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RESEARCH ARTICLE

Phytochemical Analysis and *In Vitro* Activity of Essential Oils of Selected Plants against *Salmonella enteritidis* and *Salmonella gallinarum* of Poultry Origin

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A B S T R A C T

Antibiotic resistant *Salmonella* is a major threat to poultry industry and public health. Medicinal plants are an effective alternative of antibiotics for the control and treatment of multiple drug resistant *Salmonella*. The objective of this study was to evaluate the *in-vitro* activity of essential oils of some medicinal plants against multiple drug resistant *Salmonella* of poultry origin and to determine their active ingredients. Essential oils of *Cuminum cyminum***,** *Cinnamomum zeylanicum***,** *Eucalyptus globulus***,** *Allium sativum* and *Nigella sativa* were prepared by steam distillation and their active ingredients were determined by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Activity of oils against *Salmonella enteritidis* (n=05) and *Salmonella gallinarum* (n=05) was determined by well diffusion assay. Broth microdilution assay was employed to determine the minimum inhibitory concentrations of oils. Well diffusion assay revealed that *C. zeylanicum* and *E. globulus* had better activity against salmonellae (26±7.6 mm and 16±6.8 mm, respectively) as compared to *C. cyminum, A. sativum* and *N. sativa* (8±5.9, 10±6.1, 8±4.7 mm, respectively). Minimum inhibitory concentrations of *C. zeylanicum* and *E. globulus* against *Salmonella* were 64.1±32.1 and 68.9±32.9 µg/mL, respectively. The GC-MS analysis revealed presence of diverse phytochemicals in all essential oils. Major antimicrobial phyto-constituents of essential oils of *E. globulus* and *C. zeylanicum* were eucalyptol (82.85%) and 1R-α-Pinene (13.781%), and cinnamaldehyde (64.14%) and eugenol (8.9%), respectively. It is concluded that essential oils of *C. zeylanicum* and *E. globulus* have excellent *in vitro* anti-*Salmonella* activity. It is insinuated that these extracts may be commercialized as an alternative of antibiotics for the control of Salmonellae in poultry after detailed *in vivo* evaluations.

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INTRODUCTION

Plants, being natural source of medicines, are used for the control and treatment of wide variety of ailments since pre historic times. Interest in use of plants is on the increase from last decade because of emergence of drug resistance, low toxicity and cheaper availability (Chouhan *et al.*, 2017; Hanem *et al*., 2018; Abbas *et al*., 2019a, 2019b). The *Salmonella*, Gram-negative, facultative anaerobic bacteria, is a member of Enterobacteriaceae. Use of contaminated feed and feed ingredients is a source of *Salmonella* in Poultry. Both of the animals and consumers of animal food products remain at risk of contracting *Salmonella* (Khan *et al.*, 2019). Ability of *Salmonella* to tolerate environmental conditions and capacity to form biofilm make it a persistent contaminant of food especially poultry products (Vestby *et al.*, 2009). Detection of contaminant at early stage of poultry raring or processing is an effective strategy to prevent contaminated products in market (Heymans *et al*., 2018). The *Salmonella typhimurium* and *Salmonella enteritidis* are major reason of food borne salmonellosis in human

(Vose *et al*., 2011). Major reservoir of *Salmonella enteritidis* is poultry. Food borne infections of *Salmonella* are reported throughout the world especially in developing countries. *Salmonella enteritidis* is considered as a major etiology of salmonellosis in Europe while *Salmonella typhimurium* in United States (Thorns, 2000).

