Molecular identification of *E.coli* O157:H7 and fungal causes of diarrhea in buffalo calves with evaluation of antibacterial and antifungal effect of some herbal extracts

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Abstract

The present study was undertaken to investigate the fungal and bacterial causes of buffalo calves, diarrhea and characterization of some isolates by DNA-PCR technique with a trial control by plant extracts and oils. Out of 60 cases of calves' diarrhea in private farm at Giza governorate, a total of 120 samples were collected (60 of each is fecal samples and the other are feed samples). They were subjected to mycological and bacteriological examination. The results revealed that the main species of isolated fungi were belonged to *Aspergillus sp.* namely *A.flavus* (40, 40%), *A.ochraceus* (40, 24 %) and *A.niger* (25, 36%), while; yeast of *Candida albicans* recovered from (15, 24%) of feed and feces samples, respectively. On the other hand, the main isolated species of bacteria from fecal swabs were *E.coli* (62.5%) includes *E.coli* OI57:H7 (8.3%). The most predominant fungal and bacterial strains (*A.flavus, C.albicans and E.coli)* isolated from samples were subjected for molecular characterization by PCR for accurate identification. The antibacterial and antifungal effects of oil and ethanol extract of clove, rosemary, cactus and nigella sativa on the most prevalent isolated strains were evaluated. The results suggested that the ethanol extracts of clove showed the broadest activity by inhibiting growth of all bacterial and fungal strains while the extracts of nigella sativa, cactus and rosemary inhibited the growth of strains at relatively lower levels. Whereas the rosemary oil showed the lowest activity against bacterial and fungal strains. It is concluded that these plants may provide better alternatives for the conventional antimicrobial additives in food.

Key words:

Bacteria, fungi, calve diarrhea, plant extracts, antibiotic resistance, DNA, PCR.

Introduction

The animal health in developing country represents the major role in food security for human consumption. However, diarrhea in neonatal calves is a syndrome of great an etiological complexity and is a common problem affecting animals in their first few weeks of life. Diarrhea occurs despite of partial immunity which might be gained to neonates from their dams. The disease causes loss of animal health resources and death may occur due to toxicity of causative agents **(Alhendi, 2000) and (Hassan *et al.,* 2011).** It is evidence that, the infectious agents capable of causing diarrhea in calves are

numerous; the most important bacterial enteropathogens are enterotoxigenic *E.coli* producing directly detectable toxin, salmonellae and *Y. enterocolitica* **(Milnes *et al.,* 2008)**. One of the most dangerous pathogens members of the Enterobacteriaceae is *Escherichia coli* a Gram negative bacterium which can cause serious food poisoning in human. *E. coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population. This process led to the spread of the gene encoding shiga toxin from Shigella to *E. coli* O157:H7, which carried by a bacteriophage **(Daferera *et al.,* 2003)**. It was found that calves were three time more likely to shed *E.coli* after weaning than before weaning **(Garber *et al.,* 1995)** and *E. coli* can causes gastroenteritis, urinary tract infections and neonatal meningitis. Whereas, in rare cases, virulent strains of *E.coli* causes hemolytic-uremic syndrome (HUS), peritonitis, mastitis, septicemia **(Todar, 2007).**

On the other hand, mycotic diarrhea were also detected during examination of fecal samples, nasal swabs and vaginal swabs of affected cattle and several members of genus *Aspergillus* and *Candida albicans* were recovered, **(Hassan *et al.,* 2011 and 2014).** Some fungi and its mycotoxins have been recorded to cause animal and human diseases and public health hazard **(Hassan *et al.,* 2012 and 2014).**

The large increase in the number and occurrence of antibiotic-resistant bacterial and fungal strains has prompted a renewed interest in the use of natural products which have gained a special attention in the recent years. Several plant species and herbs exert antibacterial and antifungal effects due to their essential active fractions. Some scientists revealed that there are antimicrobial and antifungal activity of essential oils: rosemary, clove, coriander, garlic, and onion against both bacteria and fungi. The composition, structure, as well as functional groups of the oils play an important role in determining their antimicrobial activity **(Omidbeygi *et al.,* 2007).** In Muslim countries Nigella sativa L. (Ranunculaceae) have been used extensively in folk medicine for treatment of different diseases, as Prophet Muhammad of Islam, stated that the black seed can heal all diseases except for death **(Bhatti and Rehman, 2009).**

Rosemary (*Rosmarinus officinalis* L.) is a spice and medicinal herb widely used around the world. Rosemary oil has an antibacterial and antifungal activity**. (Oluwatuyi *el al.,* 2004)**.

Clove (*Syzygium aromaticum*) have been used in foods since antiquity. Major antimicrobial components in clove reported to be eugenol, which have been given special attention to find their antibacterial activity against food borne pathogens. Eugenol has been reported to inhibit the growth of E. *coli* **(Mustafa, 2013)**.

Cactus (family Cactaceae) and cactus products have shown anti-inflammatory, immunomodulatory and anti-oxidative activities **(Huang *et al.,* 2009) and (Schepetkin *et al.,* 2008).**

Therefore, the aim of the present work was undertaken to investigate the prevalence of E.coli O157:H7 and some fungal causes of diarrhea with identification by traditional and molecular tools of isolates from fecal samples of diarrheic buffalo-calves and feed. The control of these pathogens by some herbal extracts was also evaluated.

Materials and Methods

1-Samples. From 60 cases of buffalo calves in private farm at Giza governorates, the animals suffered from symptoms of diarrhea, a total of 120 samples (60 of each of fecal swabs of diarrheic animal and feed samples) were aseptically collected by sterile swabs and clean sterile container. Each sample was divided into two parts one for bacteriological examination and the second part was subjected for mycological examination.

2- Bacteriological Examination of samples.

a- Isolation and identification of *E.coli.* (Koneman *et al.,* 1992) and (Quinn *et al.,* 2002): Samples were cultured onto macConkey agar medium for 24h at 37°C, then a part of single typical lactose fermenting colony was tested for sorbitol fermentation by culturing on

sorbitol macConkey agar and incubated at 37° C overnight. A single isolated colony from sorbitol macConkey agar was picked and grown in peptone water for 6 hours. Peptone water culture was used for biochemical tests.

b- Serotyping of isolated *E.coli* (Edward and Ewing, 1972): The *E.coli* somatic antigen (O157) and flagellar antigen (H7) antisera were used in serotyping which obtained from (Welcome Escherichia coli Diagnostic Antisera).

C- Evaluation the virulence of *E.coli* (O157):

1. Enterotoxin assay using mice (Robins-Brown *et al.*, 1993). Three Swiss mice of 1-4 day- old were injected with 0.1 ml of culture filtrate through the abdominal wall into the milk filled stomach (repeated for each serotype of *E.coli* (O157). Another group of 3 mice were injected with 0.1 ml of saline and kept as negative control group. After 4 hours, the mice were killed and the entire intestine was removed. The ratio of intestine weight to total body weight was calculated. A ratio equal or greater than 0.083 was recorded as positive test for enterotoxin.

2. Hemolysin detection (Beutin *et a1.*, 1989): *E.coli* O157:H7 isolates were inoculated into blood agar plates containing sheep blood 5% for the detection of B-hemolysin. After 3 hours of incubation at 37 °C, a positive B-hemolysin production was indicated by inner clear zone.

