

Foods irrespective of their origin may contain several spoilage and many potentially pathogenic bacteria which limits consumer acceptability and safety for the foods. Moreover, additives in processed foods further add to the risk. Although many of the spices added in foods have antimicrobial activity [4, 5] they may also add to microbial load of the food being loaded with bacteria [10-12]. In scenario of emergence of multiple drug resistant strains in different spheres including biotic and abiotic environment food are also not aloof of those.

Studies have shown that being time tested herbal antimicrobials may be effective against bacteria including MDR strains [13] and thus are of potentially utility. Several studies have been conducted on efficacy of different herbs and herbal products on foodborne bacteria [1, 2] but being limited with the number of strains restrict the utility. Moreover, regional variation in microbial quality of foods, the microbiota composition and different herbs and spices used and their antimicrobial quality [14] necessitate elaborate regional studies on efficacy of herbs and herbal antimicrobials on the bacteria occurring in foods.

Sandalwood oil (SWO) was the most effective antimicrobial inhibiting the maximum number of strains (50.5%) followed by LGO (45.9%), EOME (43.8%), ACME (40.4%), PO (38%), ZEME (19.6%) and AO (17.5%). However, none of the herbal preparation tested had antimicrobial activity parallel to ampicillin and observations are in concurrence to earlier observations on efficacy of herbal antimicrobials on microbial strains of different origin in India [7].

Zone of inhibition induced by SWO, EOME and AME, AO and LGO discs could positively correlate with zone of inhibition induced by ampicillin discs. The correlation indicated that similar mechanism of sensitivity/resistance might be operational in microbes for herbs and antibiotics as proposed earlier [3, 7]. However, more controlled studies are required to confirm the hypothesis.

Observations of more sensitivity of bacteria from Axone to EOME (p, 0.009) and PO (p, 0.0002) than bacteria isolated from foods of animal origin or from food handlers (p, 0.013) are important. However, earlier studies have not made similar comparison; higher sensitivity of Axone origin strains than strains of animal origin has been reported [15-18]. Therefore, it might be attributed to carryover effect on resistant strains of bacteria from animals to their products and then to the food handlers or transfer of drug resistant bacteria from hands of food handlers to animal products [19, 20].

Antimicrobial activity of agar oil, the least effective antimicrobial in the study, varied according to source of the bacteria tested. Significantly more of the bacteria of muffen origin than those of buffen (p, 0.03) and chevon (p, 0.04) origin were sensitive to agar oil. The observation cannot be explained on the basis of limited number of observations in the study. However, sensitivity of many of the *Bacillus, Staphylococcus* and *Streptococcus* but not of *E. coli* strains to AO observed in the study is in concurrence to earlier studies revealing the same fact [21].

The study indicated that instead of source of bacteria their genus was more important determinant of their sensitivity to many of the herbal preparations. More of the *Bacillus* spp. strains were sensitive to herbal preparations ($p, < 0.01$) than strains of other bacteria and observations are in concurrence to earlier studies [15-18, 21, 22]. More sensitivity of *Aeromonas* strains to LGO is also similar as reported earlier [16]. Many (95.6%) more of the *Pseudomonas* spp. strains were sensitive ACME than strains of other bacteria. Though interesting, it is in contrast to earlier observations reporting *Pseudomonas* as the most resistant bacteria tested against *Ageratum* oil [23]. It might be due to several reasons viz., regional effect, bacteria from different regions may differ in their sensitivity pattern [9, 24], difference in the source of herb etc. More common occurrence of resistance in Enterobacteriaceae strains to herbal antimicrobials preparations tested is in concurrence to earlier observations on herbal antimicrobials [15-18, 21, 22, 25]. Sensitivity of oxidase +ve strains to herbal antimicrobials tested in the study was an important observations as many of the deadly pathogens of animal are oxidase positive including *Aeromonas, Bacillus, Brucella, Pasteurella, Pseudomonas, Burkholderia* and *Campylobacter* spp. strains [26, 27]. However, the observation may be skewed due to proportionately high level of sensitivity of *Brucella* (4), *Bacillus* (94) and *Pseudomonas* (30) strains constituting > 82% of the total oxidase positive strains in the study. Similar to oxidase -ve G -ve strains, some of the oxidase positive groups including *Aeromonas* and *Campylobacter* spp. strains were rarely sensitive to herbal antimicrobial in the study. Though cannot be deduced from the study it might be associated with microhabitat of the potential pathogens, the pathogens affecting system other than gastrointestinal tract might be more prone for killing by the herbal preparations.

