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Antibacterial effect of ethanolic extracts of Moroccan plant against *Escherichia coli*

H. Douhri[1,*], I. Raissouni[1], N. Amajoud[2], O. Belmehdi[2], M. Benchakhtir[3], S. Tazi[1], J. Abrini[2], B. Douhri[2]

[1] *Materials and Interfacial systems Laboratory, Department of Chemistry, Faculty of Science, Abdelmalek Essaadi University, BP 2121, Tetuan, Morocco.*

[2] *Biology and Health Laboratory, Department of Biology, Faculty of Science, Abdelmalek Essaadi University, BP 2121, Tetuan, Morocco.*

[3] *Regional Epidemiologic Laboratory, Regional Direction of Health, Tetuan, Morocco.*

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- ✓ *Escherichia coli*;
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H. Douhri
douhri.enviro@gmail.com
+2125 39 97 24 23

Abstract

Rural populations of Morocco are still taking their water supplies from unprotected sources such as wells and springs that are usually contaminated. In order to protect citizens against diseases, water treatment is crucial. Moreover, the use of chlorine as disinfectant may produce toxic compounds (trihalomethanes) that have health risks. It is therefore necessary to find alternative methods of water disinfection. Our investigation was focused on the evaluation of efficiency of seven Moroccan plant extracts against a fecal bacterium: *Escherichia coli* (gram negative) found in the prospected springs. The *in vitro* antibacterial studies were carried out using ethanolic extracts of pomegranate (*Punica granatum* L.) and bitter orange (*Citrus* x *aurantium* L.) seed as well as fig (*Ficus carica* L.), oregano (*Origanum elongatum* (Bonnet) Emb. & Maire), lemon balm (*Melissa officinalis* L.), thyme (*Thymbra capitata* (L.) Cav.) and myrtle (*Myrtus communis* L.) leaves. The results showed that the ethanolic extract of *Origanum elongatum* leaves was the most effective against *Escherichia coli* with 30.33±2.51 mm of diameter of inhibition zone. Besides, the ethanolic extracts of *Myrtus communis, Thymbra capitata* leaves and *Punica granatum* seeds had also a significant antibacterial activity against *Escherichia coli* with diameters of inhibition of 20.16±0.76, 11.33±1.15 and 17.33±4.50 mm respectively. On the other hand, preliminary phytochemical screening showed that the most active extracts contains a significant quantity of phenols. Phenolic contents for these extracts ranged between 100.69±3.40 and 136.83±1.03 (mg GAE / g of extract).

1. Introduction

Access to safe water is an essential need to prevent health risks. It has been reported that there are approximately 663 million of people that lack access to improved drinking water and about 1.6 million people die every year from diarrhoeal diseases [1]. High occurrence of diarrhea is linked to the polluted drinking water and it is mainly manifest in children, seniors and immunocompromised individuals [2].

Most of rural populations all over the world are dependent on traditional water sources that they use without treatment. To make it safe for human consumption, water treatment is crucial [3, 4]. Even if conventional disinfection methods used in drinking water treatment contribute to the control of bacterial pathogens, investigations have revealed that the use of chlorine as disinfectant may produce toxic compounds having health risks such as cancer, hemolytic anemia and/or nervous system trouble. This situation forced the scientists to search for alternative methods of water disinfection [5, 6].

Antibacterial properties of natural substances and plant extracts were more and more reported. They have been tested on removal microorganisms, and water conditioning for human consumption [7]. In fact, plants have important antibacterial and antioxidant properties, being a rich source of compounds such as phenolics, flavonoids, terpenoids and alkaloids [8-10]. Besides, aromatic plants and their extracts have antibacterial, antifungal and antiviral properties [11, 12].

Previous researches have reported that there is a relationship between the chemical compounds and the antimicrobial activity of the plants. Indeed, Al-Mariri and Safi [11] have shown significant antibacterial activities of several plant extracts (*Origanum syriacum* L., *Thymus syriacus* Boiss., *Syzygium aromaticum* (L.)

Merr. & L. M. Perry, *Cinnamomum zeylanicum* Blume, *Laurus nobilis* L., *Juniperus foetidissima* Willd., *Allium sativum* L. and *Myristica fragrans* Houtt) against the gram-negative bacteria.