3. PCR assay (Paton and Paton, 1989): It was used for detection of shiga like toxin type 2(Stx2) in the extracted DNA of 5 E.coli O157:H7 isolates.

3- Mycological examination of samples:

a-Isolation and identification of fungi: The collected samples of fecal swabs and feed were prepared and examined for isolation and identification of fungi according to the technique recommended by **(Refai *et al.*, 2012) and (Refai and Hassan, 2013).**

b- DNA extraction and PCR amplification (Muñoz *et al.*, 2003): DNA extraction of the isolated strains of *C.albicans and A. flavus* was extracted using extraction kits QIA – amp DNA mini kit (Qiagen). Whereas, the genomic DNA were subjected to PCR amplification with oligonucleotide primers which specific for each strain.

The used forward primer for *C.albicans* was (′5 – CTGATTTATGGGTTCCTGAT- 3′) and the reverse primer was (′5 – GTTGATCAATT-GAAGTAGAATC - 3′). While, the used forward primer for *A. flavus was* (3' GTGGAGTGATTTGTCTGCTTAATTG 5') and the reverse primer was (3' TCTAAGGGCATCACAGACCTGTT 5'). The amplification was carried out in 20 ul of a mixture containing 50 ng of genomic DNA, 1.5 ul 10 x buffer, 2 mM MgCl2, 0.2 mM dNTPs, 0.5U DNA Taq polymerase (Roche Co., Germany) and 0.4 uM each primer. Amplification was initiated by incubation at 94℃ for 5 min followed by 35 cycles at 94℃ for 1 min, 62℃ for 1 min and 72℃ for 2 min at each temperature. Upon conclusion of thermal cycling, reaction mixtures held at 72℃ for 10 min.

4- Plant material: The used plant included clove, rosemary, nigella sativa and cactus which were collected locally. All the plant materials were further identified in the faculty of science, Department of Botany, Cairo University.

A- Preparation of plant oil and ethanolic extracts.

1- Cactus ethanolic extract (CEE): Three hundred grams of cactus plant were minced and homogenized. Ethanolic extraction was performed by soaking the homogenized cactus in 400 ml of 95% ethanol at room temperature overnight in well Stoppard glass bottle then filtered. Ether was evaporated at 50℃. the extract was collected and stored till used. **(Shixing *et al.*, 2011**).

2- Nigella Sativa ethanolic extract: One hundred grams of Nigella Sativa seed was ground and soaked in 200 ml of 95% diethyl ether at room temperature overnight in well Stoppard glass bottle then filtered . Ether was evaporated at 50℃ leaving a clear dark brown oily liquid , **(Hanafy and Hatem, 1991).**

3- Rosemary ethanolic extract: Green leaves were dried at temperature of 40℃ by drying oven. 500 g of dried leaves were grinded and

soaked in 200 ml 95% diethyl ether at room temperature overnight with shaking then filtered with filter paper no1. The extract was collected and stored at 4℃ till used**. (Abdullatif, 2009).**

4- Clove ethanolic extracts:

The dried clove was ground using a grinder. Twenty grams were soaked in 100 ml of 95% ethanol, and shaken at 150 rpm for 4 days at ambient temperature. The mixtures were then filtered. The filtrates were evaporated using vacuum rotary evaporator and stored till used **(Suree and Pana, 2005).**

5- Preparation of plant oil:

The small pieces of plant materials (300 g) were pressed in a commercial mill without any heat treatment and the pure oil was collected, dispensed into dark bottles, and stored at 4℃ until used. **(Hanafy and Hatem, 1991).**

6- Control antibacterial and antifungal:

A known antibacterial and antifungal drugs as Cefotaxime (30 µg/disc) and Nystatin (10 µg/ ml) were purchased from Sigma Chemical Company (USA) and used as a positive control toward bacteria and fungi, respectively.

B- Evaluation the antibacterial and antifungal potentials of prepared plants extracts and oil:

Different concentrations (10%, 20%, 30%, 50%, 70%, 100%) of oil and ethanolic extracts of plants were prepared using tween 80 **(Hanafy and Hatem, 1991)**.The antibacterial and antifungal activity of oil and ethanolic extracts of plant against *E.coli, C. albicans* and *Aspergillus* were evaluated by using agar well diffusion method **(Perez *et al.,* 1990)**. The media of tryptic soy agar for bacteria and sabouraud dextrose agar (SDA) for fungus were poured into plates containing one ml of cell suspension of bacteria and spores suspension of fungus (1.0 x 10^5 cells / ml), respectively, shaken over the table in rotary manner. After solidification, the agar plates were pored to wells of 5 mm diameter with the help of borer and filled with (100µl) of different concentrations (10%, 20%, 30%, 50%, 70%, 100%) of oil and ethanolic extracts of plants. The plates were incubated at 37°C for 24 hrs for bacteria and incubated at 25°C for 72 hrs for moulds. Antibacterial and antifungal activity was evaluated by measuring the zone of inhibition against the test organism.

Results and Discussion

Moulds and bacteria were recorded to constitute a public health hazard due to mycotoxin and bacterial toxins such as aflatoxin, ochratoxin, patulin, zearalenone and shiga toxins. These compounds cause some degree of acute toxicity when given in high amounts and are potential carcinogen. Also it appears that there is a direct correlation between dietary toxins intake and the incidence of liver cancer **(FDA, 2000) and (Bahtnager *et al.,* 2002)**. In addition, the outbreaks of food borne pathogens continue to draw public attention to food safety. So, there is a need to develop a new alternatives of plant origin has antimicrobial effects to ensure food safety.

In our study, the results revealed that the main species of isolated fungi from feed and fecal samples were belonging to Aspergillus *sp. namely A.flavus* (40, 40%), *A.ochraceus* (40, 24%) and *A.niger* (25, 36%), while, yeast of *C.albicans* recovered from (15, 24%) of feed and fecal samples, respectively (Table1).

These findings were confirmed with the result showed by **(Hassan *et al.,* 2014)** who investigated cases of diarrheic buffaloes and isolated species of bacteria including *E. coli,* Klebsiella and Enterococcus and mould as Aspergillus species and yeast species of *Candida albicans*. These bacteria and moulds affecting livestock can have devastating impact on animal productivity and death may occur due to enterotoxicity. However, **(Hassan *et al.,* 2008),** recovered many members of Aspergillus sp. and *C.albicans* from cases of diarrhea and its control by extracts of Rhamus catheritica leafs.

On the other hand, the current data in Table (2) indicated that the prevalence of sorbitol fermenting *E.coli* (S.F. E.coli) was recovered from 54.28% of all samples, where they isolated from 63.33% of fecal samples and from 45% of feed. While, the non sorbitol fermenting E.coli (N.S.F. E.coli) was 8.3% from the

total examined samples as (11.7 %, 5%) from fecal and feed samples respectively. In this concern, **(Confedera *et al.,* 1997)** recorded that in 3 studies conducted at slaughter; *E.coli* O157 was isolated in 3.6%. Also, **(Kang *et al.,* 2004)** examined 244 diarrheic calves, 24 cases (9.8%) positive for EHEC O157.

