JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

*Original Article*

# Effect of combination therapy between thyme oil and ciprofloxacin on ulcer-forming *Shigella flexneri*

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## Abstract

Introduction: *Shigella flexneri* is a Gram-negative bacteria that has the ability to invade the epithelium of the colon and cause colon ulcers.
Methodology: The ability of isolated *Shigella flexneri* from bloody diarrhea to cause colon ulcers was investigated by histopathological examination via oral administration of the bacteria to adult male albino Sprague-Dawley rats. The antibacterial activity of thyme oil, ciprofloxacin, and their combination were evaluated *in vitro* and *in vivo*.
Results: Oral administration of $12 \times 10^8$ CFU/mL of *S. flexneri* was able to cause colon ulcers. Thyme oil had the highest antibacterial activity among other investigated oils (minimum inhibitory concentration [MIC] 150 µL/L). Ciprofloxacin had the highest antimicrobial activity against *S. flexneri* (MIC 0.4 mg/L). The synergism between thyme oil and ciprofloxacin showed the maximum growth inhibition of *S. flexneri*. The synergistic activity of thyme oil and ciprofloxacin succeeded in healing the epithelial surface of the colon and decreased the inflammation of the lamina propria; it also decreased the bacterial load in the infected colon, while the commercial drug failed to heal the colon ulcer. Thyme oil, ciprofloxacin, and their combination showed different degrees of effects on the bacterial cell structure by transmission and scanning electron microscopes.
Conclusions: The combination of thyme oil and ciprofloxacin gave synergistic activity, which proved to be more effective in inhibiting the growth of ulcer-forming *S. flexneri*, healing the colon ulcer, and decreasing infiltration of the lamina propria with inflammatory cells.

**Key words:** colon ulcer; *Shigella flexneri*; thyme; ciprofloxacin.

*J Infect Dev Ctries* 2015; 9(5):486-495. doi:10.3855/jidc.6302

(Received 23 November 2014 – Accepted 14 January 2015)



## Introduction

The digestive tract is lined by a mucus membrane; a break in the mucus membrane lining results in the formation of an ulcer. Ulcers can affect the digestive tract from the mouth to the anus. Symptoms depend on the location of the ulcers. Pain is a usual feature. In cases of deeper ulcers, bleeding may be present. Ulcers can occur due to various causes, which can include infection such as bacteria resulting in stomach ulcers [1], inflammatory causes such as Crohn's disease and ulcerative colitis, or ingestion of spicy food, alcohol, or medications. In some cases, ulcers may be due to a malignancy.

Members of the genus *Shigella* are Gram-negative facultative anaerobes and are divided into four species: *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, and *Shigella dysenteriae*. These species are further divided into serotypes based on variations in their O-antigen. *Shigella* produce both an endotoxin and an exotoxin. The exotoxin is an enterotoxin, like the *E. coli* heat labile toxin and the cholera toxin, that produces diarrhea. It can also cause an ulceration of the intestine. Upon invasion of a cell, *Shigellae* produce and excrete another major virulence factor, nicotinamide adenine dinucleotide glycohydrolase (NAD glycohydrolase), which destroys all of the NAD of the human cell and virtually shuts down cellular metabolism, causing cell death [2].

There have been reports that essential oils (EOs) and antibiotics together are more strongly antimicrobial than their major components individually [3], which indicates that the minor components play an important role and may have a synergistic effect. The synergistic effect of the major components of EO and antibiotic has been reported in several studies [4].

Treating bacterial infections by antibiotics is beneficial, but their indiscriminate use has led to an alarming resistance among microorganisms and to the re-emergence of old infectious diseases. Alternative approaches to treatment of infectious diseases are being investigated; examples include the use of plant

extracts individually and/or in combination with antibiotics. This latter approach (*i.e.*, combination therapy or synergistic therapy) may lead to new ways of treating infectious diseases and could be useful for patients with serious infections caused by drug-resistant pathogens [5]. therefore warrants further investigation. A possible antibiotic for combination therapy is ciprofloxacin, which is a broad-spectrum fluoroquinolone and possesses good activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus*. It is active *in vitro* against *Citrobacter* spp., *Serratia* spp., *Klebsiella* spp., *Salmonella* spp., and *Shigella* spp. [6,7].

Natural products are a source of synthetic and traditional herbal medicine and are still used in the primary healthcare system. Antimicrobials of plant origins have enormous therapeutic potential [8]. EOs are natural products extracted from vegetal materials, which are characterized with their antibacterial, antifungal, antioxidant, and anticarcinogenic properties. Most of the antimicrobial activity in essential oils is from spices and culinary herbs; cinnamon, thyme, clove, black seed, and oregano appear to be associated with phenolic compounds.

Thyme contains a variety of flavonoids, including apigenin, naringenin, luteolin, and thymonin. These flavonoids increase thyme's antioxidant capacity [9]. Also, it plays a role in eliminating free radicals from the body and even protecting people from certain lung and oral cavity cancers. Thyme oil is used as a germ killer in mouthwashes and liniments. It is also applied to the scalp to treat baldness and to the ears to fight bacterial and fungal infections [10].

This study focused on the effect of combination therapy between traditional treatment (antibiotic) and an alternative natural product (essential oil) to treat bacterial ulcers. The synergistic activity of thyme oil and ciprofloxacin on inhibiting growth of ulcer-forming *Shigella* sp. and healing of ulcer cellular damage via antimicrobial and antioxidant activity of the combination was examined.

## Methodology

*Study design*

This prospective study included isolation of colonic ulcer-forming bacteria (*Shigella* sp.) from patients suffering from watery and bloody diarrhea. The ability of isolated *Shigella* to form colon ulcers was investigated in an animal model to examine the invasion of bacteria through the epithelial surface of the colon and to study the histopathological changes in the colon tissue due to ulcer formation. The rats were divided into six groups; each group contained 25 rats. The negative control rats were not infected with *Shigella* sp. nor treated but received saline. Infected control rats were orally subjected to *Shigella* sp. ($12 \times 10^8$ colony-forming units [CFU]/mL) for 10 days, then received saline only until the end of the experiment. The other four groups were treated (150 µL/L thyme oil, 0.4 mg/L ciprofloxacin, combination group and Azulfidine ) after receiving *S. flexneri* for 11 days.

