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Phytochemical Screening and Bactericidal Effect of Some Plant Oils of Family Apiaceae, Lamiaceae and Myrtaceae, against APEC, a Microbe of Zoonotic Importance

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Abstract

In the present investigation bactericidal activity of three different plant oils i.e fennel oil (Family – Apiaceae/Umbelliferae), thyme oil (Family – Lamiaceae) and tea tree oil (Family – Myrtaceae) has been evaluated against avian pathogenic *E.coli* (APEC) serotypes from different poultry farms situated in and around Nagpur region (MS), India. The avian pathogenic *Escherichia coli* (APEC) isolates recovered from diagnosed cases of avian colibacillosis were serotyped and examined for typical susceptibility to antimicrobials of human and veterinary significance. 146 out of 174 APEC isolates belonged to thirteen different serogroups. The rest were untypable. Antibiogram profiles of APEC using various narrow and broad spectrum antibiotics belonging to β-lactam antibacterials, quinolones, fluoroquinolones, polyketide antibiotics, aminoglycosides, rhodostreptomycins, polymyxins and cephalosporins revealed that the most resilient action was given by APEC for β-lactams, quinolones, fluoroquinolones and polyketide antibiotics, whereas sensitivity was exhibited by APEC towards aminoglycosides, rhodostreptomycins, polymyxins and cephalosporins. The phytochemical screening of oils using standard techniques revealed the presence of various bioactive compounds having clinical significance. The prominant bioactive agents found in fennel oil were Terpineol, α-Fenchone, Anethole. Terpinene-4-ol Thymol, α-Terpinene, Carvacrol and α-pinene were the major components of thyme oil that possess potent antibacterial property. The major compounds of tea tree oil were Limonene, γ – Terpinene, α- Terpinene, Cineol and α- Terpinolene. While evaluating the bactericidal action of these plant oils on APEC, it was noted that the bactericidal efficacy was in the order of thyme oil > tea tree oil > fennel oil.

Key words:
Tea tree oil, thyme oil, fennel oil, APEC, bactericidal activity.

Introduction:

Avian pathogenic *Escherichia coli* (APEC) strains cause pericarditis, perihepatitis, airsacculitis, polyserositis, septicaemia and other mainly extraintestinal diseases in chickens, and other avian species. Colisepticaemia in broilers, caused by *E.coli* is a very serious problem worldwide affecting chickens of all ages and is responsible for heavy

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economic losses to the poultry industry worldwide with an increasing evidence of its zoonotic importance. Typically, the main reason for condemnations at the poultry processing plant is septicaemia, which refers to the presence of bacteria in the bloodstream of a chicken. At slaughter, resistant strains from the gut readily soil poultry carcasses and as a result poultry meats are often contaminated with multiresistant *E. Coli* (Chaslus Dancla *et al.,*1985, Jayaratne, A. *et al.,* 1990, Turtura, *et al.,* 1990), likewise eggs become contaminated during laying (Lakhotiya, R.C. *et al.,* 1973). Thus the multiresistant faecal *E. coli* from poultry can infect humans both directly and via food. These resistant bacteria may colonize the human intestinal tract and may also contribute resistance genes to human endogenous flora (van den Bogaard, E., *et al.,* 2001). Many poultry diseases are transmissible to human, but of prime concern are the avian colibacillosis and avian salmonellosis. The spectrum of *E.coli* serovars that causes colisepticaemia is much broader. Currently serotypes O1, O2 and O78 are recognized as the most prevalent (**La Ragione, et al., 2002,),** however the number of published serotypes is increasing Antibiotics are widely used to treat clinical disease and to maintain healthy and productive animals (Freed *et al.,* 1993). Fluoroquinolones and other drugs have been approved to be used in veterinary medicine but ciprofloxacin, penicillin, and streptomycin are often used through feed. However, their excessive use in feed, and in treating infection has led to increased antibiotic resistance. Resistance to two or more classes of antimicrobials in *E. coli* is common nowadays in human (Dennesen *et al.,* 1998) and veterinary (Gonzalez and Blanco, 1989) medicine and has led to increased drug resistance to an alarming level. These resistant microorganisms may be responsible for transfer of antibiotic resistance to human pathogens.(Bebora *et al*, 1994) In addition to the human health concerns, multidrug resistant pathogens pose a severe and costly animal health problem leading to prolonged illness and decrease in productivity. These problems have highlighted the need for natural vegetal products for controlling drug resistance.