Also, it was evident from the results recorded in Table (3) that (7, 3) isolates recovered from fecal and feed samples respectively, were positive by slide agglutination test using O157 and H7 antisera. These results come in accordance with that of **(Roopnarine *et al.,* 2007)** who stated that 0.9% of the isolates of *E.coli* were positive for *E.coli* O157 antiserum. In addition, **(Dunn *et al.*, 2004)** verified that isolates positively reacted with anti- O157 and positively reacted with anti-H7, were considered to be *E.coli* O157:H7. Whereas, the results presented in Table (4) showed that 7 isolates were enterotoxigenic *E. coli* (ETEC) and caused accumulation of fluids in the intestinal tract of the injected infant mice. However, 5 *E.coli* O157:H7 isolates (71.4%) recovered from fecal samples. On the other hand, 2 *E.coli* O157:H7 isolates (66.7%) recovered from feed samples were enterotoxigenic. A ratio of the intestinal tract to the body weight at 0.083 or higher is considered positive heat stable toxin (sta). **(Sjoling *et al.,* 2006)** concluded that ETEC colonize the intestine by means of different host specific colonization factor (CFS) and produce one or both of two enterotoxins, the heat stable (ST) and heat labile (LT) toxins which are both able to cause diarrhea. Seven *E.coli* O157:H7 isolates that recovered from fecal samples were hemolytic, 2 *E.coli* O157:H7 isolates were found to be a-hemolysin positive (28.6%), while 5 *E.coli* O157:H7 isolates (71.4%) were found to be β-hemolysin positive, Table (5).

The animal diarrhea causes economic losses of animal wealth and death may occur due to enterotoxicity of causative agents **(Al hendi, 2000) and (Hassan *et al.,* 2011).** It is evidence that, the infectious agents capable of causing diarrhea in calves are numerous; the most important bacterial enteropathogens are enterotoxigenic *E.coli* (ETEC) produces detectable toxin **(Milnes *et al.*, 2008)**. Whereas, **(Hassan *et al.,* 2014)** investigated cases of diseased buffaloes and recovered species of bacteria including *E. coli, Klebsiella species and Enterococcus species* and mould species as *Aspergillus* species and yeast species of *Candida albicans.*

The DNA-PCR amplification for *E.coli* O157:H7 isolates as observed in photo (1) revealed positive amplification of 255 bp fragment of shiga toxin 2 (stx2) gene which was yielded from the extracted DNA of 4 *E.coli* O157:H7 isolates. While, no amplification could be observed with the extracted DNA of 1 *E.coli* O157:H7 isolate. While**, (Vernozy-Rozand, 1997)** recorded that of the three *E.coli* O157:H7 strains isolated from heifer fecal samples, only one strain was toxigenic, producing VT2 only. In addition, adherence of enterohemorrhagic *E.coli* (EHEC) to the intestinal epithelium is essential for initiation of infection.

On the other hand, the PCR of *C. albicans and A.flavus* that isolated from cases of diarrheic calves confirmed the identification in 90 - 100% of the isolates (Photo 2-3). The detection of fungal strain DNA by PCR was most effective in diagnosis than the traditional microscopically identification. Most of bands of DNA PCR of tested *A.flavus and C.albicans* observed that the density and size were variable depending on the virulence of isolates. The identification of *A.flavus* by PCR was similar to traditional identification (100%), whereas, 12 of 15 isolates of *C.albicans* could be detected by PCR and 3 isolates not detected at all and the accuracy of identification (80%) as traditional identification. Similar findings was reported by **(Bagyalakshmi *et al.,* 2007) and (Saleh, 2011)** who found that the accuracy of the PCR was 70.85% - 90%, where PCR proved to be a rapid diagnostic technique for detection of pan fungal genome directly from clinical specimens as the sensitive and specific nested PCR assay as well as the rapid and quantitative PCR assay might be useful for the diagnosis and monitoring of candidiasis.

The *E. coli* is resistant to many antibiotics and

this is due to overuse of antibiotics and the use of antibiotics as growth promoters in food of animals **(Huang *et al.*, 2009).** Antibiotic-resistant *E. coli* may also pass on the genes responsible for antibiotic resistance to other species of bacteria, such as *Staphylococcus aureus*. *E. coli* often carry multidrug resistant plasmids and under stress readily transfer those plasmids to other species. Indeed, *E. coli* is a frequent member of biofilms, where many species of bacteria exist in close proximity to each other. This mixing of species allows *E. coli* strains that are palliated to accept and transfer plasmids from and to other bacteria. Thus *E. coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance **(Bullerman *et al.*, 1977).** However, it was noticed recently that, an increase in the frequency of fungal infections is related with progress in mycology and decreased susceptibility of fungal strains to commonly used antifungal agents as reported by **(Nowakowska *et al.*, 2009)**. Moreover, the changes in treatment strategies and the increased use of antifungal prophylaxis **(Lass- Flörl, 2009)** and the lack of an effective fungicidal regimen as well as the development of antifungal resistant strains suggest that continued investigation is necessary to devise immunotherapeutic strategies and / or drug targets to combat fungal infection **(Warmly and Perfect, 2005).** Accordingly, alternatives to conventional antimicrobial therapy are needed due to the emergence of multi-drug resistance and also to overcome the cumulative side effects of the chemical commercial antifungal preparations as reported by **(Van Vuuren *et al.*, 2009).** Therefore, in the present work preparation and evaluation the antimicrobial potential of some plant extracts were monitored**.** The results indicated that oil and ethanolic extracts of nigella sativa, cactus, clove and rosemary showed different degrees of growth inhibition, depending on the tested bacterial and fungal strains. (Tables 7, 8, 9, 10). These results were agreed with that of **Md. *et al.*, (2008).** Also, the clove ethanolic extracts showed the broadest activity by inhibiting growth of all bacterial and fungal strains (the diameter of inhibition zone, range from (10-30 mm), while the extracts of nigella sativa, cactus and rosemary inhibited the growth of strains at lowest level. Rosemary oil showed the least activity against bacterial and fungal strains.

In general, the degree of antimicrobial properties of four plants tested against *E.coli* growth (Table 7) showed that clove ethanolic extract was at the top of all plant extracts followed by rosemary ethanolic extract, clove oil, nigella ether extract and cactus ethanolic extract, respectively. Also, our study showed that the first inhibition zone diameter of clove ethanolic extract was exhibited in the dilution concentration (20%) with inhibition zone diameter about (14 mm) while its maximum inhibition zone diameter about (30 mm) in the dilution concentration (100%). The widest zone of inhibition was exhibited by clove oil at dilution concentration of 100 % (28 mm). While for rosemary ethanolic was 24 mm at dilution concentration of 100% but rosemary oil not produce any effect. This result agreed with **(Mustafa, 2013)** who found that the degree of antimicrobial properties of clove > rosemary. The antimicrobial activity of clove is attributable to eugenol, oleic acids and lipids found in it **(Nzeako *et al.*, 2006).**

Regarding, the antifungal effects of plant extracts and oil against *Aspergillus flavus* (Table 8), the clove oil showed stronger antifungal effects than other extracts and oil . These findings were agreed with the result showed by **(Hassan *et al.*, 2008 and 2010)** and **(Yage *et al.*, 2012)** who revealed that clove oil possessed stronger antifungal activities against *A. flav-us* and *P. citrinum* and eugenol is the main constituent of clove oil. Also **(Viuda-Martos *et al.*, 2006**) found that the antifungal potential of essential oils clove was a stronger inhibitor against *A.niger* than against *A.flavus* .Whereas, Nigella sativa oil and ethanolic extract completely inhibited *Aspergillus flavus* at concentrations of 50%, 70% and 100 % with diameter of inhibition zone that ranged from (15-19 mm). The anti-fungal property of Nigella sativa is mainly related to their high

phenolic content **(Anwar *et al.,* 2007**). Also, **(Entela *et al.,* 2012)** illustrated the antifungal and antibacterial activities of ethanolic and methanolic extracts of volatiles oils of Nigella Sativa and concluded significant activities in comparison to tetracycline, cefuroxime and ciprofloxacin positive controls.