The MIC for different herbal preparations varied for different strains (Table 4) from 1 µg mL^{-1} to > 16.384 mg mL^{-1}. Variation in MIC observed under the study appeared to be similar phenomenon as observed for antibiotics [8, 9]. In the study MIC of herbal antimicrobials was the maximum for *Salmonella*, *Citrobacter, E. coli*, and *Klebsiella* strains, all the members of Enterobacteriaceae. High level of antimicrobial drug resistance in Enterobacteriaceae member has been frequently reported all over the world [9].

Observations revealed that for herbal antimicrobials (methanolic extract of *Eupatorium odoratum Ageratum conyzoides*, *Zanthoxylum rhetsa*, and essential oils of *Cymbopogon citrates, Pogostemon cablin, Santalum album* wood and *Aquilaria crassna* wood) in general, *Pseudomonas* and *Bacillus* strains were more often sensitive little or not affected by the source of isolation. However, it was evident that more of the strains of Axone (fermented soybean) origin were sensitive to all the herbs tested than the strains isolated from foods of animal origin. To understand the role of oxidase production in sensitivity of bacteria to herbal antimicrobials more organized studies are required considering the zoonotic potential of oxidase positive bacteria including large and comparable number of strains of *Aeromonas, Bacillus, Brucella, Pasteurella, Pseudomonas, Burkholderia* and *Campylobacter* species. The study further reveals that herbal antimicrobials, being edible and acceptable since ages, can be an option for use in foods to control spoilage as most of the *Pseudomonas* and *Bacillus* strains (common bacteria associated with food spoilage) were inhibited by herbal antimicrobials. However, the choice of herbal antimicrobial may depend on type of microbe causing the problem in the specific food unit or the region.

Acknowledgements

Authors are thankful to the Director and Joint Director (Research) for permitting to undertake the study. Authors are thankful to in-charges of *Brucella, Campylobacter, Salmonella*

and other specific pathogen laboratories of the Institute for providing the reference cultures. We also thanks to Dr. RK Singh, In-charge KVK, Phek, Nagaland, for helping in collection of fresh leaves of *Eupatorium odoratum*, *Ageratum conyzoides*, and seeds of *Zanthoxylum rhetsa*. Authors also thank supporting staff of Epidemiology laboratory (Mr. HC Joshi and Laikur Rahman Khan) for their untiring support throughout the study.

Conflict of Interest

There is no conflict of interest pertaining to this manuscript.

Authors Contribution

All the three authors planned and carried out the research work, data analysis and manuscript draft was done by the first author (BRS), 2nd (DKS) and 3rd author (VKOR) rechecked the analysed data and manuscript for correctness.

