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Combinative effect of *Salvia sclarea* L., *Artemisia annua* and *Dracocephalum heterophyllum* B. essential oils against *Salmonella enterica* in raw chicken

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Antibacterial properties of essential oils (EOs) extracted from *Salvia sclarea, Artemisia annua* and *Dracocephalum heterophyllum* against 17 food borne pathogens was studied. EOs of the three plants showed a broad spectrum of antimicrobial activity with different degrees of inhibition against the tested Gram-positive and Gram-negative bacteria. EOs of *Salvia* and *Dracocephalum* depicted bactericidal mode of action while that of *Artemisia* inhibited the bacteria with bacteriostatic mode. *Salmonella enterica* MTCC 733 was the most sensitive strain to *Salvia, Artemisia* and *Dracocephalum* EOs with minimum inhibitory concentration (MIC) values of 2000, 2000 and 8000 µg/ml, respectively. The antimicrobial activity of EOs individually and in combinations based on their respective MIC values against *S. enterica* was tested in raw chicken. Treatment of food sample with 20 times MIC value of *S. sclarea, A. annua* and *D. heterophyllum* EOs individually caused reduction of bacterial load to 3.36, 3.64 and 4.22 log cfu/g after 180 min. In contrast the bacterial cell loads reduced to an undetectable level by the combinative effect (*Salvia + Dracocephalum* and *Salvia + Artemisia*) of EOs at MIC value after 120 and 180 min, respectively. This study suggests that combinations of EOs could minimize application concentrations in real food system.

Key words: Antibacterial activities, essential oils, food-borne pathogens, fractional inhibitory concentration, minimum inhibitory concentration, *Salmonella enterica*.

INTRODUCTION

Foodborne diseases resulting from consumption of contaminated food with pathogenic bacteria and/or their toxins is a priority concern to public health. According to World Health Organisation (WHO) study of the state of foodborne diseases in OECD countries (Rocourt et al., 2003), salmonellosis is the most occurring bacterial foodborne disease, with more than 325,000 cases per year. In this context, the identification and evaluation of antimicrobial agents for the control of these pathogens, to assure consumers a safe, wholesome and nutritious food supply is a matter of global concern.

Chemical and synthetic compounds have been used as antimicrobials to inhibit bacteria in foods. Due to the economical impacts of spoiled foods and the consumer's concerns over the safety of foods containing synthetic chemicals, a lot of attention has been paid to naturally-derived compounds or natural products. In this context, most of the plant essential oils (EOs) are attracting interest for their potential as natural food preservatives as they have Generally Recognized As Safe (GRAS) status. The versatile composition of plant EOs and the large antimicrobial spectrum, associated with their low toxicity, make them potential natural agents for food preservation (Conner, 1993). Many *in vitro* studies report a high

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efficacy of EOs against food-borne pathogen (Smith-Palmer et al., 1998; Dorman and Deans, 2000). However, a higher concentration of EO is needed to achieve the same effect in food as compared to *in vitro* (Burt, 2004). If EOs are expected to be widely applied as antibacterials, minimum amount of EO has to be used, which does not alter the taste of food or exceed acceptable flavor thresholds (Hsieh et al., 2001; Nazer et al., 2005). Even though several studies have been conducted regarding the *in vitro* antibacterial and antifungal properties of plant EOs and extracts (Militello et al, 2011; Burt, 2004; Vagi et al., 2005), only a few studies on the activity of EOs in food systems have been reported in the literature (Holley and Patel, 2005). It is well known that the antimicrobial potency of EOs in food systems is greatly reduced when compared to *in vitro* work, as the presence of fats, carbohydrates, proteins and salts strongly influence the effectiveness of these agents (Burt, 2004). Accordingly, larger amounts of EOs are required in food systems, thus interfering with the final organoleptic properties (Lis-Balchin et al., 1998).

Combination of plant extracts and EOs may help to reduce sensory impact. Furthermore, these combinations may also control some bacteria that are known to show consistently high resistance to plant antimicrobials, such as *Pseudonomas* species (Hammer et al., 1999; Holley and Patel, 2005). There are evidences that EOs are more strongly antimicrobial than is accounted for by the additive effect of their major antimicrobial components, minor antimicrobial components appear, therefore, to play a significant role (Lattaoui et al., 1994; Paster et al., 1995). For this reason, the present study was carried out using EOs extracted from aerial parts of three high altitude medicinal plants, namely *Salvia sclarea* (SS), *Artemisia annua* (AA) and *Dracocephalum heterophyllum* (DH). To the best of our knowledge, the antibacterial activities of the three oils, against the foodborne pathogens along with the *in vivo* studies have not been reported earlier. Also, since *Salmonella enterica*, one of the most important pathogen for meat industry, was seldom included in previous antibacterial studies of EOs, this strain was used to evaluate antibacterial activity of all oils in raw chicken.

In this study, the synergistic and additive interactions between the oils in exhibiting antibacterial effect have been determined both *in vitro* and in the food system, that is, raw chicken. To the best of our knowledge, this is the first study presenting the successful use of the plant EOs in combination in inhibiting *S. enterica* in food. The purpose of this study was to create comparable, antibacterial data between *in vitro* studies and a real food system.