Control and treatment of *Salmonella* associated infections is done by employing effective hygiene strategies and antibiotics, respectively. Overuse and misuse of antibiotic has resulted in emergence of antibiotic resistance in *Salmonella* which now pose a serious threat to poultry industry as well as public health (Klemm *et al*., 2018). Activity of medicinal plants against different pathogenic organisms is well known since long. A decreased interest in use of plant based medicines and antimicrobials since discovery of antibiotics has revived with the emergence of antibiotic resistance. Now, scientists have shifted their focus on development of alternatives of antibiotic. Essential oils derived from a wide variety of medicinal plants have antibacterial activity and an effective strategy to control microorganisms in poultry. Essential oils possess some volatile compounds having diverse bioactivities including promising antimicrobial activity (O'Bryan *et al.,* 2015). Essential oils also provide many other health benefits to poultry i.e increased weight gains and enhanced immunity (Ebani *et al.,* 2018). Antimicrobial activity of essential oils depends upon their chemical constituents, functional group and their lipophilic character. Chemically terpenes are the most common class of compounds found in essential oils along with hydrocarbons. Cinnamon oil generally contains cinnamaldehyde, eugenol, limonene, linalool and cinnamic acid while leaves of *Eucalyptus* oil contain eucalyptol, pinene, carvone and cinnamic acid (Unlu *et al.,* 2010; Gouveia *et al.,* 2012). Use of essential oils against *Salmonella* has also been demonstrated previously in different reports (Adaszyńska-Skwirzyńska and Szczerbińska, 2017).

Keeping in mind the problems associated with *Salmonella* in poultry, its threat to public safety and emergence of antibiotic resistance, this study was designed to determine *in vitro* activity of essential oils of different plants against multidrug-resistant *Salmonella* of poultry origin. In addition, phyto-constituents of essential oils were also investigated.

MATERIALS AND METHODS

Indicator organism: Multidrug resistant *Salmonella* (n=10) including *Salmonella gallinarum* (S01, S07, S15, S54, S64) and *Salmonella enteritidis* (S02, S47, S62, S63, S67) of poultry origin were obtained from in house collection of Department of Microbiology, University of Veterinary and animal Sciences, Lahore. Antibiotic resistance pattern of these isolates was determined previously is given table 1 (Yasmin *et al*., 2019). All these isolates were sub-cultured and maintained on salmonella shigella agar at 37ºC.

Plant collection and extraction of essential oils: Plants (C*innamomum zeylanicum, Cuminum cyminum, Eucalyptus globulus, Allium sativum and Nigella sativa*) were collected/ procured from local markets of Lahore and identified by Government College University, Lahore. Plants were shade dried, crushed into powder using mortar and pestle. Essential Oils (EOs) were extracted by steam distillation as described elsewhere (Adinew, 2014). Extraction was carried out by immersing 300 g of dry plant material in 600 mL distilled heated continuously and essential oils were collected by using a condenser. Oils were stored in brown bottles and kept at 4ºC for further use.

Agar well diffusion assay: Activity of essential oils were determined by well diffusion assay on nutrient agar plates as described previously (Mohamed *et al.,* 2016). Briefly, exponentially growing indicator bacteria were re-suspended in phosphate buffered saline (PBS) (~0.5 McFarland) and swabbed on agar plates to obtain a uniform lawn of growth. Wells of appropriate size were made on inoculated plates, sealed and 100 µL of essential oils (100 mg/mL dimethyl sulfoxide) were added in wells. Plates were incubated and anti-*Salmonella* activity was read as zone of inhibition of growth surrounding the wells after incubation of plates at 37ºC for 24 hours.

Minimum inhibitory concentrations: Minimum Inhibitory Concentrations (MICs) of EOs were determined by broth microdilution method using 96 well microtitre plate as described elsewhere (Manandhar *et a*l., 2019). Doubly diluted essential oils (24 µg/mL-12.5 mg/mL) in MH broth (50 µL) were added in each well and inoculated with 100 µL of indicator organism (~1.5 × 10^5 CFU/ mL). Inoculum was prepared by suspending the exponentially growing indicator organism in PBS (~1 McFarland) and diluting it by 1:1000 in MH broth. Plates were incubated at 37ºC for 24 hours and MIC was read as lowest concentration of essential oils which totally inhibited the growth of indicator organism.