During the course of the experimental period, six rats of each group were randomly chosen and sacrificed at days 3, 10, 14, and 21 in order to measure the level of serum total antioxidant capacity, determine the bacterial load in the infected colon, and detect the histopathological changes in the colon by histopathological examination of the colon tissue as adopted from Wassef *et al*. [11].

*Sample collection*

Twenty-two stool samples were collected from patients suffering from watery or bloody diarrhea (between November 2011 and January 2012) at the El-Gamaa Hospital, Tanta. The stool samples were collected in clean, sterile containers and subjected to processing for isolation of bacteria.

*Processing of stool sample for isolation of bacteria*

Fecal specimens were processed based on the guidelines of Centers for Disease Control (CDC) and World Health Organization (WHO) (2003) [12]. One loopful of fecal sample was streaked on Salmonella-Shigella media (SS agar, Oxoid, Basingstoke, England) and incubated at 37°C for 24 hours aerobically. *Salmonella* sp. and *Shigella* sp. are non-lactose fermenters and form colorless colonies on Salmonella-Shigella agar. $H_2S$-positive *Salmonella* sp. produce black-center colonies. $H_2S$-negative *Shigella* sp. do not produce black-center colonies. *Shigella* was maintained in nutrient agar slants stored in a refrigerator.

*Selection of ulcer-forming bacteria*

Adult male albino Sprague-Dawley rats, weighing approximately 100 to 120 g, were given different doses of eight isolates of *Shigella* sp. (isolated from bloody diarrhea samples) orally for three to four days. Diarrhea usually appeared after 48 hours, with dysentery supervening about two days later. The animals were sacrificed, and their colons were rapidly removed and kept in 10% formalin for morphological and histopathological examinations with haematoxylin and eosin (HE) stain in order to determine the *Shigella*

isolates and the dose which is able to cause ulcers [13].

*Identification of ulcer-forming Shigella sp. by 16S rRNA gene sequence*

Polymerase chain reaction (PCR) was performed in a thermal cycler (Bio-Rad MJ Research, Hercules, USA). The 50 µL reaction mixture consisted of 20 ng of genomic DNA, 2.5 U of Taq DNA polymerase, 5 µL of 10 × Taq buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3), 200 µM dNTP, 10 pmoles each universal primers (forward primer AGA GTT TGA TCC TGG CTC AG and reverse primer GGT TAC CTT GTT ACG ACT T), and 2.0 mM $MgCl_2$. Amplification included initial denaturation at 94°C for 5 minutes, followed by 25 cycles of denaturation 94°C for 30 seconds, annealing temperature of primers at 50°C for 30 seconds, and extension at 72°C for 1 minute. A final extension at 72°C for 15 minutes was used. A total of 5 µL of the amplified product was then analyzed by submarine agarose gel electrophoresis in 1.2% agarose gel with ethidium bromide at 8 V/cm, and the PCR product were visualized under a gel doc UV transilluminator. The amplified PCR product was gel purified using the QIAGEN gel extraction kit. A total of 100 ng/µL concentration of 16S rRNA amplified product was used for the sequencing [14] by GATC Company using ABI 3730xl DNA sequence using forward and reverse primers (Sigma Scientific Services Co., Cairo, Egypt).

*Assay of antibacterial activity*

Twenty milliliters of sterilized MacConkey agar was poured into sterile petri plates; after solidification, 100 µL of fresh culture of ulcer-forming *Shigella* sp. ($1 \times 10^6$ CFU/mL) were swabbed on the respective plates and then investigated against essential oils or antibiotics to assess antibacterial activity. The wells were punched over the agar plates using a sterile gel puncher. Fifty microliters of different essential oils (clove, cucumber, chamomile, ginger, cinnamon, nigella, garlic, onion, thyme, and oregano) were aliquoted into each well. The antibiotic disks, which included ciprofloxacin, tetracycline, amoxicillin, erythromycin, nalidixic acid, and cefotaxime were placed on the surface of the plates. The plates were incubated at 37°C for 24 hours, and after incubation, the diameter of the inhibition zones were measured in millimeters and recorded as previously described [15,16].

*Determination of minimum inhibitory concentration (MIC)*

A series of culture tubes were prepared, all containing the same volume of the MacConkey medium inoculated with the same concentration ($1 \times 10^6$ CFU/mL) of test microorganism and then added different concentrations of the antimicrobial substances (for thyme oil: 0, 50, 75, 100, 125, 150, and 250 µL/L; for ciprofloxacin: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 mg/L). The cultures were incubated at 37°C for 24 hours. The lowest concentration of samples at which the subculture from the test dilution yielded no viable organisms was recorded as the minimum inhibitory concentration [17].

*Synergistic activity*

The synergistic activity was examined by a combination of the standard chosen antibiotics and essential oil by the well diffusion method (Kirby-Bauer technique) using one well in a plate of pathogenic bacteria, the well containing thyme oil 0.5 mg/mL in combination with ciprofloxacin 30 mg/L. The wells were then incubated at the standard conditions for 24 hours at 37°C, and the diameter of the inhibition zones were measured in millimeters around the wells [18].

*Determination of bacterial load in infected colon*

The colon was homogenized in a tissue grinder or homogenizer. Each organ was homogenized in 5 mL of sterilized saline solution. The homogenate was serially diluted, and 0.1 mL was plated on Salmonella-Shigella media. The plates were incubated at 37°C for 18 hours and the number of colonies was counted in order to record the number of colony-forming units per organ (CFU/organ). Number of bacteria (CFU/organ) = (average number of bacterial colonies/amount plated) × dilution [19].

*Histopathological examination*

A portion of the colon tissue was fixed in 2.5% buffered gluteraldhyde and washed three times with phosphate buffer (pH = 7.2). Post fixation with osmic acid, it was washed three times with phosphate buffer (pH = 7.2), dehydrated with serial dilution of ethanol 30, 50, 70, 90, then absolute alcohol. The specimen was then placed in acetone, and then embedded in resin araldite and polymerized in an oven at 65°C for 24 hours for histologic studies [20].

