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The Synergistic Effect of a Combination of Anise Seed Extract and Antibiotics (Erythromycin, Ampicillin, Gentamycin, Amikacin) against the Escherichia Coli Bacterium.

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ABSTRACT

The combination of plant extracts with antibiotics as resistance to microorganisms that have shown resistance to many synthetic drugs has been ongoing for a number of years. In this study, the interaction between ethanolic extract of anise seeds and four antibiotics were investigated and the inhibition effects of four antibiotics and the crude extract of anise seeds against a strain of E. coli were studied individually. Results show that the zone of inhibition was 20mm for Gentamycin, 19mm for Amikacin, and 11mm for tetracycline, while the E. coli showed resistance to the antibiotics of Ampicillin. Furthermore, the zone of inhibition when the extract alone was used was 10 mm at a concentration of 250 mg/ml and 20 mm at 500 mg/ml concentration. The bacterium showed resistance at a concentration of 1000 mg/ml. However, their synergistic effect was more efficient and induced an inhibition zone of 21.5mm in diameter. The significant zone of inhibition was reported as 21.5mm when the E. coli was exposed to a combination of Amikacin with 1000 mg/ml concentration of anise seed extract followed by 21.2mm at a concentration of 500 mg/ml with the same antibiotic and 21.1mm at a concentration of 250 mg/ml. A combination of Gentamycin with anise seed extract at the same concentrations revealed zones of inhibition as 20.2 mm, 20.1 mm and 20.1 mm respectively. A combination of the anise seed extract with tetracycline did not result in the same inhibition zone, being approximately 11.2 mm at all three concentrations. However, high resistance was shown when the E. coli bacterium was exposed to Ampicillin at the three different concentrations of anise seed extract. The results of the present study illustrate that this extract can be used alone or combined with antibiotics to fight multi-drug resistant bacteria such as the E. coli bacterium.

Keywords: Synergistic effects, anise seed, Erythromycin, Ampicillin, Gentamycin, Amikacin

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INTRODUCTION

Returning to natural medicinal sources has become more prevalent in Western medicine recently due to the increasing prevalence of side effects of drugs, drug tolerance in patients, and new recombinants in genetic elements of bacteria, the latter of which is responsible of drug resistance [1]. The difference in photochemical compounds in various species of plants gives us an opportunity to use these materials for various applications, such as in the industrial and medical fields [2]. Many plants have been evaluated, not only for antimicrobial activity, but also as resistance-modified agents. Different chemical compounds – synthetic or from natural origins – have been shown to have direct activity against many species of bacteria, improving the activity of a specific antibiotics and reversing the natural resistance of specific bacteria to given antibiotics [3]. The medical application of plants are thus crucial, but this must be done under the strict guidance of physicians to reduce the risk of possible side-effects[4].

Certain plants (i.e. anise seeds) have a long history of use in public and classical medicine and in pharmaceutical manufactory. This is because therapeutic and aromatic plants have been found to be excellent sources of different phenolic compounds, especially secondary metabolites. For instance, in recent times, a variety of studies have concluded various bioactivities of fundamental oils [5]. That said, plant extracts, under the name of antimicrobials, are barely used as intrinsic antibiotics due to their low level of activity; specifically, against gram-negative bacteria [6]. Expansion of our knowledge of available natural medicines is especially important due to the mortality and morbidity caused by infectious diseases all over the world. The World Health Organization reported that 55 million people died globally in 2011, one-third of these due to infectious diseases. Antimicrobial resistance can further increase mortality rates due to the process of bacteria acquiring and transmitting resistance after exposure to antibiotic drugs, one of the most common therapies for infectious disease[7]. Antibiotic-resistant bacteria reduce antibiotic performance and limit their therapeutic action, even for regular infections. A further problem is the decrease in studies and development of new antibacterial factors capable of inhibiting antibiotic resistance disease-causing microorganisms such as *E. coli*. Consequently, much attention should be paid to natural products which could be used as effective drugs to treat human diseases, with high effectiveness against bacteria and imperceptible side effects[2].