GC-MS analysis of selected oils: Phyto-constituents of EOs were determined by subjecting the oils to Gas Chromatography Mass Spectrometry (GC–MS) using an Agilent 6890N gas chromatography coupled to Agilent 5973N mass selective detector equipped with a flame ionization detector and fused silica capillary column HP-5MS as described previously (Kamaliroosta *et al.,* 2012). Temperature of injector and detector were adjusted at 240°C and 300°C, respectively. GC program was as follows: Initial oven temperature was held at 60°C for 1 min and ramped at 8°C min^{-1} to 200°C where it was held for 2 min, and then ramped at 10°C min−1 to 230°C and held there for 5 min. The final temperature was 260°C for 10 min. Most constituents were identified by comparison of their retention indices. Relative percentages of the constituents determined on the basis of GC peak areas.

RESULTS

Essential oils of medicinal plants (*C. zeylanicum, C. cyminum, E. globulus, A. sativum and N. sativa*) extracted by steam distillation had strong aroma. Highest EOs yield was obtained for *N. sativa* (2.3%) followed by *C. cyminum* (1.8%) *A. sativum* (1.7%), *C. zeylanicum* (1.5%) and *E. globulu1us* was (1.5%). Antimicrobial activity of EOs was determined through well diffusion assay against

Table 1: Antibiotic resistance profile of selected *Salmonella*

Salmonellae	Resistance profile
Salmonella gallinarum S01	AMP^R, GEN^R, AMX^R, TET^R, CTX^R, CAZ^R, CHL^R, CIP^R, NAL^R
Salmonella gallinarum S07	AMP^R, GEN^R, AMX^R TET^R, CHL^R, CIP^R, NAL^R
Salmonella gallinarum S15	AMP^R, GEN^R, AMX^R CRO^R, CAZ^R, CHL^R, SXT^R, CIP^R, NAL^R
Salmonella gallinarum S54	AMP^R, GEN^R, AMX^R CRO^R, TET^R, CTX^R, SXT^R CAZ^R, CHL^R, CIP^R, NAL^R
Salmonella gallinarum S64	AMP^R, GEN^R, AMX^R, CRO^R, CHL^R, CIP^R, NAL^R
Salmonella enteritidis S02	AMP^R, GEN^R, AMX^R, TET^R, CTX^R, CHL^R CIP^R, NAL^R
Salmonella enteritidis S47	AMP^R, GEN^R, AMX^R, TET^R, CAZ^R, CHL^R, SXT^R, CIP^R, NAL^R
Salmonella enteritidis S62	AMP^R, GEN^R, AMX^R, CRO^R, CTX^R, CAZ^R, CIP^R, NAL^R
Salmonella enteritidis S63	AMP^R, GEN^R, AMX^R, CRO^R, CAZ^R, CH^RL, SXT^R, CIP^R, NAL^R, CFM^R
Salmonella enteritidis S67	AMP^R, GEN^R, AMX^R, TET^R, CAZ^R, CHL^R, SXT^R, CIP^R, NAL^R

R: resistant; AMP: ampicillin; GEN: gentamicin; AMX: amoxicillin; CRO: ceftriaxone; TET: tetracycline; CTX, cefotaxime; CIP: ciprofloxacin; CHL: chloramphenicol; SXT: sulfamethoxazole; NAL: nalidixic acid; CAZ: ceftazidime; CFM: cefixime

Table 2: Antimicrobial activity of essential oils determined by well diffusion test