Studies have shown that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant activities (Stahl-Biskup & Saez, 2002). Tea tree oil is a natural product with strong antibacterial, antifungal, and anti-inflammatory activity. Fennel oil is known for its antispasmodic (Reynolds,1982), diuretic, anti-inflammatory, analgesic and antioxidant activities (Choi and Hwang,2004). Anand *et al* reported that fennel seed possesses anticancer activity. In view of this, a study was carried out to know the presence of various bioactive compounds of these oils and to study their potential as bactericidals.

Materials and methods:

Antimicrobial agents:

Plant oils-

The Essential oils of *Thymus vulgaris* (thyme oil), *Melaleuca alternifolia* (tea tree oil) and *Foeniculum vulgare* (fennel oil) were procured from a known standard company.

Antibiotics-

The antibiotics used were: Ampicillin (30µg), Nitrofurantoin (300µg), Nalidixic acid (30µg), Ofloxacin (5µg), Norfloxacin (10µ), Ciprofloxacin (30µg), Tetracycline (30µg), Oxytetracycline (30µg), Chloramphenicol (30µg), Amikacin (30µg), Streptomycin (10µg), Colistin (10µg), Gentamycin (10µg), Ceftazidime (30µg), Cephalexin (30µg).

Phytochemical Screening:

The phytochemical analysis of thyme oil was performed by using Gas chromatography and Mass spectroscopy studies using Shimadzu QP-2000 GC/MS instrument at 70eV (unless otherwise specified) equivalent to OV-1, fused silica capacity - 0.25 mm X 50 M with film thickness - 0.25 micron. The entry on the GC- MS trace such as 100-6-10-250 means that the initial temperature was 100˚C for 6 min and then heated at the rate of 10˚C per minute to 250˚C. Carrier gas (helium) flow: 2ml per minute. Identification of GC-MS spectra is based on the direct comparison of Kovates index and mass.

APEC isolates:

A total of 190 broiler chickens, 2 -6 weeks of age, were collected from different poultry farms in and around Nagpur. Using standard methods 174

bacterial isolates of APEC were collected, processed and identified based on their clinical findings observed during post mortem examination such as perihepatitis, enteritis, airsacculitis and pericarditis.

Serotyping:

E. coli isolates were serotyped at the National *Salmonella* and *Escherichia* centre, Kasauli, H.P, India.

Antimicrobial activity:

Antibiotic vulnerability pattern of APEC- Antibiotic vulnerability test was performed according to the standard procedures (Bauer *et al.,* 1966). Octadiscs of antibiotics (HIMEDIA, India) with varying potency were placed aseptically on seeded Mueller Hinton Agar plates (HIMEDIA).. Results were noted by measuring zone of growth inhibition in mm

Antimicrobial activity of the plant oils- The plant oils were tested against the entire APEC isolates (Kirby Bauer method). Results were noted by measuring zone of growth inhibition in mm using zone reader and average values of three replicates were calculated for each isolate and recorded.

Determination of MIC and MBC:

The microplate bioassay (micro-dilution broth assay) was used for the determination of minimum inhibitory concentration (MIC) using Mueller Hinton Broth. A stock solution of 5% (v/v) of the oils was prepared with Tween 80. A series of two-fold dilutions of each oil were carried out in 96-well microtitre plates over the range of 2.5% to 0.004882815%. The inocula (5µl) were then added to all the wells (except negative control) of plates, which were incubated at 37˚C for 24 h. To prepare bacterial inoculums one or two morphologically similar colonies were selected and aliquot was transferred to a test tube containing nutrient broth and incubated at 37˚C for 4hrs. The density of the suspension was standardized by McFarland 0.5 standard. . MIC was determined visually with the aid of a reading mirror. MIC was defined as the lowest concentration of oil inhibiting visible bacterial growth.

Aliquots of broth culture from MIC test plates were subcultured on the surface of solid nutrient agar plates by streaking. The plates were

incubated at 37°C for 24 hrs and MBC's were recorded after 24 hrs. Plates that did not show growth were considered to be the MBC for the oil used

Results and Discussion:

Phytochemical Screening:

Aroma chemicals and phenolic compounds present in fennel oil were Terpineol (32%), α-Fenchone (11.1%), Anethole (29%) (Table-1) which were also reported by. Shahat, *et al.,* (2011).

The phytochemical analysis of thyme oil showed the presence of 13 compounds by GC-MS. The main constituents present in *Thymus vulgaris* were Terpinene-4-ol (32.7%) followed by Thymol (18.1%), α-Terpinene (7.4%), Carvacrol (5.6%) α-pinene (3.5%), but the levels of other compounds were low. (Table-2). Similar secondary metabolites were also reported by Penalver *et al.,* 2004. The antimicrobial propertiesof thyme are due to its main components: Thymol, carvacrol (Kaloustain and Reynolds, 1984). Besides, there are several chemotypes for thyme, such as: Linalool, α-Terpineol, Thymol, Carvacrol-cymene, Terpinene-4-ol, and 1, 8-cineole, most of them are reported to show varying degree of antimicrobial activity (Carson and Riley, 1995). These findings are in concordance with the present study.