Considering *Aspergillus ochraceus,* it was inhibited by all extracts of four plants (Table 9). It is interesting to report that the antifungal effects of clove ethanolic extract was higher than all other extracts followed by cactus oil, cactus ethanolic extract, nigella sativa ethanolic extract, rosemary ethanolic extract, nigella sativa oil, clove oil and rosmary oil, respectively. Clove oil and rosemary oil showed higher antifungal effect against *C. albicans* (Table 10). Our results agreed with the findings of **Bullerman *et al.,* (1977)** who, found that clove inhibited growth of mycotoxigenic mould and subsequent inhibited its ability for toxin production.

Conclusion

It is concluded that the bacterial and fungal causes of diarrhea must be identified by traditional methods in parallel with the molecular characterization of pathogens for accurate diagnosis. The natural plants extracts and oils have antibacterial and antifungal potentials against the isolated pathogens of calves' diarrhea. These plants may be used as food additives in the processed food industry to protect meat products and other foods which easily contaminated by *bacterial and fungal strains* through fecal, oral route and during bad handlings. The ethanolic extracts and essential oils of plants can be recommended for therapeutic purpose and be used as alternative medicine.

Table (1). prevalence of fungi in feed and fecal samples collected from diarrheic buffalo calves.

Fungal species	Feed (60)		Fecal swabs (60)	
	NO.	%	NO.	%
Aspergillus sp.	31	52	24	40
A.flavus	24	40	24	40
A.ochraceus	24	40	14	24
A.niger	15	25	22	36
Mucor sp.	1	1.6	-	-
Scopulariopsis sp.	1	1.6	2	3.2
C.albicans	9	15	14	24

%: The percentage was calculated according to number of the examined cases NO: positive number

Table (2): Prevalence of sorbitol and non sorbital fermenting E.coli from fecal samples and feed samples.

Source of isolates	Number of examined samples	Number of E.coli isolates	%	E.coli isolates			
				S.F.		N.S.F.	
				NO.	%	NO.	%
Fecal samples	60	45	75	38	63.33	7	11.7
Feed samples	60	30	50	27	45	3	5
Total	120	75	62.5	65	54.28	10	8.3

S.F. = Sorbitol fermenting N.S.F= Non sorbitol fermenting.
NO. = Positive number
%: The percent was calculated according to the number of examined samples.

Table (3). Serological identification of E.coli O157:H7 isolated from buffalo-calves fecal samples and feed samples.

Source of Isolates	Number of E.coli isolates	S.F.								N.S.F.							
		O157 antisera				H7 antisera				O157 antisera				H7 antisera			
		+ve		-ve		+ve		-ve		+ve		-ve		+ve		-ve	
		NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
Fecal samples	45	0	0	38	84.4	0	0	38	84.4	7	15.5	0	0	7	15.5	0	0
Feed Samples	30	0	0	27	90	0	0	27	90	3	10	0	0	3	10	0	0
Total	75	0	0	65	86.7	0	0	65	86.7	10	13.3	0	0	10	13.3	0	0

S.F.= Sorbitol fermenting. N.S.F. = Non sorbitol fermenting.
+ve = Positive -ve = Negative
No.= Number
%: The percent was calculated according to number of E.coli isolates.

Table (4). Prevalence of enterotoxigenic E.coli O157:H7 isolated from fecal samples and feed samples.

Source of isolates	No. of E.coli O157: H7 isolates	+ve	%	-ve	%
Fecal samples	7	5	71.4	2	28.6
Feed samples	3	2	66.7	1	33.33
Total	10	7	70	3	30

No. = Number
+ve = Positve isolates
- ve= Negative isolates.
%: Percent was calculated according to number of E.coli O157:H7 isolates

Table (5). Detection of a and b hemolytic E.coli O157:H7 isolated from fecal samples and feed samples.

Source of isolates	No. of E.coli O157: H7 isolates	a	%	b	%	total +ve	%	-ve	%
Fecal samples	7	2	28.6	5	71.4	7	100	0	0
Feed samples	3	1	33.3	1	33.3	2	66.7	1	33
Total	10	3	30	6	60	9	90	1	10

a = α hemolysis β = β hemolysis +ve = Positive - ve = Negative

%: The percent was calculated according to number of examined E.coli O157: H7

Table (6). Characterization of E.coli O157:H7 isolates by PCR assay.

Number of examined isolates	Stx2* gene
1	-
2	+
3	+
4	+
5	+

* Shiga toxin type-2 gene.
- : gene absent

Photo (1). Shows agarose electrophoresis of PCR amplification of 255bp fragments of Shiga toxins (Stx2) gene from the extracted DNA of *E.coli* O157: H7 isolates lane shows 100 bp ladder marker.