*Detection of serum total antioxidant capacity (TAC)*

Serum total antioxidant capacity was measured by detection of TAC of ferric reducing antioxidant power (FRAP) assay. At low pH, reduction of ferric tripyridyl triazine (FeIII TPTZ) complex to ferrous form (which has an intense blue color) could be monitored by measuring the change in absorption at 593 nm. The change in absorbance was directly related to the combined or total reducing power of the electron donating antioxidants present in the reaction mixture [21].

*Effect of antimicrobial substance on bacterial cells*

A loopful of bacteria was transferred to a 10 mL test test tube containing MacConkey broth. Essential oils, antibiotics, and the combination of the two were incubated with bacteria at 37°C for 18 hours. Changes in morphology of the bacteria were photographed under a scanning electron microscope (model JEOL, JSM-5200 LV; JEOL, Tokyo, Japan) in the electron microscope unit of Tanta University. Changes in the ultrastructure of bacteria were photographed under a transmission electron microscope (model JEOL-JEM-100 SX electron microscope) at the electron microscope unit of the Faculty of Medicine of Tanta University. Authors please include name, city and country of manufacture.

*Statistics*

The comparison between the different parameters are analyzed by the ANOVA one-way test and Student's *t*-test using SPSS version 17.0. Significance was set at $p < 0.05$.

## Results

*Isolation of Shigella sp.*

In the present investigation, fourteen isolates of *Shigella* spp. were isolated from stool samples, six isolates from watery diarrhea, and eight isolates from bloody diarrhea.

*Selection of ulcer-forming Shigella sp.*

Only one *Shigella* sp. (isolate no. 3) of eight investigated isolates was able to cause an ulcer after three days of oral administration with $12 \times 10^8$ CFU/mL. The ability to form ulcers was detected via the invasive effect of *Shigella* spp., which was clearly noticed through focal loss of surface epithelium, infiltration of lamina propria with inflammatory cells (Figure 1), and the area of sloughed necrotic and desquamated epithelium (Figure 2).

*Identification of ulcer-forming Shigella sp.*

The 16S rRNA gene sequence of *Shigella* sp. was compared to that in GeneBank, and the phylogenetic tree was constructed. The obtained sequence proved that *Shigella* spp. (isolate no. 3) was *Shigella flexneri* strain ATCC 29903.

*Antibacterial activities of some essential oils against S. flexneri* ATCC 29903

The obtained data revealed (Table 1) that thyme oil had the highest antibacterial activity of all the investigated oils.

The effect of different concentrations of thyme oil on *Shigella flexneri* growth revealed that with increasing concentration of oil, the growth decreased until 150 µL/L (complete inhibition), as shown in Table 2.

**Figure 1.** Photomicrograph of colon showing focal loss of surface epithelium (ulcer) (arrow) and infiltration of lamina propria with inflammatory cells (star) (× 250)

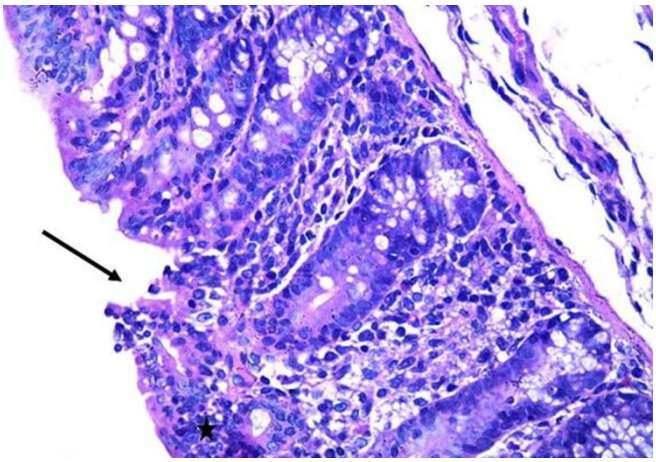


**Figure 2.** Photomicrograph of colon showing area of sloughed necrotic epithelium (star), area of desquamated epithelium (arrow) (× 400)

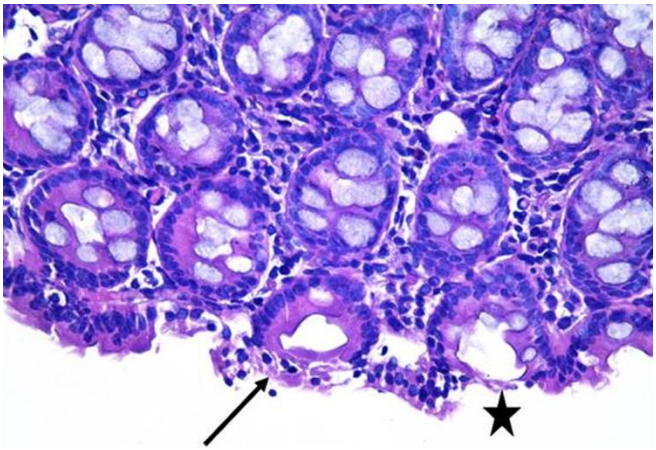


**Table 1.** Antibacterial activities of essential oils against *Shigella flexneri*

| Oils | Diameter of inhibition zone (mm) (mean ± SD) |
|---|---|
| Ginger | 0.00 ± 0.00 |
| Clove | 20.22 ± 1.30 |
| Orange | 0.00 ± 0.00 |
| Chamomile | 0.00 ± 0.00 |
| Nigella | 16.22 ± 1.79 |
| Onion | 0.00 ± 0.00 |
| Garlic | 0.00 ± 0.00 |
| Cucumber | 0.00 ± 0.00 |
| Cinnamon | 23.44 ± 1.42 |
| Thyme | 45.55 ± 3.12 |
| Oregano | 25.55 ± 1.51 |

All data represented the means of three replicates ± standard deviation; F value is 1247.163; P value is 0.000

**Table 2.** Minimum inhibitory concentrations of thyme oil against bacteria

| Concentration of oil (µL/L) | Percentage of growth reduction (%) |
|---|---|
| 0 | 0 |
| 50 | 25 |
| 75 | 50 |
| 100 | 70 |
| 125 | 80 |
| 150 | 100 |
| 250 | 100 |