MATERIALS AND METHODS

Plant

The aerial parts of *Salvia, Artemisia* and *Dracocephalum* specimens were collected from trans-Himalayan Ladakh region, India in August, 2009 and identified by comparing morphological features with the herbarium specimens in DIHAR. A voucher specimen has been deposited in the herbarium of Department of Medicinal and Aromatic Plants, DIHAR, Leh, India.

Preparation of EOs

The dried aerial parts were ground prior to the operation and then 100 g of ground powder was subjected to hydrodistillation for 5 h using a Clevenger apparatus. The distilled EOs were dried over anhydrous sodium sulphate, filtered and stored at +4°C in a dark bottle until further analysis. The plant parts used and yield of each EO is presented in Table 1.

Antibacterial activity

Bacterial strains and culture conditions

Seventeen foodborne pathogens were selected, which included 6 Gram positive species and 11 Gram-negative species (Table 2). Strains were obtained from international repository at Microbial Type Culture Collection (MTCC), India.

Active cultures for experimental use were prepared by transferring a loopful of cells from stock cultures and inoculated in Luria-Bertani (LB) broth medium at 37°C for 24 h, except for *Pseudomonas fluorescens*, which was grown at 25°C for 24 h. Cultures of each bacterial strains were maintained on LB agar medium at 4°C.

Disc diffusion

The EOs were dissolved in 10% aqueous dimethylsulfoxide (DMSO) supplemented with 0.5% Tween 80 and filter sterilized using a 0.45 µm filter. The antibacterial test was carried out by disc diffusion method using 100 µl of standardized inoculum suspension (CLSI, 2000a). To prepare standardized inoculums, bacteria were grown overnight in LB broth at 37°C with constant agitation until the density matched the turbidity of a 0.5 McFarland standard. Sterile discs (6 mm in diameter) were placed onto the Mueller Hinton Agar (MHA) (HiMedia, India) medium and 5 µl of the EO solutions (5000 µg/disc) were spotted on them. Aqueous DMSO supplemented with 0.5% Tween 80 was used as the negative control. Standard reference antibiotics, gentamicin (10 µg) and rifampicin (30 µg), each from HiMedia, India were used as positive controls for the tested bacteria. Plates loaded with 10% DMSO supplemented with 0.5% Tween 80 were taken as negative controls. Before incubation, all petri dishes were kept at 4°C for 4 h. The plates were then incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition and the diameter of the sterile disc subtracted, giving the size of each inhibition zone beyond the disc. Each assay in this experiment was replicated three times.

MIC assay

The minimum inhibitory concentration (MIC) values were studied for the bacterial strains, being sensitive to the EOs in the disc diffusion assay. A broth microdilution susceptibility assay was performed using CLSI methods for the determination of MIC (CLSI, 2000b). All tests were performed in Mueller Hinton Broth (MHB) (HiMedia, India). The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The EOs were dissolved in 10% aqueous DMSO

Table 1. List of plants and their parts used for EO extraction, and yields of the extracted oils.

Common names	Scientific names	Parts used	*Oil yield (% v/w)
French clary sage	*Salvia sclarea*	Flowering top and leaves	3.1
Sweet wormwood	*Artemisia annua*	Leaves	0.29
Zinkzer	*Dracocephalum heterophyllum*	Flowering top and leaves	0.29

*[Amount of EO (ml)/Amount of dried plant material (g)] × 100.

Table 2. Antimicrobial activity of EOs against foodborne pathogens.