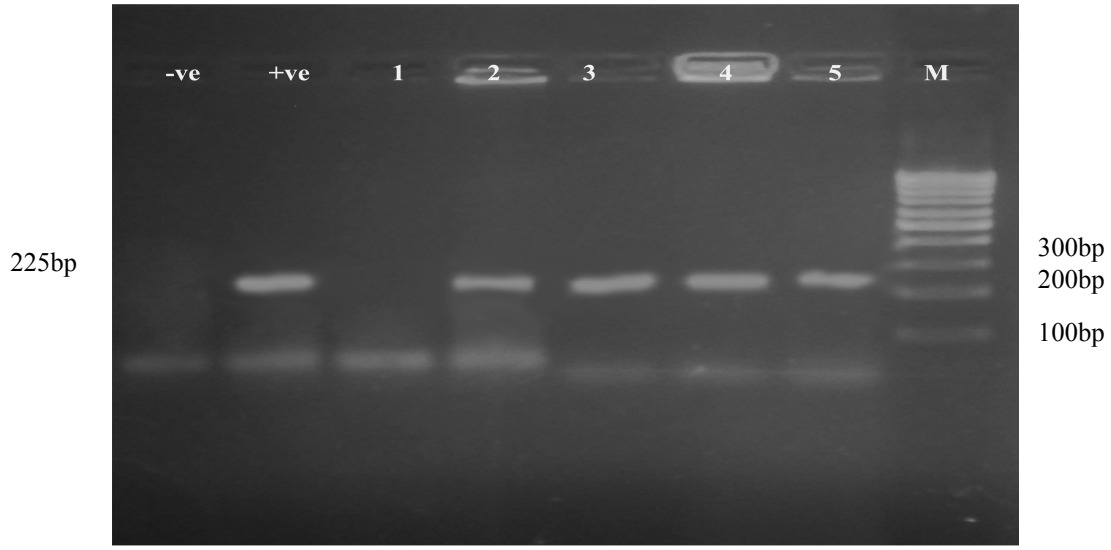
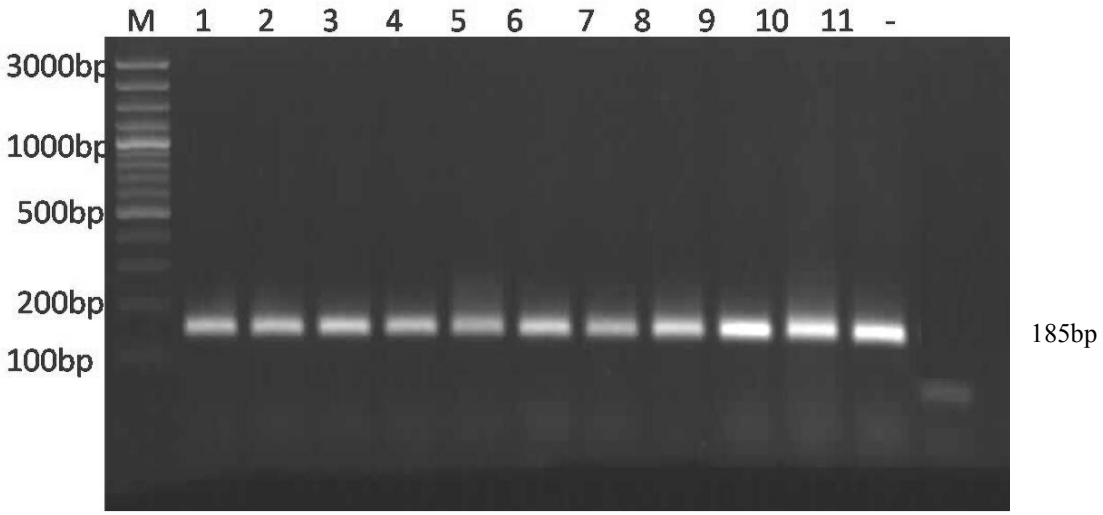
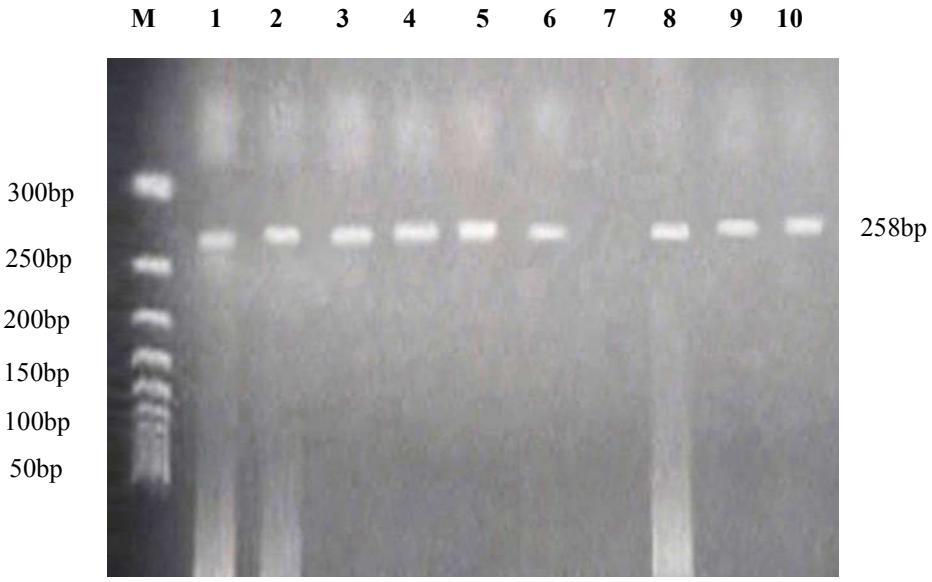


Photo (2). Detection of *A.flavus* -DNA by using PCR (DNA bands showed at 185 bp).



Lane M, molecular weight marker- Lanes 1 - 11 amplification of DNA from the isolates. Lane 12, negative control.

Photo (3). PCR for SAP gene of *C. albicans* (DNA bands showed at 258 bp).



Lane M , 100 bp molecular weight marker - Lanes 1 - 6 and 8 - 10, amplification of *C. albicans*-DNA from the isolates . Lane 7 negative control.
bp: base pairs. Lane (M): molecular size marker (in base pairs).

Table (7). Antibacterial activity of different dilution concentrations of plant extract and oil against E. coli O157:H7.

Plant	Diameter of Inhibition Zone Ø (mm)					
	conc. of plant oil and extract					
	10%	20%	30%	50%	70%	100%
Nigella S. Ethanolic extract	------	------	-----	-----	18	20
Nigella S. Oil	------	------	-----	-----	----	-----
Cactus ethanolic extract	------	------	------	------	13	18
Cactus oil	------	------	------	------	-----	-----
Clove ethanolic extract	------	14	17	21	28	30
Clove oil	------	-----	13	17	26	28
Rosemary ethanolic extract	------	-----	14	16	21	24
Rosemary oil	------	----	------	-----	-----	------

N.B. "------" no inhibitory zone, Ø diameter of inhibitory zone in mm
Cephotaxime: (30µg/disc): Control antibiotic drug (Zone of inhibition 21 mm).

Table (8). Antifungal activity of oil and ethanolic extract of plant against *Aspergillus flavus*.

Plant	Diameter of Inhibition Zone Ø (mm)					
	conc. of plant oil and extract					
	10%	20%	30%	50%	70%	100%
Nigella S. Ethanolic extract	------	------	------	16	17	19
Nigella S. Oil	------	------	------	15	16	18
Cactus ethanolic extract	------	------	------	12	14	16
Cactus oil	-----	------	------	-----	17	18
Clove ethanolic extract	-----	-----	18	20	23	30
Clove oil	-----	-----	19	25	30	35
Rosemary ethanolic extract	------	------	-----	----	12	13
Rosemary oil	------	------	------	----	11	12

N.B.- "------" no inhibitory zone, Ø diameter of inhibitory zone in mm
Nystatin: (10µg/ml): As positive control antimycotic drug (zone of inhibition 22 mm).

Table (9). Antifungal activity of oil and ethanolic extract of plant against *Aspergillus ochraceus.*

Plant	Diameter of Inhibition Zone Ø (mm)					
	conc. of plant oil and extract					
	10%	20%	30%	50%	70%	100%
Nigella S. Ethanolic extract	------	------	------	-----	15	20
Nigella S. Oil	------	------	------	----	13	18
Cactus ethanolic extract	------	------	------	17	20	22
Cactus oil	-----	------	------	12	16	23
Clove ethanolic extract	-----	-----	15	20	25	30
Clove oil	-----	-----	-----	-----	12	15
Rosemary ethanolic extract	------	------	-----	----	14	19
Rosemary oil	------	------	------	-----	10	15

N.B."------" no inhibitory zone, Ø diameter of inhibitory zone in mm
Nystatin: (10µg/ml): As positive control antimycotic drug (zone of inhibition 22 mm).