Isolate	Diameter of zones of inhibition (mm)					
	Cinnamomum zeylanicum (100 mg/mL)	*Cuminum cyminum* (100 mg/mL)	*Eucalyptus globulus* (100 mg/mL)	*Allium sativum* (100 mg/mL)	*Nigella sativa* (100 mg/mL)	Cefexime (5µg)
Salmonella gallinarum S01	33±0.58	6±1.00	12±0.58	0±0.00	0±0.00	21±0.57
Salmonella gallinarum S07	23±0.58	17±1.00	22±0.58	11±0.58	9±1.00	22±1
Salmonella gallinarum S15	27±1.53	1±2.31	16±1.15	10±1.53	8±1.53	27±0.57
Salmonella gallinarum S54	35±1.53	15±1.00	20±2.08	14±1.15	17±1.53	21±2.64
Salmonella gallinarum S64	22±1.00	1±1.15	13±7.57	20±1.53	7±0.58	20±2
Mean±S.D *Salmonella gallinarum*	28±5.8	8±7.6	17±4.3	11±7.2	8±6.05	22.06±2.81
Salmonella enteritidis S02	25±1.53	12±1.53	20±1.53	2±2.89	5±1.53	22±1.7
Salmonella enteritidis S47	11±0.58	0±0.00	13±1.00	5±0.58	6±0.58	23±2
Salmonella enteritidis S62	21±0.58	6±1.00	0±0.00	9±0.58	11±1.00	19±2
Salmonella enteritidis S63	32±1.00	9±1.00	20±1.73	16±2.00	11±1.53	15±0.57
Salmonella enteritidis S67	35±1.15	8±1.53	23±0.58	8±1.53	3±1.00	19±2.5
Mean±S.D *Salmonella enteritidis*	25±9.49	7±4.47	15±9.2	8±5.2	7±3.6	19.7±3.10
Overall Mean±S.D	26±7.6	8±5.9	16±6.8	10±6.1	8±4.7	20.9±3.14

Table 3: Minimum inhibitory concentrations of selected plants essential oils against *S. enteritidis* and *S. gallinarum*

Isolates	Minimum Inhibitory Concentrations (µg/mL)	
	Cinnamomum zeylanicum	*Eucalyptus globulus*
Salmonella gallinarum S01	64±27.7	48±0
Salmonella gallinarum S07	48±0	48±0
Salmonella gallinarum S15	64±27.7	48±0
Salmonella gallinarum S54	48±0	64±27.7
Salmonella gallinarum S64	48±0	80±27.7
MIC Range	48-64	48-80
Mean±S.D	54.4±8.7	57.6±14.3
Salmonella enteritidis S02	64±27.7	64±27.7
Salmonella enteritidis S47	64±27.7	129±57.1
Salmonella enteritidis S62	48±0	80±27.7
Salmonella enteritidis S63	129±57.1	48±0
Salmonella enteritidis S67	64±27.7	80±27.7
MIC Range	48-129	48-129
Mean±S.D	73.8±31.6	80.2±30.3
Overall MIC Range	48-129	48-129
Overall Mean±S.D	64.1±32.1	68.9±32.9

ten different multiple drug resistant isolates of *Salmonella gallinarum* and *Salmonella enteritidis* characterized previously (Yasmin *et al.*, 2019). All essential oils exhibited different levels of antimicrobial activity against these isolates. The essential oil of *C. zeylanicum* and *E. globulus* had higher activity against *Salmonella gallinarum* (35±1.53 mm and 22±0.58 mm, respectively) as compared with *C. cyminum, A. sativum, N. sativa* (17±1.00, 20±1.53, 17±1.53 mm, respectively). Similarly, essential oil of *C. zeylanicum* and *E. globulus* showed better activity against *Salmonella enteritidis* (35±1.15 mm and 23±0.58 mm, respectively) as compared to *C. cyminum, A. sativum, N. sativa* (12±1.53, 16±2.00, 11±1.53 mm, respectively) (Table 2). The essential oil of *C. zeylanicum* showed highest diameter of zone of inhibition 35±1.15 mm and 35±1.53 mm against *Salmonella enteritidis* S67 and *Salmonella gallinarum* S54 (Table 2, Fig. 1). Cinnamon oil showed higher zones of inhibition (26±7.6 vs 20.9±3.14) as compared to antibiotic cefixime (5µg). The MICs were determined for plants showing better activity by well diffusion assay i.e *C. zeylanicum* and *E. globulus*. The MICs ranged from 48-129 µg/mL against different isolates. Mean of MICs of C. *zeylanicum* and *E. globulus* essential oils were 64.1±32.1 and 68.9±32.9, respectively as shown in Table 3. Phyto-constituents of EOs of all plants are given in Table 4 and Fig. 2. Major phyto-constituents of essential oils of *E. globulus* and *C. zeylanicum* were eucalyptol (82.85%) and 1R-α-Pinene (13.781%) and cinnamaldehyde (64.14%) and eugenol (8.9%), respectively.