Indeed, a number of studies have shown that the use of plant extracts along with antimicrobials improve the minimum inhibitory concentrations of antibiotics for strains of bacteria[8]. One of the ways in which plant-derived compounds exert their potential as antibiotics is via synergism, which is a positive reaction that is created when two factors combine [9]. Therefore, in this study, ethanolic anise seed extract was used to examine its efficacy against the *E. coli* bacterium in combination with four commonly used antibiotics.

MATERIALS AND METHODS

Sample collection

A bacterial sample of *E. coli* was collected from the Microbial Museum of the Zoology Department of the University of Sebha and the seeds of anise used in the study were collected from some shops located within the city of Sebha in Libya. The anise seeds were collected and cleaned with tap water to remove dust from them, and they were left in the shade away from the sunlight. They were then milled using an electric mill to obtain the seed powder, and the powder was kept in sealed containers inside the laboratory until the extraction procedure was performed.

Growth of Bacteria Samples

The culture and preservation of bacteria was done using the synergism and antithesis tests of Muller Hinton Agar, prepared according to the production company specifications (Oxoid) by dissolving 38 grams in a liter of distilled water, sterilized in an autoclave at 121 °C for 15 minutes.

Extract Preparation

50 grams of anise seed powder was weighed, and then placed in an opaque bottle with 200 ml of ethanol solvent at a concentration of 95%. The bottle was then closed tightly and left for 24 hours (it was shaken every 6 hours to ensure complete contact between the solvent and the powder). Following this, Whatman No.1 filter paper was used to filter the resulting extract, and once again, the residues were extracted from the filtration, after which the obtained filter was collected both times and evaporated to dry at a temperature of 40 °C using a rotary evaporator[10]. After obtaining the crude extract, it was weighed and its quantity was estimated at 32.27 g, as shown in Table 1 below. The prepared concentrations used in the experiment were 250 mg/ml, 500 mg/ml and 1000 mg/ml.

Commercial Antibiotics Used

Four antibiotics tablets were obtained from the company Oxoid at the following concentrations in µg: erythromycin 15 µg, ampicillin 10 µg, gentamycin 10 µg, amikacin 30 µg. The tablets were placed on the surface of a solid medium in a petri dish, with the distance between the antibiotic and the edge of the plate being approximately 2 cm, and the distance from the other antibiotics being between 2 and 3 cm. The dishes were incubated for 24 hours at 37 °C. After incubation, the dishes were examined to discover the effects of the antibiotics on bacterial growth. If there was an area of suppression around the disc, this was then measured using a ruler with a mm scale.

The Effect of the Extract on Bacteria

Filter paper was saturated with approximately 50µ of the prepared extract concentrations using a sterile pipette. This paper was then placed on MHA media containing the bacteria samples using a sterile loop. The dishes were incubated at 37 °C for 24 hours. The appearance of an area devoid of bacterial growth around the tablets (zone of inhibition) containing the tested extract was considered evidence of the effect of the extract on the tested bacteria. If there was the absence of such a region, the test scored negative (bacteria are resistant to laboratory extract). The average diameter of the damping zone was taken using a mm scale ruler. The process was repeated three times with each sample to ensure reliability of results.

Synergistic Effect Test (Efficacy of Extraction with Antibiotics on Bacteria)

The antibiotic tablets were saturated with approximately 50µ of the prepared extract concentrations using a sterile pipette, which were then placed on MHA media containing the bacteria samples using a sterile loop. The dishes were incubated at 37 °C for 24 hours. The appearance of an area devoid of bacterial growth around the tablets (zone of inhibition) containing the tested extract was considered evidence of the effect of the extract on the tested bacteria. If there was the absence of such a region, the test scored negative (bacteria are resistant to laboratory extract). The average diameter of the damping zone was taken using a mm scale ruler. The process was repeated three times with each sample to ensure reliability of results.

RESULTS AND DISCUSSION

In this study it was shown that the anise seed extract was effective against gram negative bacteria (*E. coli*), as it was more sensitive to the extract concentrations with a diameter of inhibition zone of 20 mm at 500 mg/ml concentration. The bacteria were less affected compared with other concentrations at 250 mg/ml concentration, with a diameter of inhibition area of 10 mm, and the bacteria showed resistance at a concentration of 1000 mg/ml (see Table 1 and Fig. 1).