Test organism	Diameter of zone of inhibition (mm)				
	[1]SS	[2]AA	[3]DH	*Antibiotics	
				GEN (10 µg)	RFP (30 µg)
Gram-positive organisms					
B. cereus MTCC 430	15.0 ± 1.0^{de}	11.3 ± 2.1^{a}	27.0 ± 2.6^{g}	17.0^{bc} ± 3.0	20.0^{e} ± 0.0
E. faecalis MTCC 2729	9.7 ± 2.1^{abc}	10.3 ± 1.5^{a}	7.3 ± 1.5^{a}	20.0^{cde} ± 0.0	9.0^{a} ± 1.0
L. monocytogenes MTCC 839	8.0 ± 1.0^{a}	12.3 ± 1.5^{a}	10.0 ± 3.0^{ab}	22.0^{def} ± 0.0	12.0^{bc} ± 0.0
L. monocytogenes MTCC 657	15.3 ± 2.1^{de}	10.3 ± 2.1^{a}	21.0 ± 2.6^{f}	18.0^{bcd} ± 2.0	16.0^{d} ± 0.0
S. aureus MTCC 902	12.7 ± 3.1^{cd}	11.3 ± 1.5^{a}	14.7 ± 1.2^{de}	15.7^{ab} ± 4.0	24.3^{f} ± 1.5
S. aureus MTCC 1430	18.7 ± 3.5^{ef}	10.3 ± 2.5^{a}	15.3 ± 3.1^{de}	22.0^{ef} ± 2.5	19.3^{e} ± 2.5
Gram-negative organisms					
A. hydrophila MTCC 1739	12.3 ± 1.5^{bcd}	11.0 ± 2.6^{a}	9.0 ± 1.0^{a}	13.0^{a} ± 3.0	13.0^{c} ± 1.0
E. coli MTCC 1687	17.3 ± 2.1^{e}	10.0 ± 1.7^{a}	10.0 ± 2.0^{ab}	20.0^{cde} ± 0.0	10.0^{ab} ± 0.0
*E. coli M*TCC 443	15.0 ± 2.0^{de}	19.0 ± 2.6^{b}	14.3 ± 2.5^{cde}	23.0^{ef} ± 1.0	20.7^{e} ± 1.5
K. pneumoniae MTCC 432	10.3 ± 1.5^{abc}	13.3 ± 3.1^{a}	13.3 ± 2.5^{bcd}	25.3^{f} ± 1.5	17.0^{d} ± 1.0
P. vulgaris MTCC 426	10.3 ± 1.5^{abc}	10.7 ± 4.0^{a}	8.0 ± 1.0^{a}	17.3^{bc} ± 3.0	13.0^{c} ± 1.0
P. aeruginosa MTCC 424	8.7 ± 1.5^{ab}	10.3 ± 2.3^{a}	8.3 ± 0.6^{a}	20.0^{cde} ±2.0	9.0^{a} ± 0.0
P. fluorescens MTCC 103	11.7 ± 2.5^{abcd}	12.0 ± 2.0^{a}	10.7 ± 2.5^{abc}	23.7^{ef} ± 1.5	16.0^{d} ± 1.0
S. enterica MTCC 733	21.7 ± 2.1^{f}	20.3 ± 0.6^{b}	17.7 ± 2.1^{def}	23.7^{ef} ± 1.5	12.7^{c} ± 1.5
S. typhimurium MTCC 98	12.7 ± 2.5^{cd}	11.3 ± 3.2^{a}	15.3 ± 2.1^{de}	20.0^{cde} ± 0.0	12.0^{bc} ± 0.0
S. flexneri MTCC 1457	9.0 ±0.0^{abc}	10.0 ±1.0^{a}	9.7 ± 2.1^{ab}	19.3^{bcde} ± 3.0	12.0^{bc} ± 2.0
Y. enterocolitica MTCC 859	17.3 ± 2.1^{e}	11.3 ± 2.1^{a}	17.3 ± 2.5^{de}	23.0^{ef} ± 3.0	12.0^{bc} ± 2.0

[1]SS- *Salvia sclarea*; [2]AA- *Artemisia annua*; [3]DH- *Dracocephalum heterophyllum*; *Standard antibiotics: G, Gentamicin (10 µg/disc) and R, Rifampicin (30 µg/disc). Different superscripts in a column differ significantly (p<0.05) by Duncan's test.

supplemented with 0.5% Tween 80 and later diluted to the highest concentration (16000 µg/ml) to be tested, and then serially two-fold dilutions were made in a concentration range from 16000 to 31.25 µg/ml with MHB in 96-well microtitre plate, volume being 100 µl. The plates were dispensed with 95 µl of MHB and 5 µl of the inocula. The volume in each well was 200 µl. The plate was covered with a sterile plate sealer. Contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at 37°C for 24 h.

Same tests were performed simultaneously for growth control (MHB + inocula) and sterility control (MHB + test sample). The optical density (OD) of the sample for the respective oil solutions were calculated by subtracting the OD of the sterility control each time for a particular concentration to negate the effect of increasing concentration of oil. Microbial growth was determined by absorbance at 600 nm using the Thermo Scientific Multiskan® FC instrument (Vantaa, Finland). MIC values were determined as the lowest concentration of the EO where absence of growth was recorded.

Examination of mode of action

Each EO (at a final concentration equal to the MIC value) was added to 4.9 ml of all the cultures (10^4 CFU/ml) (Rattanachaikunsopon and Phumkhachorn, 2009). After incubation at 37°C for 24 h, 100 µl of the mixtures inoculated into 4.9 ml of fresh LB broth. As a control, 100 µl of untreated cultures at a concentration of 10^4 CFU/ml was transferred to 4.9 ml of fresh LB broth. The optical density at a wavelength of 600 nm of the tested and control cultures was determined at the time of inoculation and after incubation at 37°C for 24 h. Bacterial cells inhibited with the EOs were later transferred to fresh LB broth and the mode of action was classified as bactericidal if bacteria did not resume growth in fresh broth and bacteriostatic if the bacterial cells are able to re-grow.

Synergy studies

Combinations of EOs were qualitatively assessed using the spot-on-agar test. Fractional inhibitory concentration (FIC) indices were calculated using the checkerboard method to quantify the potential synergy of the selected oils in combination with one another.

Spot-on-agar test

The spot-on-agar test was performed based on previous work (Cintas et al., 1998) with minor modifications. 5 µl of EO combinations diluted in 10% aqueous DMSO supplemented with 0.5% Tween 80 was spotted onto MHA plates seeded with 10^7 CFU/ml of the tested pathogens. Combinations were initially assessed in a 1:1 ratio. Spotted plates were kept at 4°C for 4 h followed by incubation at 37°C for 24 h. 10% aqueous DMSO supplemented with 0.5% Tween 80 was used as control and inhibition zones were measured.

Checkerboard assay

The checkerboard method was performed using 96-well microtitre plates (Moody, 2003; Schelz et al., 2006) to obtain the FIC index. The microtitre plate assay was arranged as follows: EO_A was diluted two-fold along the x-axis, whilst EO_B was diluted two-fold along the y-axis. The final volume in each well was 100 µl comprising 50 µl of each dilution. Subsequently, 100 µl of media containing 10^7 CFU/ml of the indicator strain was added to all wells. The plates were then incubated at 37°C for 24 h. The FIC indices were calculated as $FIC_A + FIC_B$, where FIC_A and FIC_B are the minimum concentrations that inhibited the bacterial growth for EOs A and B, respectively.