**Table 3.** Antibacterial activities of antibiotics against bacteria

| Antibiotics | Diameter of inhibition zone (mm) (mean ± SD) |
|---|---|
| Cefotaxime (25 µg) | 0.00 ± 0.00 |
| Ciprofloxacin (10 µg) | 36.89 ± 2.20 |
| Erythromycin (10µg) | 8.00 ± 1.66 |
| Amoxicillin (8 µg) | 11.44 ± 1.13 |
| Tetracycline (50 µg) | 0.00 ± 0.00 |
| Nalidixic acid (10 µg) | 24.89 ± 0.93 |

All data represented the means of three replicates ± standard deviation; F value is 1191.560; P value is 0.000

**Table 4.** Minimum inhibitory concentrations of ciprofloxacin against bacteria

| Concentration (mg/L) | Percentage of growth reduction (%) |
|---|---|
| 0 | 0 |
| 0.1 | 25 |
| 0.2 | 50 |
| 0.3 | 80 |
| 0.4 | 100 |
| 0.5 | 100 |
| 0.6 | 100 |
| 0.7 | 100 |
| 0.8 | 100 |

**Table 5.** Determination of bacterial load in infected colons

| Groups | Intervals (bacterial load) (CFU/mL x $10^2$) | | | |
|---|---|---|---|---|
| | 3 days | 10 days | 14 days | 21 days |
| Infected control (not treated) | 109 ± 1.0 | 181 ± 2.0 | 206 ± 1.0 | 220 ± 0.5 |
| **Treatments** | | | | |
| Thyme oil group | 113* ± 2.6 | 177.6 ± 0.5 | 99* ± 1.0 | 72.6* ± 1.5 |
| Ciprofloxacin group | 116* ± 0.5 | 179 ± 0.5 | 121.6* ± 0.5 | 67.6* ± 0.5 |
| Azulfidine group | 107 ± 0.5 | 178 ± 2.6 | 144.6* ± 0.5 | 127* ± 1.0 |
| Synergism group | 112* ± 2.5 | 178.3 ± 1.5 | 81* ± 1.0 | 41.6* ± 1.0 |

All data represented the means of three replicates ± standard deviation; *Significant in comparison to infected control group

**Figure 3.** Histologic studies of control and experimental groups of rats. (a) A control micrograph showing normal architecture of surface epithelium of colon. (b) A control micrograph of *lamina propria* showing no infiltrating cells. (c) A part of epithelium showing marked reduction in the number of microvilli and intracellular bacilli (arrow) surrounded by a large lytic area in the tissue of *Shigella flexneri* induced rats (infected control group). (d) Photomicrograph of lamina propria showing infiltration with numerous eosinophils (infected control group). (e) EM of surface epithelium showing a focal area of affection in the form of nuclear indentation (star) of an epithelial cell and widening of intercellular spaces of desmosomes (arrow) in a rat administered thyme oil + *S. flexneri* (thyme oil group). (f) Photomicrograph of lamina propria showing infiltration with fibroblasts and eosinophils.

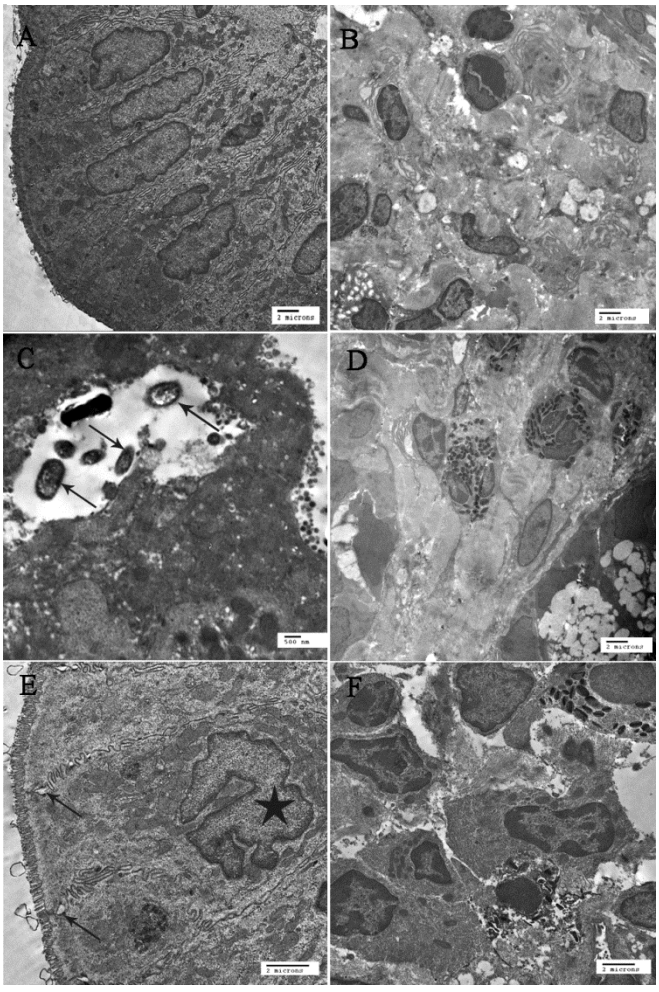
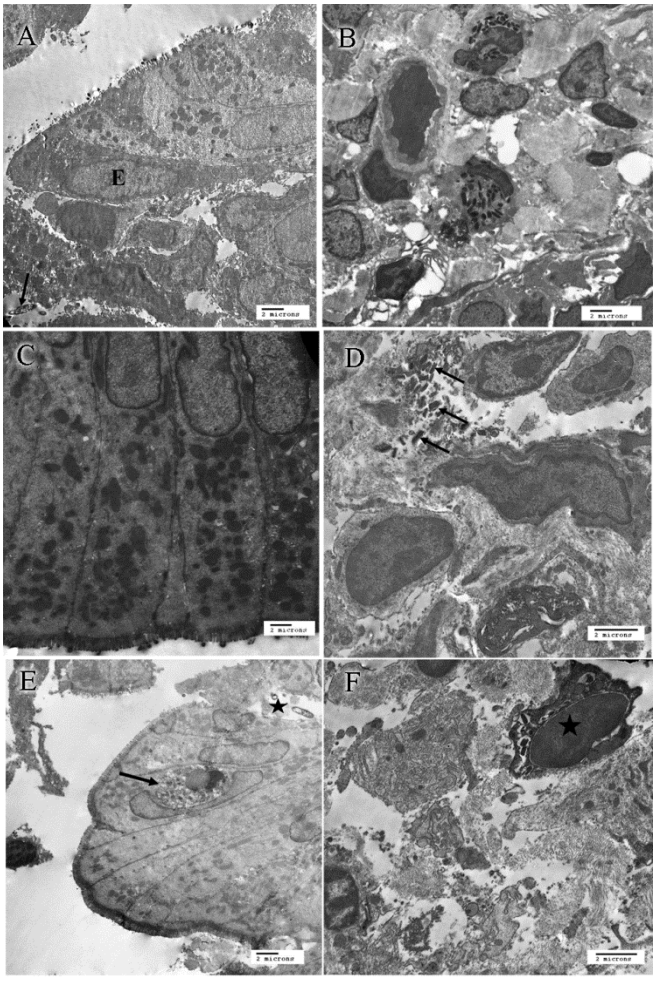