DISCUSSION

Current study was an effort to evaluate *in vitro* anti-*Salmonella* activity and determination of phyto-constituents of essential oils of medicinal plants. Essential oils were extracted by steam distillation and results revealed that yield of essential oils were in range of 1.5%-2.3% (w/w). Similar results reporting a moderate yield (<2.5% w/w) of essential oil of different plants have been reported previously as well (Ammar *et al*., 2017; Kasim *et al*., 2014). Higher yields of EOs (≥2.5% w/w) as compared to report in current study have also been reported (Li *et al*., 2013). These different or lower yield of essential oils in current can be attributed to different variety of plant, climate and soil type, plant part used for extraction, and irrigation and cultivation techniques (Teles *et al*., 2019). All the essential oils had variable activity against indicator *Salmonella*. The essential oils of *C. zeylanicum*

Table 4: Phyto-constituents of essential oils of medicinal plants as revealed by Gas Chromatography-Mass Spectrometry (GC-MS)

	Peak #	RT	Compound	Total (%)
Eucalyptus globulus	1	4.306	1R-α-Pinene	13.781
	2	5.37	B-Pinene	1.740
	3	6.957	Eucalyptol	82.856
	4	10.241	3-Cycbhexen-l-ol, 4-methyl-1-[1-methylethyl]-, [R]-	1.140
	5	12.354	4-Octen-3one, 6-ethyl-7hydroxy-	0.483
Cumminum cyminum	1	4.272	α-Pinene	2.761
	2	5.299	β-Pinene	16.005
	3	6.306	D-Limonene	1.535
	4	6.655	Benzene, 1-methyl-4-[1-methylethyl]-	12.396
	5	7.088	1,4-Cyclohexadiene, 1-methyl-4-[1-methylethyl]-	18.173
	6	12.532	Benzaldehyde, 4-[1-methylethyl]-	24.841
	7	13.473	2-Caren-10-al	24.289
Allium sativum	1	4.26	1R-α-Pinene	7.601
	2	5.836	α-Phelandrene	0.816
	3	6.051	1,3-Cyclohexadiene, 1-methyl-4-[1-methylethyl]-	1.858
	4	6.297	D-Limonene	8.762
	5	6.664	Eucalyptol	77.761
	6	8.956	Cyclohexasiloxane, dodecamethyl-	1.896
	7	10.248	3-Cyclohexen-aol, 4-methyl-1-[1-methylethyl]-	0.704
	8	11.863	Cycloheptsiloxane, tetradecamethyl-	0.602
Nigella sativa	1	3.918	Bicylo[3.1.0]hexane, 4-methyl-1-[1-methylethyl]-, didehydro derive.	11.180
	2	4.843	β-Pinene	2.737
	3	5.892	Benzene, 1-methyl-4-[1-methylethyl]-	38.273
	4	7.037	Ether, p-menth-6-en-2-ylmethyl	1.324
	5	10.282	2,5-Cyclohexadiene-1,4-dione, 2-methyl-5-[methylethyl]-	33.938
	6	10.829	Ascaridole expoide	6.506
	7	11.346	1,4-methanaoazulene, decahydro-4, 838-trimethyl-9-methylene-[1S-[1 α, 3αβ, 4α, 8 αβ]-	6.042
Cinnamomum zeylanicum	1	4.061	1R-α-Pinene	0.141
	2	5.73	Limonene	12.411
	3	6.648	Cyclohexene, 1-methyl-4-[m1-methyldene]-	0.213
	4	6.948	1,6-Octadien-3,7-dimethyl-	0.171
	5	7.431	Fenchol, exo-	0.274
	6	7.746	3Cyclohexen-l-ol, l-methyl-4-[l-methylethyl]-	1.349
	7	8.043	3Cyclohexen, l-methyl-4-[l-methylethyl]-	0.635
	8	8.488	3Cyclohexen-l-ol,4-methyl-[l-methylethyl]-	1.459
	9	8.953	3-Cyclohexene-l-methanol, α α4-trimethyl-	12.289
	10	10.319	2-Propenal, 3-phenyl-	1.207
	11	11.841	Cinnamaldehyde	64.14
	12	12.042	Eugenol	8.9