Thus, FICs were calculated as follows: FIC_A = (MIC_A combination/MIC_A alone) and FIC_B = (MIC_B combination/MIC_B alone). The results were interpreted as synergistic when the FIC index was < 0.5; additive when between 0.5 and 1.0; indifferent when between 1.0 and 2.0; and as antagonistic when the index was ≥ 2.0 (EUCAST, 2000).

Examination of antibacterial activity in food

Raw chicken meat sample was obtained from the local market and trimmed into square pieces (5 × 5 cm) and exposed to the UV lamp in a laminar flow cabinet for 15 min on both sides to minimize interference of the natural bacterial flora. Pieces of meat were minced in a sterile blender, and portions of 100 ± 0.1 g were placed in polyethylene bags. The meat samples were inoculated with *S. enterica* MTCC 733 to a final concentration of 10^8 CFU/g of meat. Prior to inoculation, oils were added to the samples in combinations (*S. sclarea* and *D. heterophyllum,* SS:DH; *A. annua* and *S. sclarea,* SS:AA) according to their respective MIC values and individually according to their respective MIC (M) and 20 times MIC (20M) values. Food samples containing only oil and ones containing only *S. enterica* served as controls. The samples were homogenized at normal speed for 5 min and examined every 30 min for a period of 3 h after bacterial inoculation for the presence of *S. enterica*. Portions of 10 g of meat were taken after every 30 min and homogenized in a plastic bag with 10 ml of phosphate buffer (pH 7) in the Stomacher for 30 s. Liquid part of the homogenate was collected and serially diluted with the phosphate buffer. Appropriate dilutions of each sample were spread on Hicrome Salmonella Agar, a selective medium for *Salmonella* (HiMedia, India). The plates were then incubated at 37°C for 24 h. Standard biochemical and serological tests for *S. enterica* were performed to confirm the identity of the isolates.

Statistical analysis

Data generated was analyzed by SPSS 11.5 and MVSP 3.1 statistical computer package. The overall means were compared with the use of Generalized Linear Model multivariate analysis and Duncan's multiple range test. Statistical significance was declared at probability of $p<0.05$. Descriptive statistics of raw values of the $\log_2$ MICs were performed for three individual and three combinations of EOs across the 17 tested pathogens. Pearson product-moment correlation coefficients for the individual and combinations of EOs were done.

RESULTS

Antibacterial activity

The *in vitro* antibacterial activity of EOs, against the employed bacteria, was qualitatively assessed by the presence or absence of inhibition zones and quantitatively by the zone diameters. *S. sclarea, A. annua* and *D. heterophyllum* oils showed a broad spectrum of antibacterial activity against the tested MTCC strains with the zones of inhibition ranging from 8.0 to 21.7, 10.0 to 20.3 and 7.3 to 27.0 mm, respectively (Table 2). Oil of *Salvia* produced potent inhibitory effect against *Bacillus cereus M*TCC 430, *Escherichia coli* MTCC 443, *Listeria monocytogenes* MTCC 657, *E. coli* MTCC 1687, *Staphylococcus aureus* MTCC 1430, and *S. enterica* MTCC 733 with the diameters of zones of inhibition of 15.0, 15.0, 15.3, 17.3, 18.7, and 21.7 mm, respectively. *E. coli* MTCC 443 and *S. enterica* MTCC 733 were found to be susceptible to *Artemisia* oil with 19.0 mm inhibition zone in the former and 20.3 mm in the latter. *B. cereus* MTCC 430 was strongly inhibited by *D. heterophyllum* oil (27.0 mm). *Enterococcus faecalis* MTCC 2729 was found to be least sensitive to all the oils tested. Both *Salvia* and *Dracocephalum* oils inhibited *Yersinia enterocolitica* MTCC 859 with same zone of inhibition (17.3 mm). All tested microorganisms were completely non-susceptible to negative controls loaded with 10% DMSO supplemented with 0.5% Tween 80. The EOs did not possess selective antibacterial activity on the basis of the cell wall differences of bacteria.

Determination of MIC

The MICs of all the EOs obtained by the microtiter broth microdilution method are presented in Table 3. MIC values for *Artemisia* oil against the tested bacteria were lower (2000 to 8000 µg/ml) than those of the other two oils (2000 to >16000 µg/ml). The high antimicrobial activity of *Artemisia* oil was confirmed by the microdilution broth assay, exhibiting MIC values of 2000 µg/ml against *E. coli* MTCC 443 and *S. enterica* MTCC 733. Except for strains of *E. faecalis* MTCC 2729 and *Aeromonas hydrophila* MTCC 1739 for which MIC value of 8000 µg/ml was obtained, rest all other strains were inhibited at

Table 3. MIC (µg/ml) of EOs against food borne pathogens tested.