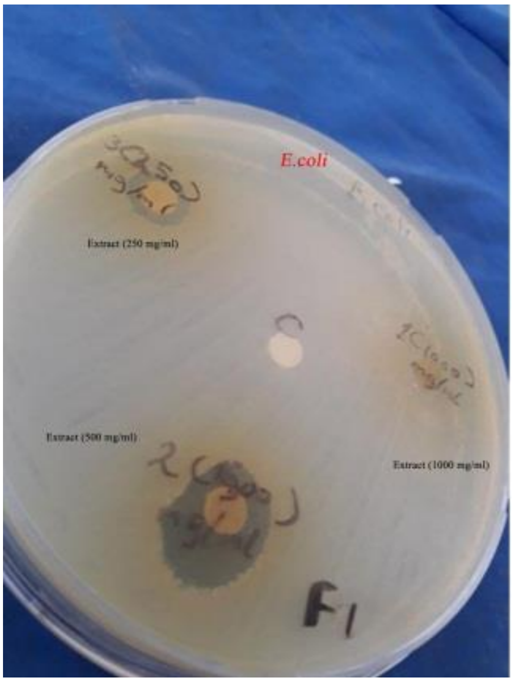
The methanol extract of *P. anisum* showed high activity with an inhibition zone of around 16 mm, while the ethyl acetate extract of *P. anisum* showed moderate activity against *E. coli*, with an inhibition zone estimated at 15 mm. In contrast, the aqueous extract showed no activity against all the tested microbes. Therefore, the various extracts of the anise have shown that there was a weak and moderation of inhibition against *E. coli* [11]. Ahmad and Beg also found that the alcoholic extract of *Punica granatum* fruit showed activity against *Staphylococcus aureus* [12], and Prashanth et al have indicated that methanolic extract of *Punica granatum* fruit peel showed activity to most microorganisms tested (*Staphylococcus aureus, Klebsiella pneumonic* and *Escherichia coli*)[13] . The same activity was shown by the methanolic and aqueous extract of *Punica granatum* leaves against the studied bacterial species that included both *Staphylococcus aureus* and *Escherichia coli* [14]. Furthermore, Naji and Jassemi have reported that ethanolic extract of ginger is effective against *E. coli* and *P. aeruginosa* [15]. In this study, ethanolic extract of anise seeds has shown the biggest effect on *E. coli.*

It is believed that the effect of anise seed oil is the result of the active substance methyl chavicol anethol present in the oil, 80-90% of it, as this substance works to change the structure of the cell wall of bacteria by making holes in the cell wall of the bacterial cell [16]. It has been observed that the insensitivity of the tested bacteria to some concentrations may be due to the decrease in the active substances in these concentrations, with the percentage of effect being directly proportional to the concentration of the extract. Thus, the higher concentration of the extract, the greater the inhibition rate. These results are in agreement with studies conducted by El-Shanawany and Deshpande [17,18]

Table 1: Size of inhibition zone of anise seed extract (using ethanol solvent) against *Escherichia coli* bacteria

Organism	Zone of inhibition in mm		
E. coli	Extract (250 mg/ml)	Extract (500 mg/ml)	Extract (1000 mg/ml)
	10	20	R

Fig 1: Effect of anise seed extract (using ethanol solvent) against *Escherichia coli* bacteria



The results of the susceptibility test to commercial antibiotics and their comparison with the anise seed extract are shown in Table 2. Fig. 2 shows that the two antibiotics, gentamycin and amikacin, were the most effective against Escherichia coli, followed by erythromycin, while the bacteria showed resistance to the antibiotic ampicillin. What distinguishes the results of this study to others is that anise seed extract, especially at a concentration of 500 mg/ml, was more effective compared to some manufactured antibiotics. For tetracycline, all the strains of E. coli and three of the four Staphylococcus aureus stains presented a resistance to this drug. For chloramphenicol, the E. coli strains, 33.1 and 16.1, and two and three strains of S. aureus showed a resistance to this drug. For levofloxacin, all the strains of E. coli showed resistance, and for ampicillin, the E. coli strains ATCC25922, 16.1, 33.1 and A2UC showed high resistance [19]. The results indicated by Ravula et al have revealed that the E. coli bacterium was found to be more resistant to ampicillin than the others. Approximately 97 strains of Escherichia coli are most resistant to tetracycline (21.45%), erythromycin (23.71%) and penicillin (32.99%). They are also sensitive to some antibiotics; namely, gentamicin (90.7%), amikacin