**Figure 4.** (a) Surface epithelium showing focal loss of microvilli, junctional complexes, vacuolated intracellular bacilli bacteria surrounded by an irregular lytic area (arrow) and shedding of some epithelial cells (e) in a rat administered ciprofloxacin + *S. flexneri* (antibiotic group). (b) Photomicrograph of lamina propria showing infiltration with eosinophils (antibiotic group). (c) Photomicrograph of surface epithelium showing intact columnar cells with intact junctional complexes, cellular organelles and numerous microvilli in a rat administered thyme oil + ciprofloxacin + *S. flexneri* (synergism group). (d) Photomicrograph of TEM of lamina propria showing extracellular release of eosinophil granules (arrow) (synergism group). (e) TEM of surface epithelium showing: a disrupted colonocyte with intracellular bacilli (star) and another colonocyte with a giant secondary lysosome (arrow) in a rat administered commercial drug + *S. flexneri* (commercial drug group). (f) EM of lamina propria showing an area of cellular debris and a macrophage (star) engulfing eosinophil granules (commercial drug group).



*Sensitivity of S. flexneri* ATCC 29903 *against commonly used antibiotics*

Among six tested antibiotics, ciprofloxacin (10 µg) recorded the highest antimicrobial activity against *S. flexneri*, as shown in Table 3. The effect of different concentrations of ciprofloxacin against *S. flexneri* revealed that by increasing the concentration of the antibiotic, the growth of *S. flexneri* decreased and complete inhibition of growth occurred at 0.4 mg/L as shown in Table 4.

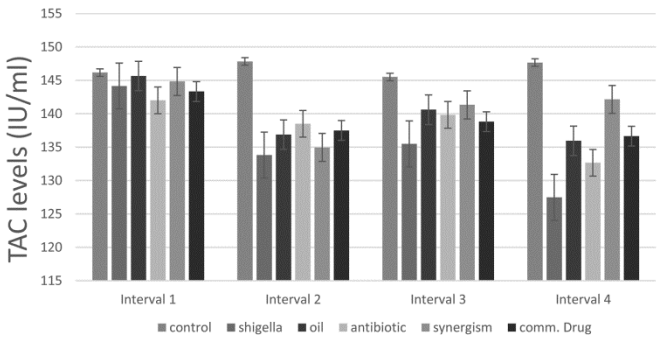
*Determination of bacterial load in infected colon*

The obtained data revealed (Table 5) that bacterial load in the infected control increased with time (21 days). All treatments were able to reduce the bacterial load with time, and the lowest bacterial count was detected after 21 days. Among different treatments, the combination between thyme oil and ciprofloxacin gave the highest reduction rate in bacterial count, up to 81%, in the infected control group.

*Histopathological examination*

The normal architecture of the colon was observed in control group as shown in Figures 3a and 3b. The rats infected with *S. flexneri* ATCC 29903 showed damage in the colon tissue and penetration of the bacteria through the epithelial cells as evidenced by pathologic changes in the architecture of the colon, namely ulceration and intense infiltration of eosinophil in the lamina propria (Figures 3c and 3d). Infected rats treated with thyme oil showed a decrease in histopathological changes in the epithelial surface and also the extracellular release of eosinophil granules (Figures 3e and 3f). The group of rats treated with ciprofloxacin showed vaculation in infiltrated bacteria within the epithelial cells in the lamina propria (Figures 4a and 4b). The combination treatment of thyme oil and ciprofloxacin showed better protection as observed by the healing of the colon ulcer and the decrease in the infiltration of the lamina propria (Figures 4c and 4d). Rats treated with commercial drugs showed that commercial drugs failed to heal the colon ulcer but decreased the inflammation that was present in the lamina propria (Figure 4e and 4f).