Test microorganism	EOs		
	[1]SS	[2]AA	[3]DH
Gram-positive organisms			
B. cereus MTCC 430	8000	4000	2000
E. faecalis MTCC 2729	16000	8000	16000
L. monocytogenes MTCC 839	8000	4000	16000
L. monocytogenes MTCC 657	4000	4000	8000
S. aureus MTCC 902	4000	4000	8000
S. aureus MTCC 1430	2000	4000	8000
Gram-negative organisms			
A. hydrophila MTCC 1739	>16000	8000	16000
E. coli MTCC 1687	8000	4000	16000
*E. coli M*TCC 443	4000	2000	8000
K. pneumoniae MTCC 432	16000	4000	8000
P. vulgaris MTCC 426	>16000	4000	16000
P. aeruginosa MTCC 424	>16000	4000	>16000
P. fluorescens MTCC 103	>16000	4000	16000
S. enterica MTCC 733	2000	2000	8000
S. typhimurium MTCC 98	4000	4000	8000
S. flexneri MTCC 1457	>16000	4000	>16000
Y. enterocolitica MTCC 859	8000	4000	8000

[1]SS- *Salvia sclarea*; [2]AA- *Artemisia annua*; [3]DH- *Dracocephalum heterophyllum*.

4000 µg/ml with *Artemisia* oil. However, MIC values of >16000 µg/ml were obtained with the *Salvia* and *Dracocephalum* oils against *A. hydrophila* MTCC 1739, *Proteus vulgaris* MTCC 426, *Pseudomonas aeruginosa* MTCC 424, *P. fluorescens* MTCC 103, *Shigella flexneri* MTCC 1457 and *P. aeruginosa* MTCC 424, and *S. flexneri* MTCC 1457, respectively.

Examination of mode of action

All the tested bacteria inhibited by *Artemisia* oil showed bacteriostatic mode of action. However, the *Salvia* and *Dracocephalum* oil exhibited a bactericidal mode of action against the tested bacteria.

Synergy studies

EO combinations were evaluated by the spot-on-agar test. All the oils in combinations with each other showed a greater efficacy than when assessed individually against few tested pathogens (results not shown). The quantitative effects of all the oils in combination are described in terms of fractional inhibitory concentration indices (FICI). The inhibitory activity of the combination of EOs was determined at 36 different combinations. FIC index ranged from 0.25 to 2.00 indicating the synergistic, additive and indifferent interactions among the combinations against the tested pathogens (Table 4). The combination of *A. annua* and *D. heterophyllum* (AA:DH) showed synergy against 3 (20.00%) species: *E. coli, P. vulgaris* and *Y. enterocolitica*; indifference against 12 (80.00%) species. The MIC of *A. annua* reduced upto 1/32, 1/8, 1/8 and *D. heterophyllum* up to ¼, ¼ and 1/8, in combination against *E. coli, P. vulgaris* and *Y. enterocolitica,* respectively. The combination *S. sclarea* with *D. heterophyllum* (SS:DH) showed synergy against one (8.33%) species, *S. enterica*; additive effect against two (16.67%) bacterial species, *S. typhimurium* and *Y. enterocolitica*; and indifferent effect against nine (75.00 %) bacterial species. Combination of *S. sclarea* with *A. annua* (SS:AA) demonstrated indifferent effect against 11 (91.67%) of the tested pathogens and an additive activity against one (8.33%) bacterial species, *S. enterica*, with an FICI of 1.00.

Pearson product-moment correlations for the six paired antibacterial agents are presented in Table 5. A positive correlation was observed between SS:AA and SS (0.744), while the next highest correlations were between SS:DH and DH (0.661), SS:DH and SS:AA (0.638) and SS:DH and SS (0.622). The multivariate modeling of the relationship between EOs was performed by principal component analysis (PCA). Eigenvectors resulting from PCAs showed 78.2% of the information contained in the six initial variables is captured with the first two variables

Table 4. FIC indices of EO combinations against food borne pathogens tested.

Test microorganisms	Oil combination	MIC of oil_A(µg/ml) in combination/alone	FIC_{oilA}	MIC of oil_B(µg/ml) in combination/alone	FIC_{oilB}	FIC Index	*Type of interaction
Gram-positive organisms							
B. cereus MTCC 430	SS:AA	8000/8000	1	4000/4000	1	2	I
	AA:DH	4000/4000	1	2000/2000	1	2	I
	SS:DH	8000/8000	1	2000/2000	1	2	I
E. faecalis MTCC 2729	SS:AA	16000/16000	1	8000/8000	1	2	I
	AA:DH	8000/8000	1	16000/16000	1	2	I
	SS:DH	16000/16000	1	16000/16000	1	2	I
L. monocytogenes MTCC 839	SS:AA	8000/8000	1	4000/4000	1	2	I
	AA:DH	4000/4000	1	16000/16000	1	2	I
	SS:DH	8000/8000	1	16000/16000	1	2	I
L. monocytogenes MTCC 657	SS:AA	4000/4000	1	4000/4000	1	2	I
	AA:DH	4000/4000	1	8000/8000	1	2	I
	SS:DH	4000/4000	1	8000/8000	1	2	I
S. aureus MTCC 902	SS:AA	4000/4000	1	4000/4000	1	2	I
	AA:DH	4000/4000	1	8000/8000	1	2	I
	SS:DH	4000/4000	1	8000/8000	1	2	I
S. aureus MTCC 1430	SS:AA	2000/2000	1	4000/4000	1	2	I
	AA:DH	4000/4000	1	8000/8000	1	2	I
	SS:DH	2000/2000	1	8000/8000	1	2	I
Gram-negative organisms							
A. hydrophila MTCC 1739	SS:AA	n.d.					
	AA:DH	8000/8000	1	16000/16000	1	2	I
	SS:DH	n.d.					
E. coli MTCC 1687	SS:AA	8000/8000	1	4000/4000	1	2	I
	AA:DH	4000/4000	1	16000/16000	1	2	I
	SS:DH	8000/8000	1	16000/16000	1	2	I
E. coli MTCC 443	SS:AA	4000/4000	1	2000/2000	1	2	I
	AA:DH	62.5/2000[d]	0.03	2000/8000[b]	0.25	0.28	S
	SS:DH	4000/4000	1	8000/8000	1	2	I
K. pneumoniae MTCC 432	SS:AA	16000/16000	1	4000/4000	1	2	I
	AA: DH	4000/4000	1	8000/8000	1	2	I
	SS:DH	16000/16000	1	8000/8000	1	2	I
P. vulgaris MTCC 426	SS:AA	n.d.					
	AA:DH	500/4000[c]	0.125	4000/16000[b]	0.25	0.375	S
	SS:DH	n.d.					
P. aeruginosa MTCC 424	SS:AA	n.d.					
	AA:DH	n.d.	-	-	-	-	-
	SS:DH	n.d.					