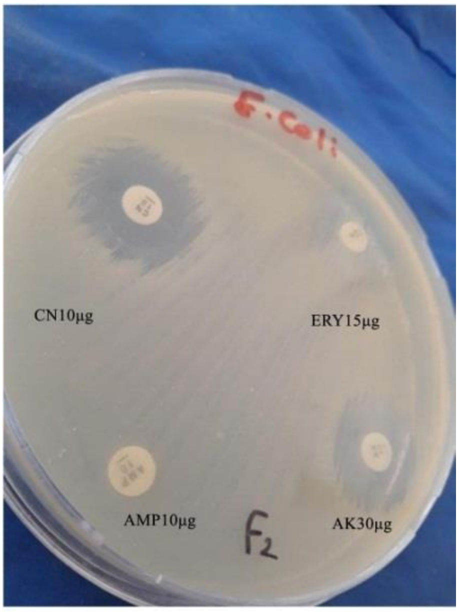
(91.75%) and nitrofurantoin (93.8%) [20]. The low resistance of Escherichia coli against the antibiotics of amikacin and gentamicin is due to the negligible use of these antibiotics in veterinary medicine and in clinical practice [21]. However, another study identified Escherichia coli as the most resistant isolates to ampicillin at 69.5%, and a large increased resistance to trimethoprim-sulfamethoxazole was ascertained by urinary microorganisms [22].

Rind and Khan assessed the sensitivity of Escherichia coli using 10 different antibiotics through the disc diffusion technique in which they found that Escherichia coli was less sensitive to chloramphenicol and gentamicin and was measured at about 53.3%, while the drugs inactive against the strains were tetracycline, with 0% showing an effect on the above bacterium[23]. In Fazlani et al.'s study, it was noted that the Escherichia coli was very sensitive to the antibiotic chloramphenicol, with a sensitivity of 100%, followed by ampicillin (76.9%), amoxicillin (69.2%) and amikacin (69.2%), and a lower susceptibility against cephalexin (61.5%), gentamycin (53.8%) and erythromycin (46.1%). The different bacterial species were found to have resistance against the antibiotic tetracycline [24].

Table 2: Size of inhibition zone of four different antibiotics against *Escherichia coli* bacteria

Organism	Zone of inhibition in mm			
E. coli	**Ampicillin 10 µg**	**Gentamycin 10 µg**	**Erythromycin 15 µg**	**Amikacin 30 µg**
	R	20	11	19

Fig 2: Sensitivity of bacteria to different commercial antibiotics



Synergistic Effects and Antibacterial Efficacy

From the results shown in Table 3 and Fig. 3, it can be concluded that the test extraction of anise seeds and standard gentamycin and amikacin have the most significant antimicrobial activity against the organism of *E. coli*. In terms of the results of the combination between anise seed extract, and gentamycin and amikacin, the zone of inhibition diameters (mm) expanded meaningfully at all three concentrations when analogized to the inhibition zones produced by standard gentamycin and amikacin alone. This high efficacy of gentamycin and amikacin when associated with the extract may be due to the efficacy affect that can be produced by the extraction of anise seeds. This demonstrates that the anise seed extract with gentamycin and amikacin has a synergistic effect on *E. coli*.