Moreover, strong antibacterial and antifungal activities of extract and compounds isolated from aerial parts of *Brillantaisia lamium* (Nees) Benth. were shown by Tamokou *et al* [13]. On the other hand, Borchardt *et al* [14] have also demonstrated wide antioxidant and antimicrobial activities of seeds from plants of the Mississippi river basin (such as *Spiraea tomentosa* L. and *Lythrum salicaria* L.*).* Given its geographical position, Morocco is reputed by a rich botanical wealth. Among the 7000 species and subspecies of the Moroccan plants, about 537 are endemic [15, 16]. Ethnobotanical data on these plants were indicated by several studies [15].

The common myrtle (*Myrtus communis*, Myrtaceae), is one of the most famous plants in Morocco [17]. It is traditionally used as an antiseptic, disinfectant and hypoglycemic agent [15, 18]. Pomegranate (*Punica granatum*, Punicaceae), is among the most important fruit cultivated in Morocco [19]. It is reputed to be a natural source of bioactive compounds with a large spectrum of bioactive properties, including anti-oxidant and antimicrobial ones [20, 21]. The common fig tree (*Ficus carica*, Moraceae), is one of the sources of remedies used in traditional medicine in Morocco [15]. Oregano (*Origanum elongatum*, Lamiaceae), is an endemic species of Morocco [16]. It is an important multipurpose medicinal plant usually useful against respiratory infections, diarrhea, urinary tract infections, and it's also used for food preservation or as an aromatic plant for its flavor [15].

The thyme (*Thymbra capitata*, Lamiaceae), is one of the most widespread North African species [22]. It is largely used for flavoring foods and culinary preparations and in folk medicine [16]. The bitter orange (*Citrus* x *aurantium*, Rutaceae), has a wide range of uses in traditional medicine and food industry [23]. The lemon balm (*Melissa officinalis*, Lamiaceae), is native to Southern Europe and the Mediterranean region. It's has many beneficial effects such as anti-bacterial, sedative, anxiety reduction and gastrointestinal treatment [24, 25].

Since ancient times, aromatic and medicinal plants of Morocco have been used in cuisine, cosmetics and traditional remedy. However, only a few species of these plants are valorized currently [18, 26].

For water supply, rural population of Morocco still prefers traditional water sources (especially springs and wells) without any treatment [27, 28]. So, the valorization of Moroccan plants in water treatment as an alternative to the chemicals has become an important priority.

The aim of this study was to evaluate the efficiency of ethanolic extracts of seven Moroccan plant: pomegranate (*Punica granatum*) and bitter orange (*Citrus* x *aurantium*) seeds as well as fig (*Ficus carica*), oregano (*Origanum elongatum*), lemon balm (*Melissa officinalis*), thyme (*Thymbra capitata)* and myrtle (*Myrtus communis*) leaves against *Escherichia coli* found in some springs of the Northern Morocco.

2. Experimental details

2.1. *Plant material*

Materials of seven plant species used in this research (Table 1) were collected in April 2016 from different locations of the north of Morocco. Taxonomic identification of plant material was authenticated by Professor Mohamed KADIRI (Laboratory of Algology and Mycology, Department of Biology, Faculty of Science, Abdelmalek Essaadi University, Tetuan, Morocco).

Table 1: Locations and parts of plants used in this study.

Sampling location	Common name	Botanical name	Plant parts used
Bni Aammart (Rif region)	Oregano	*Origanum elongatum*	Leaves
Jbel Ghorghiz (Tetuan region)	Myrtle	*Myrtus communis*	Leaves
Nekkata (Tetuan region)	Thyme	*Thymbra capitata*	Leaves
Bouzaghlal (M'diq region)	Fig	*Ficus carica*	Leaves
Rabat region	Lemon balm	*Melissa officinalis*	Leaves
Bouanane (Tetuan region)	Pomegranate	*Punica granatum*	Seeds
Bab Okla (Tetuan region)	Bitter orange	*Citrus* x *aurantium*	Seeds

2.2. *Preparation of ethanol extracts*

Plant materials were air dried under the shade and milled using an electric grinder (mesh size 50µm). Then, each plant powder sample was extracted with ethanol (with ratio of 25 mg in 100 ml solvent) with shaking at 150 rpm for 72 hours at room temperature. The extracts were filtered through a Whatman filter paper (0.2 µm pore size, 11 cm diameter) and the solvent was eliminated using a rotary vacuum evaporator (Heidolph, Laborota 4000) to obtain dry extracts. Extracts were then stored (at -12ºC) in obscurity until use for antibacterial studies.

