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The effect of thyme (*thymus vulgaris*) extract on *Escherichia coli* in diarrheic calves with study of its immunological effect

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ABSTRACT

The current study was designed to examine the antibacterial effect of thyme (*thymus vulgaris*) on *Escherichia coli* in diarrheic calves and also the effect of the thyme as immunostimulant. Collection of 100 rectal swabs from 100 diarrheic calves was done for isolation and identification of *E. coli* and serotyping of the bacterial isolates followed by their antibiotic sensitivity testing and determination of MIC and MBC of thyme oil on them. Supplementation of thyme extract to Friesian calves' milk was done and examined its effect on their immunity by antibodies titration of IgM, IgG and IgA in their serum. Results revealed that the incidence of *E. coli* was 58% and pathogenic *E. coli* was 46%. The most predominant serotype was O125. The highly resistance of the strains was against oxytetracycline (OT) and ampicillin (AMP). The thyme had antibacterial effect against *E. coli* strains by MIC ranged from 5 to 320 µg/ml, but the MBC was negative for all strains and the thyme extract at 40 mg/kg body weight improved the immunity as it significantly ($P \leq 0.05$) increased IgM, IgG and IgA titre in calves serum

1. INTRODUCTION

Diarrhea is one of the major mortality reasons in newborn calves, the incidence rate of diarrhea among calves at first month of age was ranged from 15 to 20%, this indicated that the great risk occurs during the first two weeks of calves life (Vandeputte et al., 2010). *E. coli* is one of the most important bacteria, causing diarrhea in newly-born calves during the first two weeks of life and characterized by watery faeces with rapid onset followed by high mortality (Yeshiwas and Fentahun, 2017).

The O-antigen is one of the molecules used in serotyping of *E. coli*. Up dated data described, more than 180 different O-serotypes but not even half of them have been structurally elucidated (Stenutz et al., 2006).

For several years' antibiotics were excessive used not only to control pathogens but also to enhancement the calves growth rate (Santos et al., 2015). The overuse of antibiotics in order to reduce these pathogens has led to the phenomenon of multi-drug resistant bacteria (Boskovic et al., 2013). Bacterial resistance increased worldwide and become one of the major causes of treatment failure of infectious diseases so herbal medicine using plant extracts as plant essential oils are interesting alternatives to antibiotic for the control of microbial infections (Lopez-Romero et al., 2015).

The thyme had numerous beneficial effects as antimicrobial, antioxidative, carminative, and antiseptic

properties (Baranauskienė et al., 2003). The Thyme oil is composed of many chemical compounds including thymol, caryophyllene, and pinene which are biologically active compounds used in treatment of various diseases so, the *Thymus vulgaris* can be used as a new potential source of medicinal plant extracts for the treatment of various types of illness due to presence of many bioactive compounds (Al-Asmari et al., 2017).

The essential oils enable to breakdown the lipids of the cell membrane of the bacteria and mitochondria because of their hydrophobicity characteristic and leading to cell structures disturbing and rendering them more permeable (Sikkema et al., 1994).

The essential oil from Thyme (*Thymus vulgaris* L.) has antibacterial activities against six Gram-positive and Gram-negative pathogenic bacteria: *Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus* spp, and *Pantoea* spp. The results showed that essential oil obtained from thyme has a range of MIC values from 0.33 to 2.67 mg/mL (Imelouane et al., 2009).

Also, the oregano water (OW) that has high amount of thymol (5.7 g/kg) had potential effect on level of immunoglobulin (IgA, IgG and IgM) in calves (Ozkaya et al., 2018).

Therefore, the aim of the work was to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the thyme on *E. coli* isolated from diarrheic calves and also to determine the effect of the thyme on the immune status.

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2. MATERIAL AND METHODS

2.1. Samples:

One hundred rectal swabs were collected from 100 cow diarrheic calves 0-3 months of age from El-Gemizah Animal Production Research Station farm at El-Gharbeia government using sterile swabs and inoculated in nutrient broth. All samples were collected under a septic condition and safety precautions. The clinically examined animals suffered from listlessness, abdominal pain, watery yellow diarrhea and normal temperature. Severely infected calves showed signs of severe enteritis like bloody diarrhea, stop of suckling, fever, dehydration and dry skin. Samples of fecal swabs were labeled and stored in ice box then transported rapidly to the laboratory and examined bacteriologically for isolation and identification of *Escherichia coli* (*E. coli*).