GC-MS profile of tea tree oil showed the presence of 4-terpineol (48.7%), followed by Terpinene (12.7%), γ-Terpinene (10.4%), Cineol (7.3%), p-cymene (4.1%), a-pinene (2.5%), α-Terpineol (2.0%), α-Terpinolene (1.0%), α- p-cyeme-8-ol (0.4%) (Table -3). The Compounds Limonene, γ-Terpinene, α-Terpinene, Cineol, α-Terpinolene, are responsible for antimicrobial and antifungal activities. α-terpinen-4-ol, linalool and α-terpineol are lipophilic monterpenes and the major active antimicrobial components of *Melaleuca alternifolia* (Carson and Riley, 1995; Kim *et al*, 1995; Raman *et al.,* 1995). *Melaleuca alternifolia* has the ability to kill a wide range of medically important micro-organisms, which was experimentally proved and confirmed with results of Shapiro *et al.,* 1994 and Carson *et al*, 1995.

The identification is based on the direct comparison of Kovates index and mass spectra. Variation in chemical composition of plant oils may be observed due to the origin, the environmental conditions, and the

developmental stage of the collected plant materials. Antimicrobial activity of plant oils is attributed mainly to its major components, although the synergestic or antagonistic effect of one compound in minor percentage of mixture has to be considered (Sara Burt, 2004). Therefore antimicrobial activity may vary based on the variations in the chemical compositions.

Serotyping:

Out of 174 avian pathogenic *E.coli* , 146 *E. coli* isolates from chickens with colibacillosis belonged to 13 different O serogroups: O106 (25%), O143 (15%), O153 (10%), O170 (10%), O36 (8%), O84 (8%), O55 (4%), O89 (4%), O32 (4%), O80 (3%), O39 (3%), O90 (3%), O3 (3%). However, 28 were untypable.

Antibiotic vulnerability pattern of APEC:

Antimicrobial reponse of these avian *Escherichia coli* serotypes was found to be variable (Table-4). Serotypes O106, O143 and O84 were resistant to ampicillin, Nitrofurantoin, Nalidixic acid, Ofloxacin, Norfloxacin, Ciprofloxacin, Tetracycline, Oxytetracycline. However, in addition to these antibiotics Serotype O84 and O55 showed resistance to Gentamycin and chloramphenicol also.

None of the antibiotics, except amikacin, used was cent percent effective. In this study, multiple antibiotic sensitivity and resistance pattern was observed in all of the examined strains similar to the findings of previous studies by Guerra *et al.,* and Saenz *et al., (2003).* Antibiogram profiles indicated maximum resistance to β-lactam antibacterials like ampicillin (100%), quinolones like nalidixic acid (100%), fluoroquinolones like ofloxacin (100%), norfloxacin (96%), ciprofloxacin (92%), polyketide antibiotics like tetracycline (88%), oxytetracycline (80%); nitrofurantoin (100%), and chloramphenicol (60%). These results are in concordance with those of R. Sharada et al., (2009). The most important finding of this study was the widespread resistance to quinolones and fluroquinolones *especially ciprofloxacin in these area farms.* This high presence of ciprofloxacin (a fluoroquinolone) resistance from broiler chickens is probably due to its overuse in treating colibacillosis. These findings are in concordance with the study of T. Zahraei Salehi et al., (2006). However, some of the isolates were

found to be highly sensitive to Amikacin (100%), Streptomycin (96%), Colistin (76%), Ceftazidime (76%), Cephalexin (76%) and Gentamycin (64%).

Bactericidal activity of the oils:

The bactericidal activity exhibited by the plant oils against APEC isolates showed the efficacy in the order of thyme oil > tea tree oil > fennel oil. (Table-5). The antimicrobial activity of EO is assigned to a number of small terpenoides and phenolic compounds present in it, which in pure form demonstrate high antibacterial activity (Conner, 1993) Essential oils rich in phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity (Baydar et al., 2004). The hydrophobicity of essential oils and their components enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Mitscher et al., 1987 and Knobloch et al., 1986) Extensive leakage from bacterial cells or the exit of critical molecules and ions will thus lead to death (Sikkema et al., 1994) The main advantage of natural agents is that they do not enhance the antibiotic resistance, a phenomenon commonly encountered with the long-term use of synthetic antibiotics (Nenad, *et al.,* 2007).