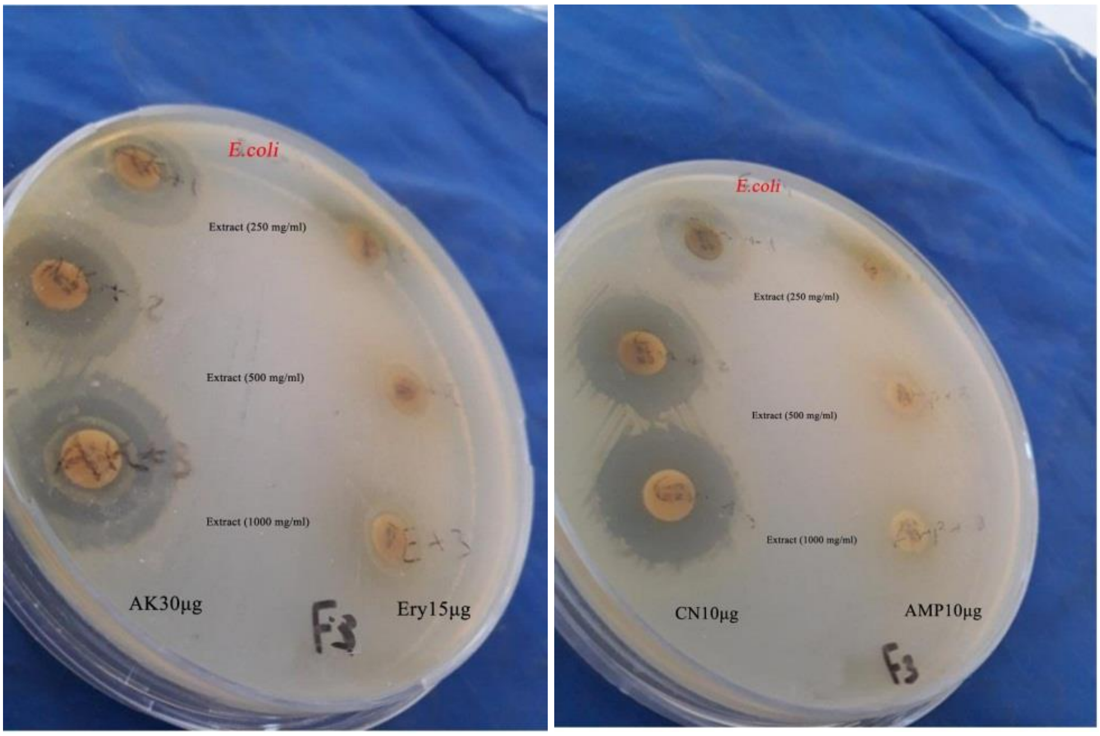
These results are in agreement with many studies that have shown that when mixing plant extract with an antibiotic from the beta-lactam group, it gives a synergistic effect, especially on *E. coli* [25]. For instance, Ravula et al showed through their results that there is a significant effect of the test extract (methanolic extract of *Vernoniaanthelmintica* seeds MEVA) with the antibiotic streptomycin mixed against three strains of bacteria (*E. coli*, *P. aeroginosa, S. aureus*). When comparing the diameter of the inhibition zones in their study, there was a significant increase between the inhibition zone produced by streptomycin alone and that produced by the combination of the extract with streptomycin. The extract thus had an additional effect. This proves that streptomycin and seeds extract of *Vernoniaanthelmintica* have synergistic activity against these three types of bacteria [20]. In another related study, Olajuyigbe and Afolayan found synergistic effects resulting from the combination of *Acacia mearnsii* extract with many antibiotics, such as amoxicillin, ciprofloxacin, chloramphenicol, metronidazole and tetracycline against *E. coli* (ATCC 25922) bacteria. They concluded that the combination of the antibiotics cephradine, amoxicillin, tetracycline, and anise waste extract may be effective against microbes, especially bacteria that shows resistance to drugs as they showed a highly synergistic effect against *Escherichia coli* [26]. A positive result was seen with the combination of chloramphenicol and amoxicillin with the SAWRE-facing *Escherichia coli* bacteria. Furthermore, the capacity of tetracycline has been raised by the SAWRE, so it might be become more effective against the isolates than more resistance of the *Escherichia coli* [27], However, the *Escherichia coli* has shown a resistance to the essential oil of *Pimpinella anisum* [28].