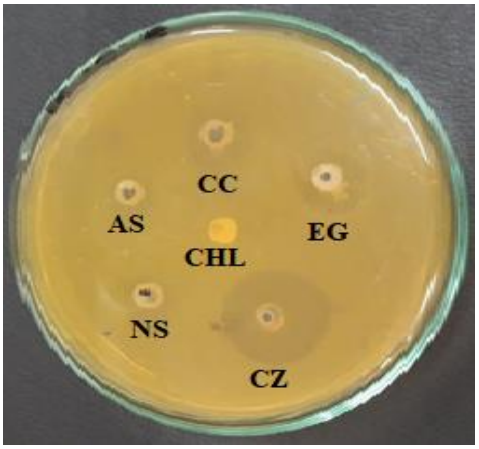

Fig. 1: Representative activity of different essential oils against *Salmonella gallinarum* S15. CC: *Cuminum cimminum* (safaid zera); *Cinamomum zylanicum* (cinnamon), *Eucalyptus globulus* (sufauda); *Nigella sativa* (black seed); *Allium sativum* (garlic); Chloramphenicol (30 µg).

and *E. globulus* had profound activity against *Salmonella gallinarum* and *Salmonella enteritidis* as revealed by both well diffusion assay and broth microdilution assay. Antibacterial activities of essential oils of all these plants against different bacteria including different *Salmonella enteritidis* and *Salmonella typhimurium* or other serovars have been reported previously as well (Patel *et al.,* 2018; Cabarkapa *et al.,* 2019; Ebani *et al.,* 2019). Previous studies also reported inhibitory effect of cinnamon against food borne *Salmonella* (Alsaiqali *et al*., 2016). Our results are in accordance with another study which reported that cinnamon oil is effective at its lowest concentration (1 µL/mL) against all tested bacterial and fungal strains (Tarek *et al*., 2014). Another study has also reported higher antibacterial activity of essential oils of cinnamon as compared to other essential oils (Zhang *et al*., 2018). To best of our knowledge, it is the first study from Pakistan which reports activity of plant essential oils against multiple drug resistant *Salmonella gallinarum* of poultry origin. The GC-MS analysis revealed that major component of essential oil of *C. zeylanicum* is cinnamaldehyde which is coherent to a previous study by Jorjani *et al.* (2017) which showed that antimicrobial activity of cinnamon oil was due to cinnamaldehyde which was found as a major component. Percentage of cinnamaldehyde found in *C. zeylanicum* in this study is remarkably higher (64.14% vs 52.3%) as compared to a previous study by Kazemi and Mokhtariniya (2016) while lower than (64.14% vs 91.82) as reported by Pooja *et al.* (2013). A wide range of phytochemicals has been reported from *E. globulus* previously (Gouveia *et al*., 2012). In this study, eucalyptol and 1R-α-Pinene were found to be major components of *E. globulus* which is consistent with previous studies as well which also agrees that antimicrobial activity of *E. globulus* can be due to α-pinene and eucalyptol(1,8-cineole) components present in the oil (Mekonnen *et al.,* 2016; Park *et al.,* 2016).