2.3. *Biochemical characterization of Escherichia coli*

The indicator bacteria used in this research were isolated from seven water samples of springs from locations with different degrees of environmental quality in Northern of Morocco. After purification, cultures of fecal coliform bacteria were subjected to detailed biochemical study in order to confirm *Escherichia coli*. In fact, bacteria have been identified as described by Kumar and Sristava [29] using the following tests: indole production, methyl red test, Voges-proskauer and citrate reactions (Table 2).

Table 2: Biochemical characterization of bacteria.

Organisms	Citrate test	Indole test	Methyl Red test	Voges-Proskauer test
Escherichia coli	-	+	+	-
Enterobacter aerogenes	+	-	-	+
Escherichia freundii	+	-	+	-
Shigella spp.	-	-	+	-
Klebsiella pneumoniae	+	-	-	+
Klebsiella oxytoca	+	+	-	+
Serratia marcescens	+	-	-	+
Proteus vulgaris	-	+	+	-
Proteus mirabilis	+	-	+	-
Citrobacter freundii	+	-	+	-
Citrobacter koseri	+	+	+	-

Adapted from Kumar and Sristava [29]

2.4. *Antibiotic resistance test*

The confirmed *Escherichia coli* isolates (from water samples) were subjected to the antibiotic susceptibility test. The disk diffusion test was carried out on the isolates according to the National Committee for Clinical Laboratory Standards, USA [30]. Strains were maintained on an inclined agar medium at 4°C. Before use, the bacteria were revived by two subcultures in an appropriate culture medium at 37°C for 12 hours (final inoculum concentrations 10^6 CFU/ml). The inoculum suspension of each bacterial strain was swabbed on the entire surface of freshly prepared Muller-Hinton Agar. Susceptibility to the following antibiotic discs was tested in *Escherichia coli* strains: Tobramycin (30 µg), Amikacin (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Nalidixic Acid (30 µg), Amoxicillin (10 µg), Cefotaxime (5 µg), Ceftazidime (10 µg), Cefoxitin (30 µg), Amoxiclav (30 µg) and Cephalothin (30 µg). Plates with antibiotic discs were then incubated at 37°C for 24 hours and sensitivity compared to the control culture.

2.5. *Antibacterial Activity Assay*

The confirmed *Escherichia coli* isolates (from water samples) were also subjected to antibacterial assay of plant extracts. In fact, the antibacterial activity was evaluated by agar-well diffusion assay making a basal layer with 10 ml of Muller-Hinton Agar [31]. After the solidification of agar plates, sterile 8mm diameter cylinders were placed. Then, 6 ml of LB medium in superfusion containing 0.8% agar were inoculated by a fresh culture of *Escherichia coli* (final concentration 10^6 CFU/ml). Plant extracts were diluted in ethanol (250 µg / ml). After solidification of the medium, wells were filled with 50 µl of diluted extracts. After incubation at 4°C for 2 hours, the plates were transferred at 37°C for 24 h.

Posteriorly, the plates were examined for bacterial growth inhibition (indicated by a clear zone around the wells). The size of any inhibition zone was measured and the antibacterial activity was expressed in terms of their average diameter of inhibition zones. The absence of such a zone was interpreted as the absence of inhibitory activity. Each extract was tested in triplicate.

2.6. *Phytochemical properties of extracts*

The reconstituted extracts were examined for the polyphenol contents by using Folin-Ciocalteu method as described by Singleton and Rossi [32]. First, the extracts were diluted (1 mg / ml). Then, 100 µl of each diluted extract were placed in test tubes and 500 µl of Folin-Ciocalteu reagent (previously diluted 10 times in distilled water) was added. After incubation for 1hour at room temperature, 2ml of 2% sodium carbonate solution (Na_2CO_3) were added to the mixture. The tubes were then mixed and placed in dark for 30 minutes at room temperature. Thereafter, the absorbance was read at 760 nm.