The synergistic effect might be produced by the creation of a special complex more effective at inhibiting a specific strain of pathogen; either by inducing its death or lysis, or by inhibition of the synthesis of the cell wall [29]. Indeed, several scientists have studied and experimented with the synergistic effects of mixing antibiotics with many different plant extracts [30]. In the present study, the combination of anise seeds extract with antibiotics showed significant antibacterial effectiveness against the Gram negative bacteria *E. coli*. The increased concentrations of anise extract were more effective in terms of growth inhibition and viability of *E. coli*, so the anise seed extract may act as a modulating agent for antibiotic activity.

Table 3: Minimal inhibition zone of different antibiotics after the association of the anise seeds extract against *Escherichia coli* bacteria

	Zone of inhibition in mm		
	Concentrations of extraction		
Antibiotic	**Extract (250 mg/ml)**	**Extract (500 mg/ml)**	**Extract (1000 mg/ml)**
Ampicillin	10	R	R
Gentamycin	20.1	20.1	20.2
Erythromycin	11.2	11.2	11.2
Amikacin	21.1	21.2	21.5

Fig 3: Antibacterial efficacy of a combination of anise seed extract and antibiotics against *Escherichia coli* bacteria



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REFERENCES

[1] Al-Terehi, M. N., Al Saadi, A. H., Zaidan, H. K., Al Ameri, Q. M. A., & Ewadh, M. J. (2012). In vivo study of antimutagenic and antioxidant activity of Glycyrrihza glabra root extract. Research in Pharmacy.

[2] Gibbons, S. (2004). Anti-staphylococcal plant natural products. Natural product reports, 21(2), 263-277.

[3] Nwaogu, L. A., Alisi, C. S., Ibegbulem, C. O., & Igwe, C. U. (2007). Phytochemical and antimicrobial activity of ethanolic extract of Landolphia owariensis leaf. African Journal of Biotechnology, 6(7).

[4] Aligiannis, N., Kalpoutzakis, E., Mitaku, S., & Chinou, I. B. (2001). Composition and antimicrobial activity of the essential oils of two Origanum species. Journal of agricultural and food chemistry, 49(9), 4168-4170

[5] Mbwambo, Z. H., Moshi, M. J., Masimba, P. J., Kapingu, M. C., & Nondo, R. S. (2007). Antimicrobial activity and brine shrimp toxicity of extracts of Terminalia brownii roots and stem. BMC complementary and Alternative Medicine, 7(1), 9.

[6] Adwan, G., Abu-shanab, B., & Adwan, K. (2009). In vitro activity of certain drugs in combination with plant extracts against Staphylococcus aureus infections. African journal of biotechnology, 8(17).

[7] Kalaivani, R., Devi, V. J., Umarani, R., Periyanayagam, K., & Kumaraguru, A. K. (2012). Antimicrobial Activity of Some Important Medicinal Plant oils against Human Pathogens. Journal of Biologically Active Products from Nature, 2(1), 30-37.

[8] Betoni, J. E. C., Mantovani, R. P., Barbosa, L. N., Di Stasi, L. C., & Fernandes Junior, A. (2006). Synergism between plant extract and antimicrobial drugs used on Staphylococcus aureus diseases. Memórias do Instituto Oswaldo Cruz, 101(4), 387-390.

[9] González-Lamothe, R., Mitchell, G., Gattuso, M., Diarra, M. S., Malouin, F., & Bouarab, K. (2009). Plant antimicrobial agents and their effects on plant and human pathogens. International journal of molecular sciences, 10(8), 3400-3419

[10] Nikolić, M., Vasić, S., Đurđević, J., Stefanović, O., & Čomić, L. (2014). Antibacterial and anti-biofilm activity of ginger (Zingiber officinale (Roscoe)) ethanolic extract. Kragujevac Journal of Science, (36), 129-136.

[11] Mohamed, H. S. A. A., Abdelgadir, W. S., & Almagboul, A. Z. (2015). In vitro antimicrobial activity of Anise seed (Pimpinella anisum L.). Int J Adv Res, 3(1), 359-67.

[12] Ahmad, I., & Beg, A. Z. (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of ethnopharmacology, 74(2), 113-123.

[13] Prashanth, D., Asha, M. K., & Amit, A. (2001). Antibacterial activity of Punica granatum. Fitoterapia, 72(2), 171-173.