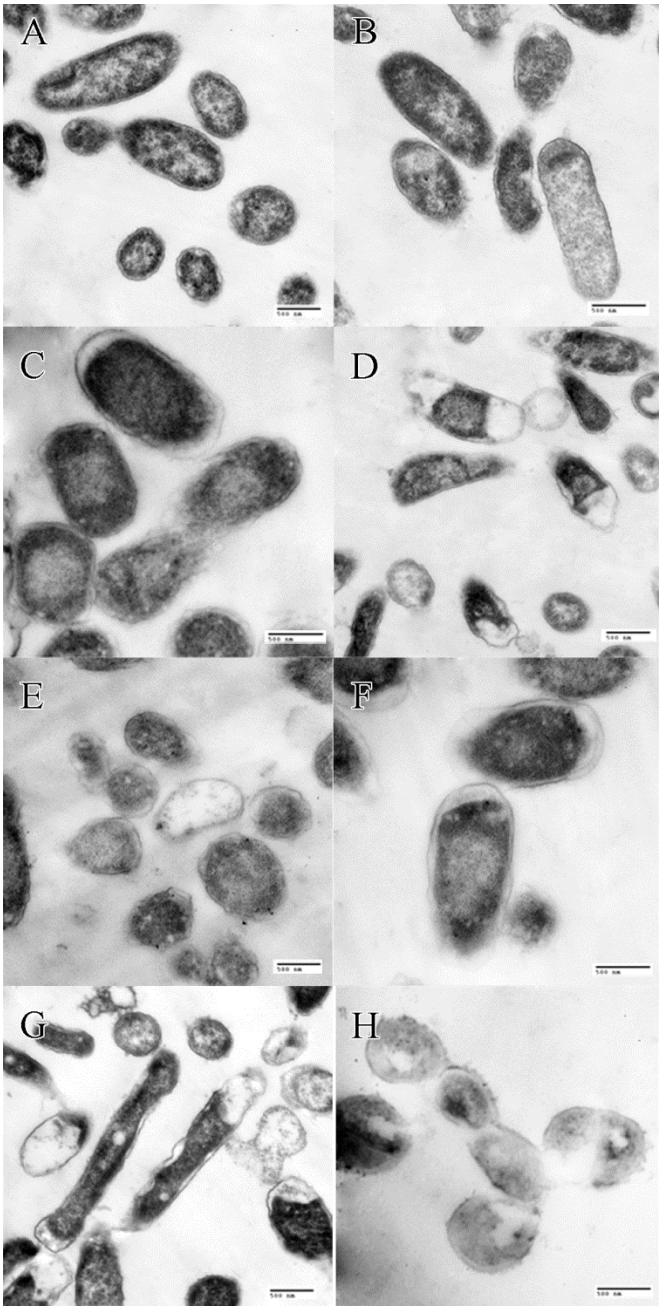
**Figure 5.** Serum TAC in the different time intervals of different groups




*Determination of serum total antioxidant capacity (TAC)*

Serum TAC had been measured at different times.

**Figure 6.** Photographs of transmission electron microscope reveal the effect of antibacterial substances on *Shigella flexneri*; (a-b) represents control; (c-d) represents effect of thyme oil; (e-f) represents effect of ciprofloxacin; (g-h) represents effect of synergism between thyme oil and ciprofloxacin.



The obtained results revealed that no significant changes had been recorded among different groups after 3 days. The TAC was significantly decreased in all groups compared to non-infected controls after 10 days. A significant increase in the TAC was observed after 14 and 21 days of thyme oil and combination treatments compared with the infected control group.

*Effect of antimicrobial substances and the combination between them on bacterial cells*

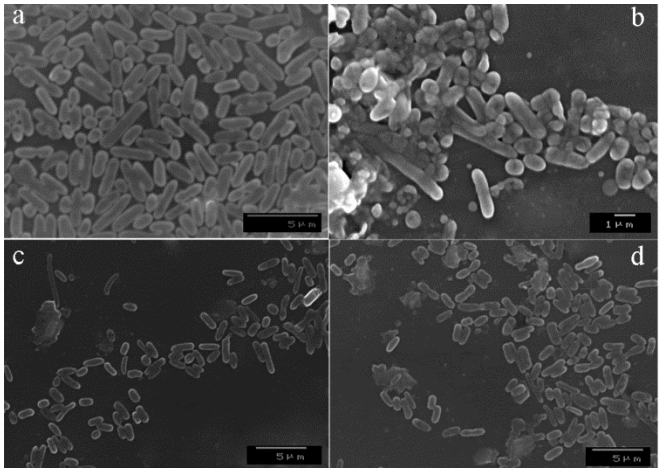
Rupture in the bacterial cell, shrinking of the bacterial content with irregularity, and distortion in the cell wall were the results of using antimicrobial substances as shown in Figure 6 (c, d, e, f, g, h) and Figure 7 (b, c, d) compared to normal cells shown in Figure 6 (a, b) and Figure 7 (a). Also, the results revealed that all previously mentioned effects occurred with more frequently after treatment with synergistic mixture followed by thyme oil, and the lowest percentage of effects was observed with ciprofloxacin.

## Discussion

This study aimed to discover a new approach to the treatment of colon ulcers resulting from infection with *Shigella flexneri* ATCC 29903 via combination therapy of two substances. The synergistic activity of a new combination between thyme oil and ciprofloxacin inhibited bacterial growth, healed cellular damage of colon ulcers, and reduced inflammation.

Pathogenesis of *S. flexneri* was based on the bacteria's ability to invade and replicate within the colonic epithelium, which resulted in severe inflammation and epithelial destruction [22]. *S. flexneri* is highly infectious, requiring as little as 100 cells to cause disease in adult volunteers [23]. This low infective dose was in part attributed to *S. flexneri*'s ability to survive the low acidity of the host's stomach, via an up-regulation in acid resistance genes [24]. Once *Shigella* reached the colon, they began to invade the mucosa, penetrating, replicating within, and spreading between the mucosal epithelial cells. This behavior and the subsequent inflammatory response of the host destroyed the colonic epithelial layer, generating the clinical symptoms of shigellosis [25]. Based on previous studies, the present study choose ulcer-forming *Shigella* for to its ability to penetrate and damage surface epithelium. Histopathological examinations with HE stain showed the invasive effect of *Shigella* sp. by its causing focal loss of surface epithelium (ulcer), infiltration of lamina propria with inflammatory cells, and areas of sloughed necrotic epithelium.

**Figure 7.** Photographs of scanning electron microscope reveal the effect of antibacterial substances on *Shigella flexneri*; (a) represents control; (b) represent effect of thyme oil; (c) represent effect of ciprofloxacin; (d) represent effect of synergism between thyme oil and ciprofloxacin.




*Shigella flexneri* bacilli invaded the surface epithelium, multiplied, migrated laterally from cell to cell, and entered the lamina propria. Finally, superficial ulceration and bleeding occured. Histopathological examination in the present study showed that *S. flexneri* succeeded in the penetration of the epithelial surface of the colon and surrounded itself by an irregular lytic area, which resulted in marked reduction in microvilli and eosinophillic infiltration in the lamina propria. Similarly, Wei *et al*. [2] found that *Shigellae* produced and excreted another major virulence factor, nicotinamide adenine dinucleotide glycohydrolase (NAD glycohydrolase), which destroyed all of the NAD of the human cells and virtually shut down cellular metabolism, causing cell death. In this way, *Shigella* penetrated the epithelial cells.