2.7. *Statistical Analysis*

Our results were expressed as mean ± standard error of triplicate. The statistical significance between phenolic content and antibacterial effect of extracts was evaluated by one-way ANOVA test using GraphPad Prism software version 6.00 (San Diego, CA, USA). The chosen level of significance was $P < 0.05$.

3. Results and discussion

3.1. *Biochemical characterization of Escherichia coli*

The IMViC tests are commonly used in bacterial identification [33, 34]. The present investigation highlights the identification of *Escherichia coli* and summarizes the results of biochemical tests (IMViC tests) in the Table 3. In fact, data showed that the indole, citrate, Methyl Red (MR) and Voges-Proskauer (VP) reactions were typical of *Escherichia coli* in more than 25% of tested strains. On the other hand, our results showed also the presence of other strains such as *Escherichia freundii* (6%), *Enterobacter aerogenes* (3%), *Citrobacter koseri* (3%), *Klebsiella oxytoca* (3%) and *Shigella* spp. (3%).

This is probably due to wastewater infiltrations, human and⁄or animal pollution. Similar results were found by many investigators such as Odonkor and Ampofo [35] and Arshad *et al* [36]. Coleman [37] has highlighted the correlation between the gastrointestinal deseases and the consumption of contaminated water in Ontario.

Table 3: Results of biochemical tests (IMViC tests).

Samples	Sample 1					Sample 2					Sample 3					Sample 4	
Strains	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17
Citrate	+	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	+
Indole	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+
MR	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-
VP	-	-	+	+	-	+	+	+	+	-	-	-	+	-	+	+	+

Table 3 (Continued)

Samples	Sample 4			Sample 5					Sample 6					Sample 7				
Strains	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35
Citrate	-	-	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+
Indole	-	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	+	-
MR	+	+	+	+	-	+	+	+	-	-	-	+	+	-	+	+	+	+
VP	-	-	+	-	-	+	-	+	+	-	-	-	+	-	+	+	-	-

(MR) Methyl Red test; (VP) Voges Proskauer test

(+) positive reaction ; (-) negative reaction.

3.2. *Antibiotic resistance test*

Thanks to the external covering around the cell wall, Gram-negative bacteria are more resistant to antibiotics than Gram-positive [38]. Antibiotic resistance rates in *Escherichia coli* continue to proliferate and constitute a significant danger to citizen's health [37, 39]**.** In this study, a disk diffusion test with isolates of *Escherichia coli* from culture was carried. Results for the antibiotic sensitivity test against twelve antibiotics are exposed in Table 4. The diameters of inhibition zones were measured and values interpreted according to categories of susceptible or resistant.

The antibiogram results of *Escherichia coli* isolates revealed resistance to more than one antimicrobial agent commonly used. Four, one, two and three isolates were resistant to cefoxitin (30 µg), ceftazidime (10 µg), cephalothin (30 µg) and amoxicillin (10 µg) in the same order. Therefore, cefotaxime (5 µg) was the most effective antibacterial agent against *Escherichia coli* isolates. Recent investigations have reported the multi-resistance of *Escherichia coli* to antibiotics [40]. Similar observations have been reported by Coleman [37]. Kaur and Awari [41] have shown a resistance of *Escherichia coli* and *Klebsiella* spp. to multiple antibiotics. Our findings were similar to those obtained by Wambugu *et al* [42] in *Escherichia coli* strains isolated from different sections of Athi River Water (Kenya). Such resistance was also shown by Fanuncio and Nuñeza [39] in *Escherichia coli* strains isolated from water hand pumps (Philippines).

Table 4: Variation of the susceptibility testing of *Escherichia coli* isolates (Results are expressed as mean±standard error).