[14] Ghosh, A., Das, B. K., Roy, A., Mandal, B., & Chandra, G. (2008). Antibacterial activity of some medicinal plant extracts. Journal of Natural Medicines, 62(2), 259-262.

[15] Naji, T., & Jassemi, M. (2010). Comparison between antibacterial effects of ethanolic and isopropyl: hexan (7: 3) extracts of Zingiber officinale Rose. World Acad. Sci. Eng. Technol, 4, 673-676.

[16] Al-Kassie, G. A. M., Mohammed, M. F., Hamood, M. F., & Jameel, Y. J. (2008). The Effect of Anise and Rosemary on the Microbial Balance. International Journal of Poultry Science, 7(6), 610-612.

[17] El-Shanawany, M. A. (1996). Medicinal plants used in Saudi traditional medicine. King Abdel Aziz City for Science and Technology, Riyadh, 144.

[18] Deshpande, S. N. (2013). Preliminary Phytochemical Analysis and In Vitro Investigation of Antibacterial Activity Of Acacia Nilotica Against Clinical Isolates. Journal of Pharmacognosy and phytochemistry, 1(5).

[19] Sanhueza, L., Melo, R., Montero, R., Maisey, K., Mendoza, L., & Wilkens, M. (2017). Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against Staphylococcus aureus and Escherichia coli. PLoS One, 12(2), e0172273.

[20] Ravula, G., Goluguri, S. R., & Konanki, K. (2012). Evaluation of antibacterial effect of vernonia anthelmintica seed extract and its synergistic effect with antibiotics on resistant bacterial strains. International Journal of Pharmacognosy and Phytochemical Research, 4(3), 79-81.

[21] Odonkor, S. T., & Addo, K. K. (2018). Prevalence of multidrug-resistant Escherichia coli isolated from drinking water sources. International journal of microbiology, 2018.

[22] Ilić, T., Gračan, S., Arapović, A., Čapkun, V., Šubat-Dežulović, M., & Saraga, M. (2011). Changes in bacterial resistance patterns in children with urinary tract infections on antimicrobial prophylaxis at University Hospital in Split. Medical science monitor: international medical journal of experimental and clinical research, 17(7), CR355.

[23] Rind, R., & Khan, T. S. (2000). Antibiogram sensitivity of bacterial organisms identified from surgical and non-surgical wounds of Animals. Pak. J. Biol. Sci, 3(10), 1719-1723.

[24] Fazlani, S. A., Khan, S. A., Faraz, S., & Awan, M. S. (2011). Antimicrobial susceptibility of bacterial species identified from mastitic milk samples of camel. African Journal of Biotechnology, 10(15), 2959-2964.

[25] Sibanda, T., & Okoh, A. I. (2008). In vitro evaluation of the interactions between acetone extracts of Garcinia kola seeds and some antibiotics. African Journal of Biotechnology, 7(11).

[26] Olajuyigbe, O. O., & Afolayan, A. J. (2012). Synergistic interactions of methanolic extract of Acacia mearnsii De Wild. with antibiotics against bacteria of clinical relevance. International journal of molecular sciences, 13(7), 8915-8932.

[27] Ibrahim, M. K., Mattar, Z. A., Abdel-Khalek, H. H., & Azzam, Y. M. (2017). Evaluation of antibacterial efficacy of anise wastes against some multidrug resistant bacterial isolates. Journal of Radiation Research and Applied Sciences, 10(1), 34-43.

[28] Al-Bayati, F. A. (2008). Synergistic antibacterial activity between Thymus vulgaris and Pimpinella anisum essential oils and methanol extracts. Journal of ethnopharmacology, 116(3), 403-406.

[29] Ahmed, Z., Khan, S. S., Khan, M., Tanveer, A., & Lone, Z. A. (2010). Synergistic effect of Salvadora persica extracts, tetracycline and penicillin against Staphylococcus aureus. African Journal of Basic & Applied Sciences, 2(1-2), 25-29.

[30] Ibezim, E. C., Esimone, C. O., Nnamani, P. O., Onyish, I. V., Brown, S. A., & Obodo, C. E. (2006). In vitro study of the interaction between some fluoroquinolones and extracts of kola nitida seed. African Journal of Biotechnology, 5(19).