2.2. Bacteriological examination of the samples:

Swabs were inoculated separately into nutrient broth and incubated at 37 °C for 18 ± 2 h under aerobic condition as described previously by Quinn *et al.* (2002). A loopful from the broth of each sample was streaked onto nutrient agar, MacConkey's agar and Eosin Methylene Blue agar. The inoculated plates were incubated at 37°C for 24 hrs. A loopful of colony was stained with Gram's stain for microscopic examination.

2.2.1. Biochemical Identification of *E. coli*:

Biochemical identification of *E. coli* was done according to Koneman *et al.* (1997) and Quinn *et al.*, (2002) including Indole test, Methyl red test, Voges -Proskauer test, Citrate utilization test, Triple sugar iron, Sugar fermentation test, urease test, Oxidase test, Catalase test, Nitrate Reduction test, Eijkman test.

2.2.2. Detection of pathogenic and hemolytic *E. coli*:

2.2.2.1. Cultivation on congo red agar (CRA):

A loopful from each isolate was cultivated on the surface of the agar and incubated at 37°C for 24 hours. The reaction was recorded at 18, 24, 48 and 72 hours. Appearance of red colonies within 72 hours was recorded as a positive reaction (invasive bacteria), while Negative colonies (noninvasive bacteria) did not bind the dye and remained white even after 72 hours (Berkhoff and Vinal 1986).

2.2.2.2. Cultivation on Blood agar (MacFaddin, 1980):

A loopful from each isolate was cultivated on the surface of the blood agar and incubated at 37°C for 24 hours to determine the hemolytic *E. coli* strains (appears as translucent white or yellow colonies surrounded by halo zone)

2.2.2.3. Serological identification of *E. coli*:

The isolates of *E. coli* were sero-grouped using Sifin antisera (Berlin, Germany) by using slide agglutination test as describe by Edwards and Ewing (1972). Polyvalent and monovalent diagnostic *E. coli* antisera were used. According to somatic (O) they included (8) vials of polyvalent and (43) vials monovalent antisera.

2.3. Antibiotic sensitivity test by disk diffusion method.

The following antibiotic disks: gentamycin 10µg, ampicillin 10 µg, ciprofloxacin 5 µg, chloramphenicol 30 µg, erythromycin 15 µg, oxytetracycline 30 µg, amoxicillin

clavulanic acid 30 µg, cefotaxime 30 µg were used to examine the sensitivity of isolated *E. coli* strains according to Finegold and Martin (1982) and interpreted according to CLSI (2016) but erythromycin according to CLSI (2011).

2.4. Determination of MIC of thyme oil:

The oil was extracted from the thyme by hydrodistillation method of extraction as the dried aerial parts of the plant were submitted to hydrodistillation for 3 hrs using Cleverger type apparatus, according to the European Pharmacopoeia (1996). The essential oil was collected and stored at 4 °C until used for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of thyme oil against the *E. coli* strains according to CLSI (2009) by weighting 1.024 g of essential oil in sterile 15 ml falcon tube, next add 5% DMSO till reach 10 ml and mix well using a vortex, then transfer 2 ml from it to another 15 cc falcon tube contain 8 ml Muller Hinton Broth II then the prepared medium (Muller Hinton Broth II) were dispensed (100 µl/well) into sterile U bottom 96 well micro titer plates, by using multichannel pipette to dispense 100 µl of medium into all wells of micro-titer plate, labeled the plates and lid. Next 100 µl of a before mentioned prepared essential oil were dispensed into the wells of column one only (far left of the plate). Using multichannel pipette set at 100 µl, mix the essential oil and Muller Hinton Broth II into the well in column 1 by sucking up and down 8-10 times (don't splash) then withdraw 100 µl from column 1 and add them to column 2 this makes column 2 a twofold dilution of column 1, mix up and down 8-10 times then transfer 100 µl to column 3, repeat the procedure down to column 11 only and discard 100 µl from column 11 rather than putting in column 12(control positive), final essential oil concentration were ranged from 5-5120 µg/ml then bacterial inoculums was Prepared, suspensions equivalent to the no 3.0 McFarland turbidity standard were adjusted by visual examination using McFarland card and inoculation of approximately 4.5 x10⁵ CFU/ml were prepared with sterile 0.85 % NaCl .After preparation of bacterial inoculums, bacteria poured into sterile Petri dish. With multichannel pipette 100 µl of each of bacterial inoculums was dispensed to each well then cover by lid and incubation to the plates at 35° c for 16-18 h. The MIC before adding the resazurin indicator was defined as the lowest concentration of the antimicrobial agent that inhibited visible growth. A change indicates the reduction of and, therefore bacterial growth .The MIC after adding resazurin indicator was defined as the lowest essential oil concentration that prevented the color change of resazurin from blue to pink (Palomino *et al.* , 2002).Determination of MBC of essential oil on the susceptible isolates by the following method: From each susceptible isolate take 100 µl from last 3 wells that showing inhibition of bacteria, dispense those 100 µl into sterile Eppendorf tube containing 900 µl of normal physiological saline and mix well, next 100 µl from last tube were transferred to another sterile Eppendorf containing also 900 µl of normal physiological saline then take 100 µl from each Eppendorf and culture them into Petridis containing nutrient agar. So that each well becomes cultured on two dishes and so every susceptible isolate become cultured on six dishes. Incubation of dishes at 37 °C for 24 hr then they were examined for bacterial growth. MBC defined as the lowest concentration of the antimicrobial agent that prevents visible growth/kill bacteria.