Antibiotics / Strains	Amoxicillin (10 µg)	Cephalothin (30 µg)	Amoxiclav (30 µg)	Cefotaxime (5 µg)	Chloramphenicol (30 µg)	Ceftazidime (10 µg)
	Diameters of inhibition zones (mm)					
E. coli 1	3.93±0.11	7.92±0.13	5.02±0.06	69.96±1.05	4.86±0.32	7.06±0.11
E. coli 2	2.06±0.12	0.00±0.00	2.83±0.28	29.67±0.57	11.93±0.28	7.85±0.25
E. coli 3	6.02±0.06	5.89±0.18	3.16±028	7.16±0.28	14.03±0.05	5.93±0.11
E. coli 4	11.9±0.17	8.92±0.12	10.33±0.57	15.5±0.86	11.96±0.06	8.88±0.20
E. coli 5	0.00±0.00	5.86±0.36	3.00±0.01	13.55±0.77	14.9±0.17	7.99±0.01
E. coli 6	0.00±0.00	0.00±0.00	10.18±0.31	25.33±1.15	9.9±0.17	10.34±0.70
E. coli 7	13.96±0.06	2.77±0.48	14.62±0.54	10.41±0.52	15.03±0.05	0.00±0.00
E. coli 8	0.00±0.00	4.83±0.28	4.16±0.28	11.00±0.01	1.92±0.12	6.16±0.28
E. coli 9	4.80±0.34	0.00±0.00	5.8±0.34	13.01±0.52	15.13±0.23	4.99±0.01

Antibiotics / Strains	Tobramycin (30 µg)	Ciprofloxacin (5 µg)	Cefoxitin (30 µg)	Amikacin (30 µg)	Gentamicin (10 µg)	Nalidixic Acid (30 µg)
	Diameters of inhibition zones (mm)					
E. coli 1	7.10±0.36	7.13±0.23	0.00±0.00	5.9±0.17	7.00±0.10	8.90±0.05
E. coli 2	16.03±0.35	19.96±2.05	0.00±0.00	15.83±0.28	18.03±0.05	17.99±0.11
E. coli 3	7.90±0.17	10.03±0.05	4.84±0.26	6.96±0.05	10.90±0.17	9.98±0.03
E. coli 4	7.93±0.20	0.83±0.15	0.00±0.00	7.03±0.05	8.93±0.11	8.96±0.05
E. coli 5	9.06±0.11	9.50±0.50	2.06±0.11	6.96±0.06	7.95±0.08	2.03±0.05
E. coli 6	10.80±0.34	22.50±1.32	0.00±0.00	15.83±0.28	17.66±0.57	15.83±0.28
E. coli 7	4.90±0.17	12.37±0.54	2.21±0.25	4.70±0.26	8.90±0.17	3.81±0.28
E. coli 8	7.73±0.25	4.60±0.40	7.83±0.28	1.77±0.39	8.03±0.05	87.89±0.27
E. coli 9	7.80±0.23	19.16±0.76	5.83±0.28	7.9±0.36	11.13±0.23	11.80±0.34

3.3. *Antibacterial activity assay*

The *in vitro* antibacterial activity of the studied plant extracts against *Escherichia coli* was evaluated quantitatively and qualitatively by the presence or absence of inhibition zones. Our results (Table 5) showed a significant inhibitory effect exhibited by the ethanolic extract of *Origanum elongatum* leaves with the diameter of 30.33±2.51 mm (the largest inhibition zone).

Table 5: Antibacterial activity of the studied plant extracts against *Escherichia coli* (Results are expressed as mean±standard error).

Plant extracts	**Diameters of inhibition zones (mm)**
Origanum elongatum	30.33±2.51
Citrus x *aurantium*	0.00±0.00
Myrtus communis	20.16±0.76
Punica granatum	17.33±4.50
Ficus carica	0.00±0.00
Melissa officinalis	0.00±0.00
Thymbra capitata	11.33±1.15

Moreover, the ethanolic extract of *Myrtus communis* and *Thymbra capitata* leaves, and *Punica granatum* seeds demonstrated also a strong antibacterial activity against *Escherichia coli* with diameters of inhibition of 20.16±0.76, 11.33±1.15 and 17.33±4.50 mm respectively. These antibacterial activities were very highly significant ($P<0.001$). Nevertheless, the other three plants (*Citrus* x *aurantium*, *Ficus carica* and *Melissa officinalis*) did not have bacteriostatic and bactericidal activities. Therefore, this may be due to the resistance of bacteria strains tested (*Escherichia coli*).