Table (10). Antifungal activity of oil and ethanolic extract of plant against *C.albicans.*

Plant	Diameter of Inhibition Zone Ø (mm)					
	conc. of plant oil and extract					
	10%	20%	30%	50%	70%	100%
Nigella S. Ethanolic extract	7	9	10	12	14	15
Nigella S. Oil	-----	7	8	10	13	18
Cactus ethanolic extract	------	------	------	11	12	15
Cactus oil	-----	------	9	10	12	13
Clove ethanolic extract	-----	-----	10	12	16	22
Clove oil	15	16	18	18	20	23
Rosemary ethanolic extract	7	9	10	12	14	18
Rosemary oil	9	11	13	15	16	19

N.B. "------" no inhibitory zone, Ø diameter of inhibitory zone in mm
Nystatin: (10µg/ml): As positive control antimycotic drug (zone of inhibition 22 mm).

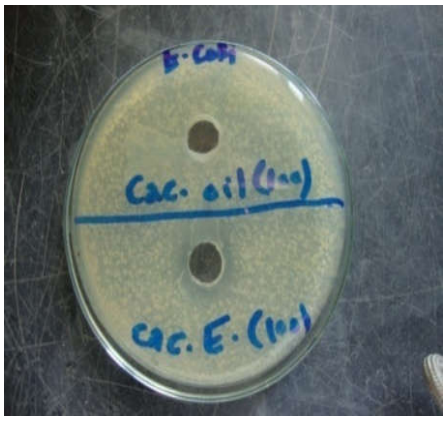


Figure 1. ethanolic extract of *cactus* (100%) showing zone of inhibition (18mm) while cactus oil not showing zone of inhibition against *E.coli* .

Figure2. rosemary, cactus and nigella sativa oil (100%) not showing zone of inhibition while ethanolic extract of cactus (100 %) showing zone of inhibition (18 mm) against E.coli.

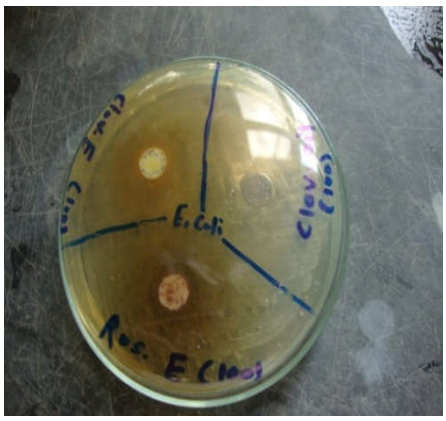


Figure3. ethanolic extract of *rosemary* (100%) showing zone of inhibition (24 mm) while oil and ethanolic extract of clove showing zone of inhibition (28,30 mm respectively) against *E.coli*.

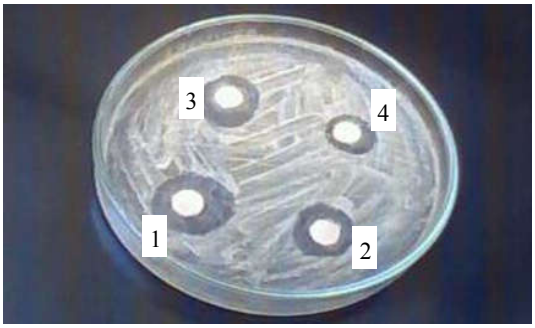


Figure 4: ethanolic extract of *cactus* (100%) (1) showing zone of inhibition (15 mm), while cactus oil: 100% (2), 70% (3), 50% (4) showing zone of inhibition13, 12, 10 mm respectively against *C.albicans.*

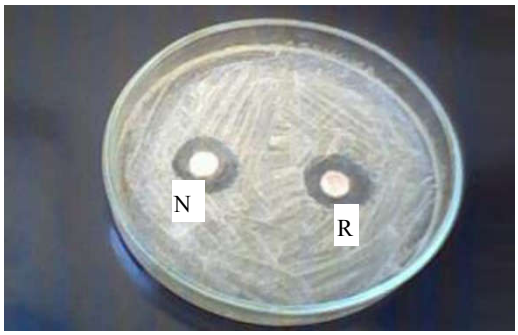


Figure 5: ethanolic extract of rosemary (R) and Nigella S. oil (N) (100%) showing zone of inhibition (18 mm) against *C.albic*ans.

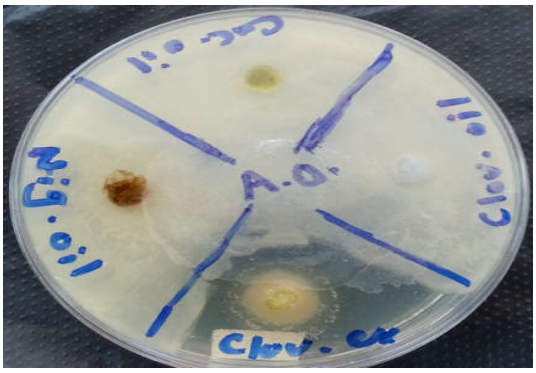


Figure 6. ethanolic extract of clove (100%) showing zone of inhibition (30 mm) while nigella sativa, cactus oil, clove oil (30 %) not showing zone of inhibition against *A.ochraceous.*

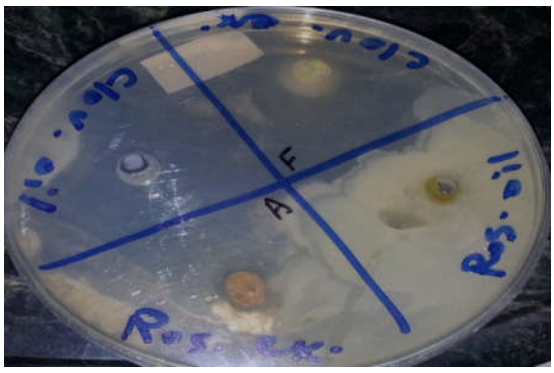


Figure7. clove oil and ethanolic extract (50%) showing zone of inhibition (25, 20 mm respectively) while rosmary oil and ether (50%) not showing zone of inhibition against *A.flavus.*

Reference

Abdullatif, M. (2009). Studying the Effect of Ethanolic Extract of Dried Leaves of Rosemary (*Rosmarinus officinalis*) on the Growth and Aflatoxin B1 production of *Aspergillus flavus*. J. OF Damascus University " Agriculture " 25, Issue 1, 121-134.

Alhendi, A.A.B. (2000). Preliminary observation on *E.coli* infection in neonatal calves in eastern province of Saudi Arabia. Pakist. Vet. J., 20 (1): 18-25.

Anwar Maraqa, Najwa F. Al-Sharo'a, Husni Farah And Wafa M. Elbjeirami (2007). Effect Of Nigella Sativa Extract And Oil On Aflatoxin Production By Aspergillus Flavus .Turk J Biol. 31, 155-159.

Bhatti, I.U., and F.U. Rehman, (2009). Effect of prophetic medicine kalonj (Nigella sativa L.) on lipid profile of human beings: an in vivo approach, World Appl. Sci. J. 6: 1053-1057.

Bahtnager, D.; Yu, J. and Ehrlich, K. (2002). Toxins of filamentous fungi. In: Breitenbach, M., Crameri, R., Lehrer, S. (Eds.). Fungal Allergy and Pathogenicity Chem Immunol., 81. Karger, Basel, pp. 167– 206.

Beutin, L.; Motenegro, M.A.; Orskov, I.; Orskov, F.; Prada, J.; Zimmermann, S. and Stephan, R. (1989). "Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of Escherichia coli". J. Clin. Microbiol., 27 (11): 2559-2564.