2.5. Determination the effect of thyme extract on immune status of calves:

A total of 15 newly born male and female healthy calves were used in this study with average live body weight of 35.07 kg at birth. Animals were divided into three groups, five suckling calves in each. Calves in the 1st group were fed on two whole cow milk meals at 6.0 a.m. and 6.0 p.m. and the starter was given with good quality berseem hay (*Trifolium alexandrinum*) after the second week of age this group considered as a control group. Calves in 2nd group were fed on the same meal of control group and supplemented with thyme extract (TE) at a level of 20 mg/kg B. Wt. according to Vakili *et al.* (2013). Calves in the 3rd group were supplemented with the double dose (40 mg/kg BW of TE). Immunoglobulins (IgG, IgM and IgA) concentrations in blood serum samples were recorded at the start experimental age (3 days of age), 30, 60, 90 d of age and 105 d (weaning age) using the quantitative ELISA (Bovine IgG, IgM, and IgA ELISA Quantitative kit, Bethyl laboratories, UK) as described by Killingsworth and Savory (1972).

2.6. Statistical analysis

Statistical analysis was done by using SPSS analysis program (SPSS ver. 15, 2010). The data of the presented experimental work were analyzed. The significant differences which were detected were performed at ($P \leq 0.05$) using Duncan Multiple Range Test (Duncan, 1955).

3. RESULTS

Bacterial isolation and identification

The bacteriological examination of 100 fecal samples recovered that the number of *E. coli* isolates was 58 with an infection rate 58% divided into 46% pathogenic *E. coli* isolates and 12% nonpathogenic.

Sero-grouping of *E. coli* isolates.

By using monovalent antisera 3 identified serogroups of *E. coli* were O₁₂₅, O₁₅₈ and O_{86a} as five isolates were O₁₂₅ (50%), four isolates were O_{86a} (40%) and one isolate was O₁₅₈ (10%).

Antibiotic sensitivity test for *E. coli* isolates.

E. coli strains, were resistant to oxytetracycline (90%) followed by ampicillin (80%), chloramphenicol (60%) and cefotaxim (30%), while *E. coli* strains were 100% sensitive to gentamycin and ciprofloxacin followed by 40% to amoxicillin/clavulanic acid and cefotaxime as shown in table (1).

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of thyme oil against *E. coli*.

MIC and MBC of thyme oil against *E. coli* isolates shown in table (2). Thyme oil was found to be effective against *E. coli* O₁₅₈ by MIC 40 µg/ml, *E. coli* O_{86a} by MIC ranged from 5 to 80 to 320 µg/ml and *E. coli* O₁₂₅ by MIC ranged from 5 to 80 to 160 to 320 µg/ml. while found to be effective against reference strain of *E. coli* by MIC 320 µg/ml. The MBC was negative in all strains even in the reference strain.

The effect of thyme extract on the immune status of calves.

Table 1 Antibiotic sensitivity to *E. coli* strains:

NO of Sample	<i>E. coli</i> strains	Antibiotic disks								
		OT 30µg	CIP 5µg	CTX 30µg	AMP 10µg	AMC 30µg	CN 10µg	E 15µg	C 30µg	
1	O ₁₂₅	R	S	I	R	I	S	I	R	
2	O ₁₂₅	R	S	R	R	I	S	I	S	
3	O _{86a}	R	S	R	I	S	S	I	S	
4	O _{86a}	R	S	I	R	S	S	I	R	
5	O ₁₂₅	S	S	S	S	S	S	I	I	
6	O _{86a}	R	S	R	R	I	S	I	R	
7	O ₁₂₅	R	S	S	R	I	S	I	S	
8	O _{86a}	R	S	S	R	I	S	I	R	
9	O ₁₂₅	R	S	I	R	I	S	S	R	
10	O ₁₅₈	R	S	S	R	S	S	I	R	
Result	S	1	10	4	1	4	10	1	3	
	I	-	-	3	1	6	-	9	1	
	R	9	-	3	8	-	-	-	6	

OT: Oxytetracycline. CIP: Ciprofloxacin. CTX: Cefotaxime. AMP: Ampicillin AMC: Amoxicillin/Clavulanic acid. GN: Gentamycin. E: Erythromycin. C: Chloramphenicol. R: Resistant. I: Intermediate. S: Sensitive

The results presented in table (3) revealed that, blood immunoglobulins IgG, IgM and IgA levels of calves at the beginning of experimental work (pretreatment period) did not differ significantly ($P \leq 0.05$) among groups.