References

1. Tiwari BK, Valdramidis VP, Bourke P, Cullen P. 2011. Application of plant based antimicrobials in food preservation. In: Rai M, Chikindas M (eds) Natural Antimicrobials in Food Safety and Quality. CAB International, Wallingford, UK, pp 204-223.
2. Tajkarimi M, Ibrahim SA. 2012. Phytochemicals as antimicrobial food preservatives. In: Patra AK (eds), Dietary Phytochemicals and Microbes. Springer, Netherlands, pp 207-235.
3. Vadhana P, Singh BR, Bhardwaj M, Singh SV. 2015. Emergence of herbal antimicrobial drug resistance in clinical bacterial isolates. *Pharm Anal Acta* 6: 434. doi: 10.4172/21532435.1000434.
4. Chopra RN, Nayar SL, Chopra CI. 1956. Glossary of Indian medicinal plants. CSIR, New Delhi, India.
5. Agarwal SS, Tamrakar BP, Paridhavi M. 2005. Clinically useful herbal drugs. Ahuja Book Company Pvt. Ltd., New Delhi, India.
6. Singh BR. 2009. Labtop for microbiology laboratory. Lambert Academic Publishing, AG & Co. KG, Berlin, Germany.
7. Singh BR. 2014. Multiple-herbal-antimicrobial-resistance (MHAR) in microbes of animals, birds, fish, food, lizard and water origin. In: Proceedings of International Conference and 28th Annual Convention of IAVMI-2014 on "Challenges and Opportunities in Animal Health at the Face of Globalization and Climate Change". Department of Veterinary Microbiology and Immunology, DUVASU, Mathura, India, pp 26-29.
8. Singh BR. 2013. Antimicrobial sensitivity assay and antimicrobial chemotherapy in Animals: a practical approach. In: Singh BR, Somvanshi R (eds) Diseases of Animals: Diagnosis and Management, Indian Veterinary Research Institute, Izatnagar, India, pp 7-31.
9. Clinical and Laboratory Standards Institute (CLSI). 2015. Performance standards for antimicrobial disk susceptibility tests, 12th edition (M02-A12). CLSI, Wayne, Pennsylvania, USA.
10. Drew AK, Myers SP. 1997. Safety issues in herbal medicine: implications for the health professions. *Med J Aust* 166(10): 538-541.
11. Ogunshe AAO, Kolajo TT. 2006. *In vitro* phenotypic antibiotic resistance in bacterial flora of some indigenous orally consumed herbal medications in Nigeria. *JRTPH* 5: 9-15.
12. Brown JC, Jiang X. 2008. Prevalence of antibiotic-resistant bacteria in herbal products. *J Food Prot* 71(7): 1486-1490.
13. Nostro A. 2006. Activity of plant extracts and plant-derived compounds against drug-resistant microorganisms. In: Ahmad I, Aqil F, Owais M (eds) Modern Phyto Medicine, Wiley-VCH, Weinheim, Germany, pp 199-231. doi: 10.1002/9783527609987
14. Cimanga K, Kambu K, Tona L, Apers S, De Bruyne T, et al. 2002. Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *J Ethnopharmacol* 79(2): 213-220. doi: 10.1016/S0378-8741(01)00384-1
15. Singh BR, Singh V, Singh RK, Toppo S, Haque N, et al. 2011. Antimicrobial effect of *Artemisia vulgaris* essential oil. *Natural Products: An Indian J* 7: 5-12. doi: 10.5958/j.0975-4261.4.2.003
16. Singh BR, Singh V, Singh RK, Ebibeni N. 2011. Antimicrobial activity of lemongrass (*Cymbopogon citratus*) oil against microbes of environmental, clinical and food origin. *Int Res J Pharm Pharmacol* 1(9): 228-236.
17. Singh BR, Singh V, Singh RK, Toppo S, Haque N, et al. 2012. Antimicrobial activity on common pathogens in essential oil of aerial parts of *Selinum wallichianum*. *Natural Products: An Indian J* 8: 233-237.
18. Singh BR, Singh V, Singh RK, Toppo S, Haque N, et al. 2012. Comparative evaluation of antimicrobial effect of *Artemisia vulgaris* essential oils extracted from fresh and dried herb. *Med Plant* 4: 76-82. doi: 10.5958/j.0975-4261.4.2.003
19. Humphrey T, Jorgensen F. 2006. Pathogens on meat and infection in animals – establishing a relationship using *Campylobacter* and *Salmonella* as examples. *Meat Sci* 74(1): 89-97. doi: 10.1016/j.meatsci.2006.04.026
20. Bhardwaj M, Singh BR, Murugan MS, Prasannavadhana, Dubey S. 2015. Emergence of carbapenemase producing pathogens in animals. *Pharm Anal Acta* 6(6): 379. doi: 10.4172/2153-2435.1000379.
21. Chen H, Yang Y, Xue J, Wei J, Zhang Z, et al. 2011. Comparison of compositions and antimicrobial activities of essential oils from chemically stimulated agarwood, wild agarwood and healthy *Aquilaria sinensis* (Lour.) Gilg trees. *Molecules* 16(6): 4884-4896. doi: 10.3390/molecules16064884
22. Singh BR. 2013. Evaluation of antibacterial activity of sage (*Salvia officinalis*) oil on veterinary clinical isolates of bacteria. Noto-are Med. 15341289.
23. Dayie NT, Newman MJ, Ayitey-Smith E, Tayman F. 2008. Screening for antimicrobial activity of *Ageratum conyzoides*: a pharmaco-microbiological approach. *Internet Journal of Pharmacology* 5(2): 1-6.
24. Singh BR. 2011. Drug resistant bacteria in the environment: In: Garg SR (ed) Environmental Security: Human and Animal Health, 1st edition. IBDC Publishers, Lucknow, India, pp 313-326.
25. Garg P, Grewal A. 2015. *In vitro* antibacterial activity of *Ageratum conyzoides* L (Asteraceae). *World J Pharmacy Pharmaceut Sci* 4(7): 893-897.
26. Carter GR. 1975. Diagnostic procedures in veterinary microbiology, 2nd edition. Charles C Thomas Publishers, Springfield, USA.
27. McVey DS, Kennedy M, Chengappa MM. 2013. Veterinary microbiology, 3rd edition. Wiley-Blackwell, Oxford, UK.