Table 4. Contd.

P. fluorescens MTCC 103	SS:AA	n.d.	1	16000/16000	1	2	I
	AA:DH	4000/4000					
	SS:DH	n.d.					
S. enterica MTCC 1457	SS:AA	1000/2000[a]	0.5	1000/2000[a]	0.5	1	A
	AA:DH	2000/2000	1	8000/8000	1	2	I
	SS:DH	250/2000[c]	0.125	2000/8000[b]	0.25	0.375	S
S. typhimurium MTCC 98	SS:AA	4000/4000	1	4000/4000	1	2	I
	AA:DH	4000/4000	1	8000/8000	1	2	I
	SS:DH	1000/4000[b]	0.25	2000/8000[b]	0.25	0.50	A
S. flexneri MTCC 1457	SS:AA	n.d.					
	AA:DH	n.d.	-	-	-	-	-
	SS:DH	n.d.					
*Y. enterocolitica M*TCC 859	SS:AA	8000/8000	1	4000/4000	1	2	I
	AA:DH	500/4000[c]	0.125	1000/8000[c]	0.125	0.25	S
	SS:DH	1000/8000[c]	0.125	4000/8000[a]	0.5	0.625	A

[1]SS: [2]AA- *Salvia sclarea + Artemisia annua*; [2]AA: [3]DH- *Artemisia annua + Dracocephalum heterophyllum.* *S- synergistic; A- Additive; I- Indifferent; n.d.- not done. [a]MIC/2 (µg/ml); [b]MIC/4 (µg/ml); [c]MIC/8 ; [d]MIC/32 (µg/ml).

Table 5. Pearson product-moment correlations between individual and combinations of EOs.

	SS	AA	DH	SS:AA	AA:DH	SS:DH
SS	1					
AA	0.511	1				
DH	0.577	0.418	1			
SS:AA	0.744	0.568	0.546	1		
AA:DH	0.459	0.594	0.541	0.521	1	
SS:DH	0.622	0.408	0.661	0.638	0.549	1

(Figure 1).

Effects of oils on *S. enterica* in food

Based on disc diffusion and MIC determination assays, all the three oils were examined for antibacterial activity against *S. enterica* MTCC 733, the most sensitive strain to all of the oils, in food systems. Sensitivity of *S. enterica* MTCC 733 was studied to the combinations of oils resulting in complete inhibition after 3 h treatment and comparing them to those obtained from the experiments performed with individual activity of the oils. Over the 3 h observation period, a significant increase in the number of bacterial cells was observed from 7.04 to 8.46 log CFU/g in the growth control (Table 6). On the other hand, decrease in the number of log CFU/g was observed when the EOs were used even at the MIC values. The combination SS:DH caused a rapid reduction of the bacterial cells in the first 90 min after treatment to undetectable level (complete inhibition) while the bacterial cell loads were reduced from 6.89 log CFU/g o 4.60 log CFU/g by the former and from 6.96 to 5.76 log CFU/g by the latter, after 180 min when applied individually at MIC values. Even the concentrations of 20 times the MIC values of both these oils, added individually, did not cause the complete eradication of the bacterial population. Population of *S. enterica* on chicken treated with the MIC value and 20 times the MIC value of *Artemisia* oil was reduced by 4.83 and 3.64 log CFU/g after 180 min. But in combination with *Salvia* oil, SS:AA, a quick repression of the inoculums was observed within 150 min. Further increasing the treatment time to 180 min caused the bacterial population to decrease below the detection limit. Treatment with any of the oils used singly resulted in no decline of the population of *S. enterica* to below detection limit.

DISCUSSION

Plant-derived Eos due to their content of anti-microbial compounds possess potential as natural agents for food preservation. Their antimicrobial activity is assigned to a number of small terpenoid and phenolic compounds which, due to their lipophillic character, accumulate in bacterial membranes causing energy depletion (Conner, 1993). Polyphenols have been used in food industry to extend the shelf-life of foods containing oxidizable lipids such as vegetable oils, animal fats,

Table 6. Effectiveness of oils in inactivating *S. enterica* MTCC 733 (log cfu/g) inoculated on raw chicken meat.