The results obtained from the present study revealed that thyme oil had the greatest antibacterial activity of origanum oil, cinnamon oil, clove oil, and nigella oil against *S. flexneri*. Also, antioxidative properties of thyme helped to reduce oxidative stress in the colon during ulcer formation and led to a reduction of inflammation in the colon, consequently helping to heal the surface epithelial cells of the colon. These results were in agreement with Abo-Ghalia *et al*. [26] and Venturini *et al*. [27], who detected antibacterial activity of thyme oil. Thymol has been shown to exhibit multiple biological activities, including anti-inflammatory [28], immunomodulating [29], antioxidant [30], antibacterial [27], and free radical scavenging properties [31].

Studying the effects of ciprofloxacin and levofloxacin administration on some oxidative stress markers in rats revealed that ciprofloxacin and levofloxacin induced oxidative stress [32]. Also, the studies of Dhamidharka *et al*. [33] and Pouzauaud *et al*. [34] reported that the generation of reactive oxygen species by fluoroquinolones resulted in cellular damage to the liver and kidneys due to increase the oxidative stress.

The WHO recommends the use of ciprofloxacin as the first-line antibiotic in suspected *Shigella* dysentery but also suggests that this choice should be based on sensitivity patterns of *Shigella* strains recently isolated in the area [35]. The present study used different antibiotics against *S. flexneri* in order to inhibit its growth. Ciprofloxacin, tetracycline, amoxicillin, erythromycin, nalidixic acid, and cefotaxime were tested against *S. flexneri*. Ciprofloxacin had the highest antibacterial activity against *S. flexneri*, with 75% reduction in the growth. It decreased the bacterial load in infected colons. Ciprofloxacin decreased the total antioxidant capacity because it increased the oxidative stress in the cells; it therefore failed to heal the colon ulcer, and it did not affect the inflammation of the colon. Histopathological examination showed the vaculation in penetrated bacterial cells in the colon epithelium due to the antibacterial activity of ciprofloxacin. There was focal loss of microvilli and junctional complexes, which indicates that ciprofloxacin had no effect in healing the colon ulcer.

*In vitro* synergistic effects of different spices and herbs (*Rosmarinus officinalis*, *Coriandrum sativum*, *Micromeria fruticosa* (L.), *Cumium cyminum*, *Mentha piperita*) with gentamicin, cephalothin, ceftriaxone, and nystatin against 13 microbial species were investigated [36]. The combination of plant extracts and antibiotics may reduce drug resistance. Similar to previous findings, the present study found that a combination of thyme oil and ciprofloxacin was able to inhibit the growth of *S. flexneri* better than did using each method alone. The synergistic activity between thyme oil and ciprofloxacin decreased the bacterial load in the infected colon due to the enhanced antibacterial activity of thyme oil and ciprofloxacin. Also, the combination increased the total antioxidant capacity due to the antioxidative properties of the thyme oil so that it decreased cellular damage, helped to heal the colon ulcer, and also decreased the inflammation of the colon. The synergistic activity of combination therapy could help in the healing of cellular damage. The commercial drug Azulfidine was used as an anti-inflammatory drug to decrease the symptoms of colon ulcers. The histopathological examination of the colon tissue treated with Azulfidine showed no effect in disruption of colonocyte and had no effect on intracellular bacilli; it only decreased the infiltration in the lamina propria of macrophage engulfing eosinophil granules. These results are in agreement with Loftus *et al*. [37], who studied the effect of Azulfidine as an anti-inflammatory drug.

The changes in the bacterial cells in response to combination therapy of thyme oil and ciprofloxacin were recorded by an electron microscope. Changes on the bacterial cell wall surface included irregularity, rupture and shrinking of the bacterial cells, lysis, and distortion of some of the cells. The previous recorded effects had occurred on the bacterial cells treated with ciprofloxacin or thyme oil rather than synergism between them. The effect of synergistic combination between thyme oil and ciprofloxacin on the bacterial cells was better or stronger than using ciprofloxacin or thyme oil alone.

## Conclusions

The synergistic combination of ciprofloxacin and thyme oil revealed remarkable antibacterial, anti-inflammatory, and healing activity against colon ulcers resulting from *Shigella flexneri* ATCC 29903 infection.

### References

1. Chen M, He C, Meng X, Yuan Y (2013) Association of *Helicobacter pylori* babA2 with peptic ulcer disease and gastric cancer. World J Gastroenterol 19: 4242-4251.
2. Wei J, Goldberg MB, Burland V, Venkatesan MM, Deng W, Fournier G, Mayhew G, Plunkett G, Rose D, Darling A, Mau B, Perna NT, Payne SM, Runyen-Janecky L, Zhou S, Schwartz DC, Blattner FR (2003) Complete genomic sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. Infect Immun 71: 2775-2786.
3. Lachowicz KJ, Jones GP, Briggs DR, Bienvenu FE, Wan J, Wilcock A, Coventry MJ (1998) The synergistic preservative effects of the essential oils of sweet basil (*Ocimum basilicum* L.) against acid-tolerant food microflora. Letters Appl Microbiol 26: 209-214.
4. Ipek E, Zeytinoglu H, Okay S, Tuylu BA, Kurkcuoglu M, Husnu CB (2005) Genotoxicity and antigenotoxicity of *Origanum* oil and carvacrol evaluated by Ames *Salmonella*/microsomal test. Food Chem 93: 551-556.
5. Chanda S, Rakholiya K (2011) Combination therapy: Synergism between natural plant extracts and antibiotics against infectious diseases. Phytochem Pharmacol Microbiol 22: 520-529.
6. Habbal O, Hasson SS, El-Hag AH, Al-Mahrooqi Z, Al-Hashmi N, Al-Bimani Z, Al-Balushi MS, Al-Jabri AA (2011) Antibacterial activity of *Lawsonia inermis* linn (Henna) against Pseudomonas aeruginosa. Asian Pac J Trop Biomed 1: 173-176.