The values of IgG clearly increased significantly ($P \leq 0.05$) in G3 compared to G1 or G2 and no significant difference in G2 calves compared to those in G1, this trend reflect the significant effect of thyme extract at 40mg/kg body weight at all periods.

Calves in G3 showed the highly significant values of IgM levels in compared to control ones. Also, calves in G2 reached a significant ($P \leq 0.05$) increase of their IgM values as compared to those in G1 but the differences between G2 and G3 was insignificant except post 30 days of treatment stage, data showed G3 recorded the highest value (2.72 g/l) compared to G2 (2.04 g/l) or G1 (1.77 g/l).

Immunoglobulin type A levels was clearly higher in G3 followed by G2 and finally G1 and there was no significant difference between G1 and G2, while the values at 60 and 90 days post treatment, there were clearly significant ($P \leq 0.05$) increase in the serum IgA levels of calves in G3 compared to those in G1 but the G2 had the moderate values

Table 2 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of thyme oil against *E. coli* isolates:

NO of sample	<i>E. coli</i> strains	MIC (µg/ml)	MBC (µg/ml)
1	<i>E. coli</i> O ₁₅₈	40	Negative
2	<i>E. coli</i> O _{86a}	80	Negative
3	<i>E. coli</i> O _{86a}	80	Negative
4	<i>E. coli</i> O _{86a}	5	Negative
5	<i>E. coli</i> O _{86a}	320	Negative
6	<i>E. coli</i> O ₁₂₅	80	Negative
7	<i>E. coli</i> O ₁₂₅	160	Negative
8	<i>E. coli</i> O ₁₂₅	320	Negative
9	<i>E. coli</i> O ₁₂₅	5	Negative
10	<i>E. coli</i> O ₁₂₅	160	Negative
11	Reference strain	320	Negative

Table 3 Means and standard errors of immunoglobulin G (g/l) of growing calves at different experimental periods.

Period	Treatment group			Overall mean
	G1	G2	G3	
Pretreatment	25.13±0.23	25.18±0.31	25.33±0.19	25.21±0.13
Post 30d	20.83±0.77 ^b	21.13±1.16 ^b	23.93±0.26 ^a	21.96±0.60
Post 60d	16.83±0.97 ^b	17.68±0.69 ^b	20.58±0.46 ^a	18.36±0.62
Post 90d	17.80±1.01 ^b	19.13±0.38 ^b	22.60±0.46 ^a	19.84±0.71
Weaning	18.40±1.03 ^b	20.15±0.46 ^b	23.95±0.32 ^a	20.83±0.78

a and b: Means denoted with the same superscripts are not significantly ($P \leq 0.05$) different

Table 4 Means and standard errors of immunoglobulin M (g/l) of growing calves at different experimental periods.

Period	Treatment group			Overall mean
	G1	G2	G3	
Pretreatment	1.94±0.12	1.97±0.13	1.93±0.10	1.95±0.06
Post 30d	1.77±0.09 ^b	2.04±0.13 ^b	2.72±0.17 ^a	2.17±0.14
Post 60d	1.54±0.08 ^b	2.02±0.04 ^a	2.13±0.09 ^a	1.90±0.09
Post 90d	1.25±0.11 ^b	2.02±0.07 ^a	2.07±0.14 ^a	1.78±0.13
Weaning	1.18±0.08 ^b	1.80±0.05 ^a	1.94±0.11 ^a	1.64±0.11

a and b: Means denoted with the same superscripts are not significantly ($P \leq 0.05$) different.

Table 5 Means and standard errors of immunoglobulin A (g/l) of growing calves at different experimental periods.