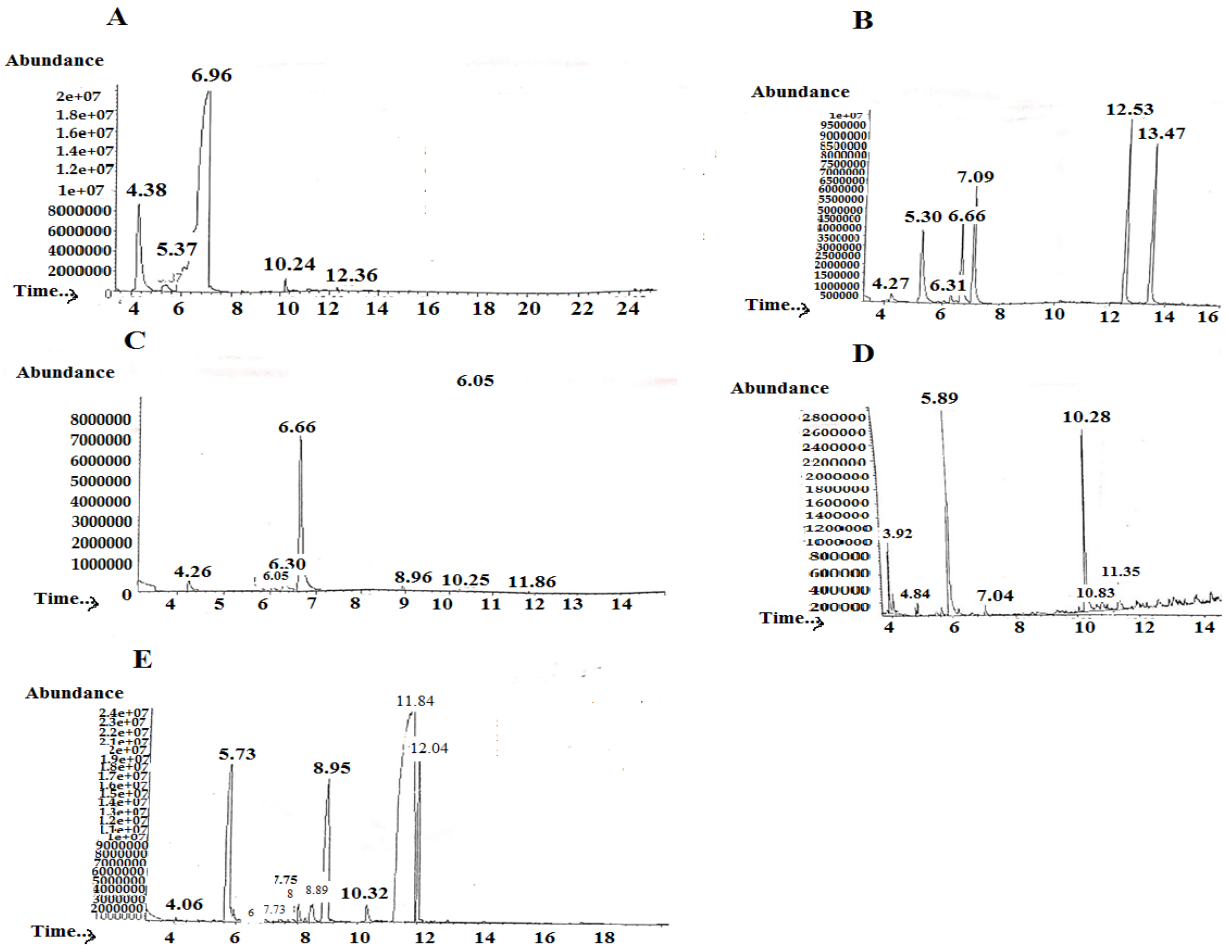

Fig. 2: Gas chromatography mass spectrometry analysis profile of plant extracts (A) *Eucalyptus globulus* (B) *Cumminum cyminum* (C) *Allium sativum* (D) *Nigella sativa* (E) *Cinnamomum zeylanicums.*

Conclusions: It is concluded that essential oils of *C. zeylanicum* and *E. globulus* have diverse phyto-constituents and profound *in vitro* activity against *Salmonella enteritidis* and *Salmonella gallinarum* of poultry origin. It is insinuated that these nutraceuticals may further be evaluated for their potential to inhibit *Salmonella* in poultry and use as an alternative of antibiotics.

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Authors contribution: MN, AAA and KA contributed to the conception of the research. SY collected the samples and conducted experiments. MN, MAA and ARB analyzed data and wrote the manuscript.

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