Time (min)	Growth control	[1]SS (2000 µg/ml) + [3]DH (8000 µg/ml)	[1]SS (2000 µg/ml) + [2]AA (2000 µg/ml)	[1]SS (µg/ml)		[2]AA (µg/ml)		[3]DH (µg/ml)	
				2000	40000	2000	40000	8000	160000
0	7.04 ± 0.01[a]	6.45 ± 0.04[d]	6.54 ± 0.04[e]	6.89 ± 0.02[g]	6.80 ± 0.04[g]	6.67 ± 0.04[g]	6.63 ± 0.04[g]	6.96 ± 0.03[f]	6.90 ± 0.05[g]
30	7.27 ± 0.01[b]	5.81 ± 0.15[c]	6.36 ± 0.06[e]	6.51 ± 0.06[f]	6.13 ± 0.03[f]	6.20 ± 0.05[f]	6.50 ± 0.05[f]	6.80 ± 0.04[e]	6.36 ± 0.04[f]
60	7.34 ± 0.04[b]	3.80 ± 0.18[b]	5.63 ± 0.31[d]	6.33 ± 0.04[e]	5.59 ± 0.02[e]	6.09 ± 0.05[e]	5.94 ± 0.03[e]	6.40 ± 0.08[d]	6.22 ± 0.02[e]
90	7.45 ± 0.07[c]	1.63 ± 0.06[a]	4.87 ± 0.15[c]	6.16 ± 0.04[d]	5.17 ± 0.03[d]	5.45 ± 0.02[d]	5.23 ± 0.03[d]	6.25 ± 0.01[c]	5.84 ± 0.03[d]
120	7.93 ± 0.06[d]	ND[z]	3.36 ± 0.08[b]	5.56 ± 0.09[c]	3.82 ± 0.02[c]	5.22 ± 0.03[c]	4.71 ± 0.17[c]	5.94 ± 0.04[b]	5.40 ± 0.02[c]
150	8.12 ± 0.10[e]	ND[z]	1.67 ± 0.10[a]	5.32 ± 0.05[b]	3.75 ± 0.02[b]	4.93 ± 0.03[b]	4.33 ± 0.04[b]	5.82 ± 0.04[a]	4.44 ± 0.04[b]
180	8.46 ± 0.06[f]	ND[z]	ND[z]	4.60 ± 0.03[a]	3.36 ± 0.05[a]	4.83 ± 0.10[a]	3.64 ± 0.04[a]	5.76 ± 0.04[a]	4.22 ± 0.03[a]

[1]SS- *Salvia sclarea*; [2]AA- *Artemisia annua*; [3]DH- *Dracocephalum heterophyllum,* [z]ND- Not detected. Different superscripts in a column differ significantly ($p<0.05$) by Duncan's test .

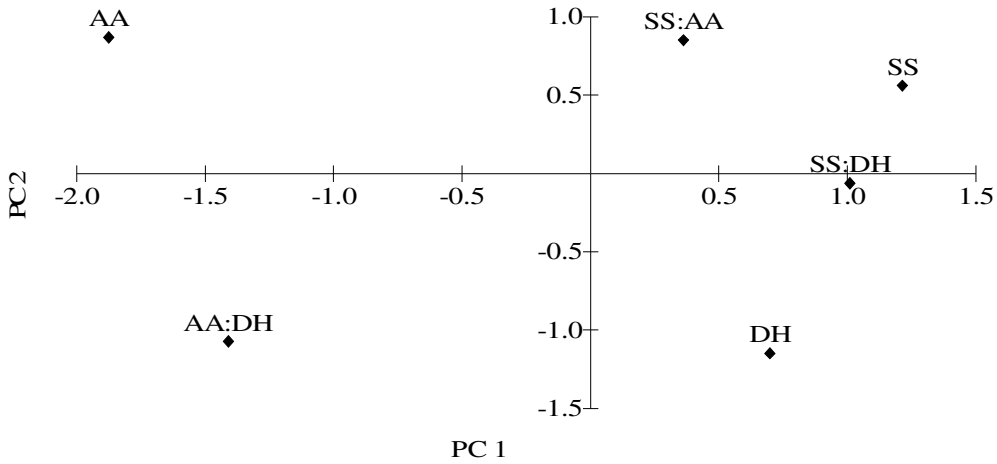

Figure 1. Principal components analysis (PCA) of the relationships among individual and combinations of EOs.

flavourings and processed meat (Korekar et al., 2011).

Many reports have described the antimicrobial activity of EOs against foodborne pathogens including *E. coli* 0157:H7 (Burt and Reinders, 2003), *L. monocytogenes* (Singh et al., 2003), *Klebsiella pneumoniae* (Sokmen et al., 2004), *S. aureus* (Bajpai et al., 2007), *B. cereus* (Rahman and Kang, 2009), and *Vibrio cholerae* (Rattanachaikunsopon and Phumkhachorn, 2009). However, the antimicrobial activities of the three EOs under study, namely, *S. sclarea, A. annua* and *D. heterophyllum* against the deadly foodborne pathogen *S. enterica* and their potential use as a preservative against the bacterium in food has never been reported before.