Period	Treatment group			Overall mean
	G1	G2	G3	
Pretreatment	0.30±0.03	0.31±0.02	0.30±0.04	0.30±0.02
Post 30d	0.37±0.04 ^b	0.43±0.03 ^b	0.59±0.03 ^a	0.46±0.03
Post 60d	0.42±0.03 ^b	0.49±0.04 ^{ab}	0.60±0.04 ^a	0.50±0.03
Post 90d	0.57±0.03 ^b	0.62±0.03 ^{ab}	0.69±0.04 ^a	0.62±0.02
Weaning	0.66±0.03 ^b	0.71±0.03 ^b	0.83±0.03 ^a	0.73±0.03

a and b: Means denoted with the same superscripts are not significantly ($P \leq 0.05$) different

4. DISCUSSION

The clinically examined animals showed mild to severe diarrhea with or without dehydration and weakness signs. The diarrhea was watery in some cases and pasty in other cases and sometimes has foul odor.

Its color ranged from green to yellow to bloody with or without mucous. The infection rate of *Escherichia coli* (*E. coli*) in the present study was 58% which was agreed with results obtained by Abou el-ella *et al.* (2013) who isolated *E. coli* from calves of 0-4 days-old with an incidence of 57.1%. On the other hand, higher incidence 75.6% was mentioned by El-Seedy *et al.* (2016) and lower percentage 17.4% was detected by Izzo *et al.* (2011).

In the present study, the result of serotyping of *E. coli* strains recorded that five isolates were O₁₂₅ (50%) four isolates were O_{86a} (40%) and one isolate was O₁₅₈ (10%) the results come in accordance with Mosaad *et al.* (2008) agreed with Mosaad *et al.* (2008), who isolated *E. coli* O₁₂₅, O₈₆ from newly born diarrheic calves and Hakim *et al.* (2017), who identify *E. coli* O₁₂₅ (6.9%) and O₁₅₈ (5.2%) from diarrheic calves.

Sensitivity test results showed that *E. coli* isolates were 90% resistant to oxytetracycline, and this result was to certain extent near to the result obtained by Karczmarczyk, *et al.* (2011) who recorded resistance rate against tetracycline 99% But lower resistance 63.21% was detected by Srivani *et al.* (2017). The results showed that 7/10 of isolates (70%) were multidrug resistant to 3 or more antibiotics and this may be due to change in the microbial metabolism and their genetic structure (Blanco *et al.*, 1998) or due to the overuse of antibiotics (Boskovic *et al.*, 2013). Ciprofloxacin showed 100% sensitivity, and this agreed with Masud *et al.*, (2012), who recorded 100% sensitivity to Ciprofloxacin and

disagreed with Mashak (2018), who recorded 71.42% resistance.

In the present study, MIC and MBC of thyme oil against *E. coli* was determined. The results revealed that thyme oil was found to be effective against *E. coli* strains by MIC ranged from 5 to 320 µg/ml. The MBC was negative in all strains even in the reference strain and this result was similar to that detected by Irena *et al.*, (2009) who founded that thyme had the highest antibacterial activities against tested food borne bacteria strains *E. coli* and *S. Enteritidis* by (MIC 640 µg/cm). Santurio *et al.* (2014) found that the geometric means of the MICs was 627.7 µg.m/L and MBCs was 990.2 µg/mL against *E. coli* strains for the thyme essential oil and the MICs was 2786 µg.m/L and MBCs was 2540 µg/mL for the thymol. The antibacterial activity of thyme oil returned to its contents of thymol and carvacrol, as reported by Helander *et al.* (1998), who demonstrated that thymol and carvacrol have inhibitory effects on the growth of enteric bacteria *E. coli* O157:H7 and *Salmonella* Typhimurium as they have outer membrane disintegration activity and they increased the permeability to ATP through cytoplasmic membrane.

The current results showed that highly significant ($P < 0.05$) increase in the serum IgM, IgG and IgA values in calves treated by thyme extract in G3 compared to the control which were agreed with Ozkaya *et al.* (2018), who said that the oregano water (OW) showed a high amount of carvacrol (994.3 g/kg) and thymol (5.7 g/kg) (the same components of thyme) and examined the effect of supplementing milk replacer (MR) with aromatic oregano (*Origanum onite L.*) water (OW) on calves and the results showed that the immunoglobulins (IgA, IgG and IgM) values of calves were significantly higher than those of the calves in the Control group.

EOs have a therapeutic potential property that can use in treatment a variety of animal diseases, they act through stimulation of blood circulation, they reduce pathogenic bacterial counts and improve the immunity response by increasing the nutrient digestion and raising essential nutrients availability from intestine (Zeng *et al.*, 2015).

5. CONCLUSIONS

It was concluded that the thyme was effective on *E. coli* as bacteriostatic agent by MIC ranged from 5 to 320 µg/ml but the MBC was negative for all strains and also it improved the immune status of calves as the thyme extract at a level of 40 mg/kg body weight improved the immunity as it significantly ($P \leq 0.05$) increased their serum antibodies titre of IgG, IgA and IgM.

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