Bullerman, L.B.; f.Y. Lieu and sally a. Seier (1977). inhibition of growth and aflatoxin production by cinnamon and clove oils. Cinnamic aldehyde and eugenol. Journal of food science 42 (4), 1107–1109, July.

Confedera, G.; Marangon, S.; Chapman, P.A.; Zuin, A. and Caprioli, A. (1997). "A typical strains of verocytotoxin-producing Escherichia coli O157 in beef cattle at slaughter in Veneto region, Italy". J. Vet. Med. Series B, 44 (5): 301-306.

Daferera, D.J., B.N. Ziogas, M.G. Polissiou, (2003). The effect of plant essential oil on growth of Botryis cinerea, Fusarium sp and Clavibacter michiganesis ssp. Michigansis, Crop Prot., 22: 39-44.

Dunn, J.R.; Keen, J.E.; Del Vecchio, R.; Wittum, T.E. and Thompson, R.A. (2004). Escherichia coli O157:H7 in a cohort of weaned preconditioned range beef calves, J. food Prot. 67, 2391-2396.

Edward, P.R. and Ewing, W.H. (1972). "Identification of Enterobacteriaceae". Minneapolis, Burgess publishing Co. PP. 209. Burgess publishing Co., Atlanta USA 3rd Ed.

Entela Haloci, Stefano Manfredini, Vilma Toska, Silvia Vertuani, Paola Ziosi, Irma Topi and Henri Kolani (2012). Antibacterial and Antifungal Activity Assesment of Nigella Sativa Essential Oils . World Academy of Science, Engineering and Technology 66 ,2012 stonger activity than methanolic one.

Food and Drug Administration (FDA) (2000). Conference on mycotoxins in animal feeds, grains and food related to human and animal health. Rockville, Maryland.

Garber, L.P.; Wells, S.J.; Hancock, D.D.; Doyle, M.P.; Tuttle, J.; Shere, J.A. and Zhao, T. (1995). Risk factors for faecal shedding of *E.coli* O157: H7 in dairy calves. J.A.V.M.A., 207: 46-49.

Hanafy, M.S. and Hatem, M.E. (1991). Studies on the antimicrobial activity of Nigella Sativa seeds. J. Ethnopharmacol., 34, 275-278.

Hassan, A.A.; El-Barawy, A.M. and El-Mokhtar, M., Nahed (2011). Evaluation of biological compound of streptomyces species for control of some fungal diseases. J. of American Science, 7 (4), 752-760.

Hassan, A.A.; El Shorbagy, M.M. and El-Barawy, A.M. and Manal, A. Hassan. (2008). Study the availability of using buckthorn(*Rhamnus cathartica*) plant extract in laboratory control of some bacterial and fungal diseases. The 5 th Scientific Congress, Minufiya Vet. J.Vol.5 (1):27-39.

Hassan, A.A.; Howayda, M. El Shafei; Rania, M. Azab. (2010). Influence of solar simulator, gamma irradiation and laser rayes

on the growth and aflatoxin production of A. flavus and A. parasiticus. 4th Sci. Congr. Of Egypt. Soc. For Anim. Manag. 25-28 Oct., 2010:1-17

Hassan, A.A.; Manal, A. Hassan; Rasha, M.H. Sayed El-ahl and A.S. Darwish (2012). Prevalence of yeast infections in small ruminants with particular references to their treatment by some natural herbal extracts. Bulletin of Environment, Pharmacology and Life Sciences. Volume 1, Issue 3, February 2012: 12-22.

Hassan, A.A.; M.A. Rashid; Noha H. Oraby; S. El-Araby Kammal and M.M. Minshawy (2013). Using of molecular biology techniques for detection of *Cryptococcus Neoformans* in respiratory disorders in cow with references to its control by nanoparticles of iron oxide (Fe_2O_3). Egypt. J. of Appl. Sci., 28 (12) 2013, 433-448.

Hassan, A.A.; Noha, H. Oraby; Aliaa. A.E. Mohamed and Mahmoud H.H. (2014). The possibility of using Zinc Oxide nanoparticles in controlling some fungal and bacterial strains isolated from buffaloes. Egypt . J. of Appl. Sci., 29 (3) 2014 ,58-83.

Huang X., Li Q., Li H., Guo L. (2009). Neuroprotective and antioxidative effect of cactus polysaccharides in vivo and in vitro. Cell Mol. Neurobiol., 29(8): 1211-1221.

Kang, SJ.; Ryu, SJ.; Chae, JS.; Eosk; Woo, GJ. and Lee, JH. (2004). "Occurrence and characteristics of enterohemorrhagic Escherichia Coli O157 in calves associated with diarrhea". Vet. Microbiol., 98 (3-4): 323-328.

Koneman, E.W.; Schreckenberger, P.C.; Allen, S.D.; William, M.J. and Washington, C.W. (1992). "Color Atlas and Textbook of Diagnostic Microbiology". 4th Ed. J. B. Lippincott Company Pheladelphia.

Lass-Flörl, C. (2009). The changing face of epidemiology of invasive fungal disease in Europe**.** Mycoses., 52(3):197-205.

Md. Mahfuzul hoque, M.L. Bari, vijay k. Juneja, and S. Kawamoto (2008). Antimicrobial activity of clove and cinnamon extract Against food born pathogensm and bacteria with their essential oils. .Rep. Nat, l. Food Res. Inst. no. 72, 9-21.

Milnes, A.S.; Stewart, I.; Clifton-Hadley and F.A.; Davies (2008). Intestinal carriage of verocytotoxigenic *Escherichia coli* O157, *Salmonella*, thermophilic Campylobacter and *Yersiniaenterocolitica,* in cattle, sheep and pigs at slaughter in Great Britain during 2003, Epidemiology Infect 136: 739-751.

Muñoz, C.B.; Boldo, X.M.; Tanaca, L.V. and Rodriguez, C.H. (2003). Identification of *Candida* spp. by randomly amplified polymorphic DNA analysis and differentiation between *C. albicans* and *C. dubliniensis* by direct PCR methods. J. Clin. Microbiol., 41 (1): 414-420.

Mustafa, H.R. (2013). Antimicrobial activity of plant essential oils against the growth of Escherichia coli . IOSR Journal Of Pharmacy (e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219.

Nowakowska, D.; Gaj, Z.; Sobala, W. and Wilczyński, J. (2009). Evaluation of susceptibility to antifungal agents of fungal strains isolated from pregnant women with diabetes and healthy pregnant women. Ginekol Pol., 80 (4): 274-279.

Nzeako, B.C.; **Zahra S.N. Al-Kharousi and Zahra Al-Mahrooqui (2006).** Antimicrobial Activities of Clove and Thyme Extracts. Sultan Qaboos Univ. Med J. June; 6(1): 33–39.

Oluwatuyi, M.; Kaatz, G.W. and Gibbons, S (2004). Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochemistry, London/Detroit, 65 (24): 3249-3254.

Omidbeygi M., Barzegar M., Hamidi Z. and Naghdibadi H., (2007). Antifungal activity of thyme, summer savory and clove essential oils against Aspergillus flavus in liquid medium and tomato paste. Food Control, 18, 1518–1523

Paton, A.W. and Paton, J.C. (1998). "Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for stx1, rfb 0111 and rfb O157". J. Clin. Microbiol., 36 (2): 598-602.