7. Marquez B, Pourcelle V, Vallet CM, Mingeot-Leclercq MP, Tulkens PM, Marchand-Bruynaert J, Van Bambeke F (2014) Pharmacological Characterization of 7-(4-(Piperazin-1-yl)) Ciprofloxacin Derivatives: Antibacterial Activity, Cellular Accumulation, Susceptibility to Efflux Transporters, and Intracellular Activity. Pharm Res 31: 1290-1301.
8. Salau AO, Odeleye OM (2007) Antimicrobial activity of *Mucuna pruriens* on selected bacteria. African J Biotechnol 6: 2091-2092.
9. Bagamboula CF, Uyttendaeleand M, Debevere J (2004) Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri*. Food Microbiol 21: 33-42.
10. Jorge N, Veronezi CM, Del Ré PV (2014) Antioxidant Effect of Thyme (*Thymus vulgaris* L.) and Oregano (*Origanum vulgare* L.) Extracts in Soybean Oil Under Thermoxidation. J Food Processing and Preservation 11: 19-29.
11. Wassef JS, Keren DF, Mailloux JL (1989) Role of M cells in initial antigen uptake and in ulcer formation in the rabbit intestinal loop model of Shigellosis. Infect Immun 57: 858-863.
12. Centers for Disease Control, World Health Organization (2003) Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World. Geneva: WHO.
13. Bancroft JD, Gamble M (2008) Theory and Practice of Histological Techniques, 6th edition. Churchill Livingstone, Elsevier, Philadelphia. 725 p.
14. Singh V, Chaudhary D, Mani I (2012) Molecular characterization and modeling of secondary structure of 16S rRNA from Aeromonas Veronii. Int J Appl Biol Pharm Tech 3: 253-260.
15. Daniyan SY, Muhammad HB (2008) Evaluation of the antimicrobial activities and phytochemical properties of extracts of Tamaridus indica against some diseases causing bacteria. Afr J Biotech 7: 2451-2453.
16. Mahalingam R, Bharathidasan V, Ambikapathy J, Panneerselvam A (2011) Studies on antibacterial activity of some medicinal plant against human pathogenic micro-organism. Asia J Plant Sci Res 1: 86-90.
17. Ferreira MJU, Ascenso JR, Valderia L, Duarte A, Frade JP, Freitas G (2003) Evaluation of antiviral and antimicrobial activities of triterpenes isolated from Euphorbia segetalis. Nat Prod Res 17: 375-380.
18. Betoni JE, Mantovani RP, Barbosa LN, Sasti LC, Fernandez Jr. A (2006) Synergism between plants extract and antimicrobial drugs used on Stphylococcus aureus diseases. Mem Inst Oswaldo Cruz 101: 387-390.
19. Neuenhahn M, Schiemann M, Busch DH (2010) DCs in mouse models of intracellular bacterial infection. Methods Mol Biol 595: 319-329.
20. Bozzola JJ, Russell LD (1999) Electron Microscopy: Principles and Techniques for Biologists, 2nd edition. Boston: Jones and Bartlett. 670 p.
21. Benzie IFF, Strain JJ (1999) Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol 299: 15-27.
22. Jennison AV, Verma NK (2004) *Shigella flexneri* infection: pathogenesis and vaccine development. FEMS Microbiol Rev 28: 43-58.
23. DuPont HL, Levine MM, Hornick RB, Formal SB (1989) Inoculum size in shigellosis and implications for expected mode of transmission. J Infect Dis 159: 1126-1127.
24. Small P, Blankenhorn D, Welty D, Zinser E, Slonczewski JL (1994) Acid and base resistance in Escherichia coli and Shigella flexneri: role of rpoS and growth Ph. J Bacteriol 176: 1729-1737.
25. Philpott DJ, Edgeworth JD, Sansonetti PJ (2000) The pathogenesis of *Shigella flexneri* infection: lessons from in vitro and in vivo studies. Philos Trans R Soc Lond B Biol Sci 355: 575-586.
26. Abo-Ghalia HH, Al-Khalifa MT, El-Mokadem AM, Ghanem KA (2004) Antimicrobial activity of essential oils of some medical plants. N Egypt J Microbiol 9: 221-241.
27. Venturini ME, Blanco D, Oria R (2002) In vitro antifungal activity of several antimicrobial compounds against *Penicillium expansum*. J Food Protect 65: 834-839.
28. Braga PC, Dal Sasso M, Culici M, Bianchi T, Bordoni L, Marabini L (2006) Anti-inflammatory activity of thymol: inhibitory effect on the release of human neutrophil elastase. Pharmacology 77: 130-136.
29. Suzuki Y, Furuta H (1988) Stimulation of guinea pig neutrophil superoxide anion-producing system with thymol. Inflammation 12: 575-584.
30. Aeschbach R, Löliger J, Scott BC (1994) Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. Food Chem Toxicol 32: 31-36.
31. Fujisawa S, Kadoma Y (1992) Effect of phenolic compounds on the polymerization of methyl methacrylate. Dent Mater 8: 324-326.
32. Afolabi OK, Oyewo EB (2014) Effects of Ciprofloxacin and Levofloxacin Administration on Some Oxidative Stress Markers in the rats. Int J Biol Vet Agricult Food Eng 8: 31-39.
33. Dhamidharka VR, Nadeau CL, Cannon HW, Harris H, Rosen S (1998) Ciprofloxacin overdose: Acute renal failure with prominent apoptotic changes. Am J Kid Dis 31: 710-712.
34. Pouzauaud F, Dutot M, Martin C, Debray M, Warnet JM, Rat P (2006) Age-dependent effects on redox status, oxidative stress, mitochondrial activity and toxicity induced by fluoroquinolones on primary cultures of rabbit tendon cells. Comp Biochem Physiol C Toxicol Pharmacol 143: 232-241.
35. World Health Organization (2005) The treatment of diarrhea. A manual for physicians and other senior health workers, 4th edition. Geneva: WHO 50 p.
36. Toroglu S (2011) In-vitro antimicrobial activity and synergistic/antagonistic effect of interactions between antibiotics and some spice essential oils. J Environ Biol 32: 23-32.
37. Loftus EV Jr, Kane SV, Bjorkman D (2004) Systematic review: short-term adverse effects of 5-aminosalicylic acid agents in the treatment of ulcerative colitis. Aliment Pharmacol Ther 19: 179-189.



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**Conflict of interests:** No conflict of interests is declared.