Many researchers have reported that EOs are slightly more active against Gram-positive than

Gram-negative bacteria as Gram-negative organisms are less susceptible to the action of antibacterials (Shelef et al., 1984; Vaara, 1992). Our results agree with the observation of Dorman and Deans (2000) that the susceptibility of bacteria to plant volatile oils and the Gram reaction appears to have little influence on growth inhibition. Differential antibacterial activity was observed between two strains of same species, namely, *S. aureus* and *L. monocytogenes* among all the oils tested. These results are in agreement with those reported by Laciar et al. (2009) who reported that within bacterial species, EO efficacy was dependent on the strain and in some cases on the strain origin.

The disc diffusion assay and MIC determination assay are rapid and practical approaches to screen large numbers of potential antibacterials, but do not account for the potential effects of a food matrix. The hydrophobicity of EO components is known to limit the value of diffusion tests for estimating the antimicrobial potency accurately (Holley and Patel, 2005). Rajkovic et al. (2005) reported that carvacrol, which inhibited the growth of *B. cereus* and *Bacillus circulans* in nutrient broth, failed to exhibit any antimicrobial properties when combined with potato puree. Interference between food matrices (juice and dip) and the antimicrobial potency of chitosan hydrolysates was also observed (Rhoades and Roller, 2000). The ability of all the three oils to inhibit *S. enterica*, a Gram-negative bacterium, makes it more interesting for use to prevent food-related illness caused by *S. enterica* and other Gram-negative bacteria, which cannot be inhibited by nisin, the only bacteriocin accepted by the Food and Agriculture Organization (FAO)/World Health Organization (WHO) in 1969 as a food preservative. The activity of the oils would be expected to relate to the respective composition of the plant volatile oils, the structural configuration of the constituent components of the volatile oils and their functional groups and possible synergistic interactions between components. Nazer et al. (2005) found that thymol in combination with other aromatic compounds led to improved inhibition, but no real synergistic effect was demonstrated between compounds against *Salmonella*. In our study, the synergistic interaction was observed in relation to *S. enterica* MTCC 733 at 1/8 and 1/4 MIC values of *Salvia* and *Dracocephalum* oils, respectively, in combination and 1/2 MIC value of both *Salvia* and *Artemisia* oil, which demonstrates the potential of these high altitude medicinal plants as a candidate of natural preservative ingredient in food.

Effect of EOs is known to be reduced in a food matrix system as a result of interaction with different components of food, thus requiring much larger concentrations to reduce the bacterial populations (Farbood et al., 1976; Smith-Palmer et al., 2001). Most of the published work points out the need to use a high concentration of EO in food systems, typically from 2 to 100 times the determined *in vitro* MIC value, depending on the food characteristics (Burt, 2004). Rattanachaikunsopon and Phumkhachorn (2009) reported 24-fold higher use of Elephant Garlic Oil in food as compared to the *in vitro* conditions against *V. cholerae*. Arora et al. (2012) reported the use of 40 times the MIC value to reduce the *L. monocytogenes* load to 1.69 log cfu/g on exposure to the methanolic extract of seabuckthorn leaves on sliced carrots after 60 min. The ratio has been recorded to range from 10-fold (in pork liver sausage) to 100-fold (in soft cheese) (Burt, 2004). In this regard, results obtained in this study indicate the technical viability of using all the three oils in combinations to prolong the shelf life of the food, as relatively low concentrations of oils were sufficient to reduce the bacterial load. Treatment with combinations of EOs added simultaneously to chicken inoculated with *S. enterica* showed increasing sensitivity of the bacteria, which was higher than that observed when the oils were added individually, even at higher doses. The present study indicates that the combinations of EOs (SS:DH and SS:AA) result in a synergistic and additive antibacterial effect on the growth of *S. enterica* cells.

Conclusion

This study indicates the potential of *Salvia*, *Artemisia* and *Dracocephalum* oils to serve as natural antibacterials against *S. enterica*. Their effectiveness over a wide range of food-borne pathogens contributes to their advantages as food preservatives and prospective alternative to currently used chemical-based inhibitors. EOs of *Salvia* and *Dracocephalum* depicted bactericidal mode of action while that of *Artemisia* inhibited the bacteria with bacteriostatic mode. Combinations of plant EOs were assessed for synergistic activity, as this would allow lower concentrations of EOs to be used, thereby achieving the twin aims of reducing any undesirable organoleptic impact, as well as controlling food-borne pathogens in food. Treatment of food sample with 20 times MIC value of *S. sclarea, A. annua* and *D. heterophyllum* EOs individually caused reduction of bacterial load to 3.36, 3.64 and 4.22 log cfu/g after 180 min. In contrast the bacterial cell loads reduced to an undetectable level by the combinative effect (*Salvia + Dracocephalum* and *Artemisia + Salvia*) of EOs at MIC value after 120 and 180 min, respectively. This study suggests that combinations of EOs could minimize application concentrations in real food system. This study is the first report on the antibacterial activities of the three EOs against *S. enterica* in a real food system. The design is not only practical but also less laborious and economical for the real life and the results are useful for the meat processing industry where they can reduce their time and cost in disinfecting the animal carcasses. However, further works are warranted for the evaluation of toxicity and safety of the oils using animal models and

it is also required to isolate and characterize, antibacterial principles to elucidate their structure and function relationships.

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