Conclusion:

The findings clearly demonstrates that these oils are rich in various bioactive agents some of which have a potential to be used as bactericides in commercial preparations and food industry. Hence additional *in vivo* studies and clinical trials would be needed to justify their efficacy.

Table 1: Identification of molecular mass of different compounds present in the Essential oil *Foeniculum vulgare* of using **GC-MS** analysis

Peak#	Scan No.	Compound	Retention Time (min)	% Area	Identification
1	820	Camphane	27.3	1.6	KI, MS
2	867	Cis-sabinene hydrate	28.86	2.3	KI MS
3	908	Terpineol	30.23	32.7	KI, MS
4	958	α-Fenchone	31.9	11.1	KI
5	1066	Anethole	35.5	9.3	KI, MS
6	1123	Methyl carvacrol	37.3	8.8	KI, MS
7	1167	Anethole	35.53	29.1	KI, MS
8	1330	Gvaiol	44.3	5.1	KI, MS
			Total	100%	

Table 2: Identification of molecular mass of different compounds present in the Essential oil of *Thymus vulgaris* using GC-MS analysis

Peak#	Scan No.	Compound	Retention Time (Min)	% Area	Identification
1	2	α-pinene	0.03	3.5	MS
2	10	p-cymene	0.3	1	MS
3	364	Unkhown	12.1	2.8	
4	372	Unknown	12.36	3.7	
5	385	Carvacrol methyl ether	12.8	9.7	MS
6	395	α-Terpinene	13.13	7.4	MS
7	1004	Terpinene-4-ol	33.43	32.7	MS
8	1049	Carvacrol	34.93	3.7	KI,MS
9	1136	Thymol	37.83	18.1	KI,MS
10	1162	Carvacrol	38.7	5.3	KI,MS
11	1168	Carvacrol	38.9	5.6	KI,MS
12	1208	Carvacrol	40.23	4.6	KI,MS
13	1237	Carvacrol	41.2	1.9	KI,MS
			Total	100%	

KI = Kovates index; MS =Camparison of Mass Spectra

Table 3: Identification of molecular mass of different compounds present in the Essential oil of *Melaleuca alternifolia* using **GC-MS** analysis

Peak#	Scan No.	Compound	Retention Time (Min)	% Area	Identification
1	430	Limonene	14.3	0.5	ISO 4730 (2004)
2	781	γ-Terpinene	20.01	10.4	ISO 4730 (2004)
3	826	Citral-A	27.5	2.4	KI
4	865	α-Terpinene	28.8	12.7	MS
5	894	Gamma Terpinene	29.76	2.9	MS
6	1033	4-terpineol	34.4	48.7	MS
7	1051	Cis-Sabinene hydrate	35.01	2.3	KI
8	1195	P-cyeme-8-ol	39.8	0.4	MS
9	1276	Aromadendrene	42.5	1.7	KI
10	1341	P-cymene	44.66	4.1	MS, KI
11	1376	a-pinene	45.83	2.5	MS,KI
12	1409	Sabinene	46.93	0.9	MS,KI
13	1448	α-Terpineol	48.23	2	MS
14	1464	Bromy acetate	48.76	0.4	KI
15	1608	Cineol	53.56	7.3	KI
16	1669	α-Terpinolene	55.6	1	MS
			Total	**100%**	

Table – 4: Antibiotic vulnerability profile of avian *Escherichia coli* isolates (n = 174)

Sr. No.	Antimicrobial agent	Disc potency(µg)	% of Resistant isolates	% of Sensitive isolates
1.	Amikacin	30	Nil	100
2.	Streptomycin	10	4	96
3.	Colistin	10	24	76
4.	Cephalexin	30	24	76
5.	Ceftazidime	30	24	76
6.	Gentamycin	10	36	64
7.	Chloramphenicol	30	60	40
8.	Oxytetracycline	30	80	20
9.	Tetracycline	30	88	12
10.	Ciprofloxacin	30	92	8
11.	Norfloxacin	10	96	4
12.	Nitrofurantoin	300	100	Nil
13.	Ofloxacin	05	100	Nil
14.	Nalidixic acid	30	100	Nil
15.	Ampicillin	30	100	Nil

Table 5: Antimicrobial activity of thyme, tea tree and fennel oil against Ciprofloxacin resistant APEC

Essential oil	Zone of Inhibition (ZOI, mm)	Minimum inhibitory concentration (MBC %)	Minimum bactericidal concentration (MBC %)
Fennel oil	14±1	0.75%	1.5%
Thyme oil	28±2	0.03%	0.15%
Tea tree oil	22±3	0.15%	0.3%

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