Perez, C.; Pauli, M. and Bazergue, P. (1990). An antibacterial assay by agar well diffusion method. Acta Bio. Et. Med. Exp. 15, 113-

115.

Quinn, P.J.; Carter, M.E.; Markey, B.K. and Carter, M.E.; Donelly, W.J.C. and Leonard, F.C. (2002). "Clinical Veterinary Microbiology and Microbial disease". 1st Lowa State University Press Blackwell Microbiology". Mosby Year Europe Limited Lynton House, London, 109-126.

Refai, M.K.; Heidy Abo El Yazied and M. El Hariri (2012). Monograph of yeast (Updated). http://Cairo academic edu./ Egypt, Mohamed Refai/ Monograph.

Refai, M.K. and Hassan, A.A. (2013). Monograph On Mycotoxigenic Fungi and Mycotoxins in food and feeds with synopsis of the authours done on Mycotoxigenic Fungi and Mycotoxins in Foods and Feeds.http:// Cairo academic edu./ Egypt, Mohamed Refai/ Monograph.

Robins-Brown, R.M.; Takeda, R.M. and Fasano, T. (1993). "Assessment of enterotoxin production by Yersinia enterocolitica and identification of a novel heat stable enterotoxin produced by a non invasive Yersinia enterocolitica strain isolated from clinical material". Infect. Immun., 61(2): 764 -767.

Roopnarine, R.R.; Ammons, D.; Rampersad, J. and Adesiyun, A.A. (2007). "Occurrence and characterization of verocytotoxigenic Escherichia coli (VTEC) strains from dairy farms in Trinidad". Zoonoses public Health., 54 (2): 78-85.

Saleh, A. Hala (2011). Genotypic identification and characterization of yeasts with particular references to recent approaches for their control. PhD thesis of Veterinary Sciences in (Bacteriology, Immunology & Mycology), Faculty of Vet. Med., Cairo Un.

Schepetkin IA, Xie G, Kirpotina LN, Klein RA, Jutila MA, Quinn MT (2008). Macrophage immunomodulatory activity of polysaccharidesisolated from *Opuntia polyacantha*. Int. Immunopharmacol., 8(10): 1455-1466.

Sjoling, A.; Oadri, F.; Nicklasson, M.; Begum, Y.A.; Wiklund, G. and Svennerholm. A.M. (2006). "In vivo expression of the heat stable (est A) and heat labile (elt B) toxin genes of enterotoxigenic Escherichia coli (ETEC)". Microbes Infect., 8 (12-13): 2797-2802.

Shixing C., Ming-Zhong S.1, Bo Wang, Lihong H., Cuili Z. and Yi Xin (2011). Wound healing effects of cactus extracts on second degree superficial burned mice. Journal of Medicinal Plants Research Vol. 5(6), pp. 973-978, 18 March.

Suree, N. and Pana, L. (2005). Antibacterial activity of crude ethanolic extracts and essential oils of spices against salmonellae and other enterobacteria . KMITL Sci. Tech. J. , 5 (3) Jul. – Dec.

Todar, K., (2007). Pathogenic *E. coli*. Online Textb ook of Bacter iology. Unive rsity of Wisconsin-Madison Department of Bacterio l o g y .http://www.textbookofbacteriology.net/e.coli.html. Retrieved on 2007-11-30.

Van Vuuren, S.F.; Suliman, S. and Viljoen, A.M. (2009). The antimicrobial activity of four commercial essential oils in combination with conventional antimicrobials. Lett Appl Microbiol., 48 (4): 440-6. Epub 2009 Feb 2.

Vernozy- Rozand C. (1997). Detection of Escherchia coli O157:H7 and other verocytotoxin-producing E. coli (VTEC) in food. J. Appl. Microbiol., 82, 537 – 551.

Viuda-Martos, M., Y. Ruiz-Navajas, J. Fernández-López1 And J.A. Pérez-Álvarez (2006). Antifungal Activities Of Thyme, Clove And Oregano essential oils . Journal of Food Safety 27, 91–101.

Wormley, F.L.J.r. and Perfect, J.R. (2005). Immunology of infection caused by *Cryptococcus neoformans* . Methods Mol Med., 118: 193-8.

Yage X., Qingliian X., Xihong L., Zhenmin C., and Juan Y. (2012). Antifungal activities of clove oil against rhizopus nigricans, aspergillus flavus and penicillium citrinum in vitro and in wounded fruit test. Journal of Food Safety 32 (1) :84–93.

التوصيف الجزيئي لعتره الايشريشيا كولاي (اتش ٧ :او ١٥٧ **) و لبعض الفطريات المسببه للإسهال في عجول الجاموس مع تقييم الأثر المضاد للبكتيريا و الفطريات لبعض مستخلصات الأعشاب.**

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الملخص العربي

استهدفت هذه الدراسه ايضاح المسببات البكتيريه و الفطريه للاسهال بعجول الجاموس و توصيف بعض العترات بإستخدام الحامض النووي و تفاعل البلمره المتسلسل مع الإشارة الي التحكم في نموها بإستخدام مستخلصات الاعشاب و زيوتها.

فمن خلال ٦٠ حالة اسهال بالعجول في مزرعة بمحافظة الجيزة، تم تجميع ١٢٠ عينة (٦٠ عينة من كل من العلائق و البراز) و قد خضعت العينات للفحص البكتيري و الفطري و قد أفادت النتائج أن الفصائل الاساسيه للفطريات المعزوله كانت ضمن نوع الاسبرجيللس شامله الاسبرجيللس فلافس (٤٠% ،٤٠%) و الاسبرجيللس اوكراشيس (٤٠%، ٢٤%) و الاسبرجيللس نيجر (٢٤%، ٣٦%) ، بينما عزل عتره الكانديدا البيكانس بنسبه (١٦%،٢٤%) من عينات العلائق و البراز علي التوالي. و من ناحيه اخرى العتره البكتيريه الرئيسيه التي تم عزلها من عينات البراز كانت الايشيرشيا كولاى بنسبه (٦٢.٥%) و عتره الايشيرشيا كولاى(اتش ٧ :او ١٥٧) بنسبه (٨.٣%) . اهم العترات الفطريه و البكتيريه المعزوله من العينات كانت الاسبرجيللس فلافس و الكانديدا البيكانس و الايشيرشيا كولاى خضعت للتصنيف الجزيئى باستخدام تفاعل البلمره المتسلسل .كما تم تقييم تأثير الزيوت و مستخلص نباتات القرنفل و الروز مارى و الصبار و حبه البركه كمضاد بكتيرى و مضاد فطرى.كما ان النتائج اثبتت ان المستخلص الايثانولى للقرنفل له التأثير الاعلى لتثبيط نمو معظم عترات الفطريات و البكتيريا المعزوله بينما مستخلص حبه البركه و الصبار و الروزمارى كان تأثيرهم المثبط علي نمو الفطريات أقل مستوى وكذلك كان زيت الروز مارى الاقل تأثير علي نمو عترات الفطريات و البكتيريا و من ذلك كله نستنتج أن هذه النباتات تعطى أفضل بدائل للمضادات الميكروبيه التقليديه المضافه للعلائق.