Several researches had attributed the antibacterial activity of extracts to the hydrophobic character of phenolic content [20, 43- 45]. Our results confirm the findings of Saeidi *et al* [46] concerning the alcoholic extract of *Myrtus communis* against *Escherichia coli*. However, they are not in agreement with results found by Al Askari *et al* [47] that showed an important antimicrobial activity of phenolic compounds of the ethanolic extracts of leaves of *Ficus carica* collected from different regions in Morocco against *Escherichia coli*.

3.4. *Determination of total phenolic content*

Among the ethanolic plant extracts investigated, total phenolic content varied widely from plant to another and ranged from 23.20±1.24 to 136.83±1.03 (mg GAE / g of extract).

In fact, our results (Figure 1) showed that the ethanolic extracts of *Origanum elongatum* and *Myrtus communis* leaves contain a significant quantity of phenols: 136.83±1.03 and 134.92±0.48 (mg GAE / g of extract) respectively. On the other hand, values of polyphenols contents were about 105.11±1.89 (mg GAE / g of extract) for *Thymbra capitata*, 100.69±3.40 (mg GAE / g of extract) for *Punica granatum*, 52.99±1.26 (mg GAE / g of extract) for *Ficus carica*, 24.05±0.71 (mg GAE / g of extract) for *Citrus* x *aurantium* and 23.20±1.24 (mg GAE / g of extract) for *Melissa officinalis*. The variation of polyphenol contents was very highly significant (P<0.001).

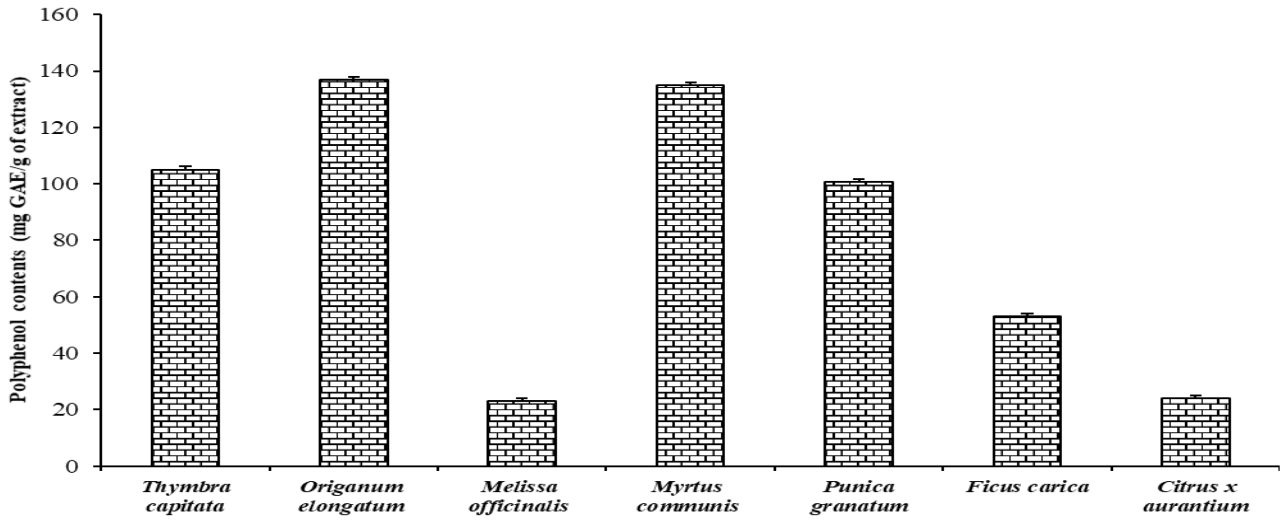

Figure 1: Polyphenol contents in the studied plant extracts (Each point represents the mean ± standard error).

The quantity of phenolic compounds found in the studied extracts should explain their different antibacterial effects. It's has been reported that a total phenolic content higher than 20 mg GAE/g dry weight could be considered as very high [48].

Conclusions

Our results showed that there is an obvious correlation between the phenolic compounds level and the antibacterial activity. We can conclude that the ethanol extracts of *Origanum elongatum*, *Myrtus communis*, *Thymbra capitata leaves*, and *Punica granatum seeds* were very effective against the fecal bacteria *Escherichia coli* (gram negative). So, the use of these extracts in water treatment seems to be very promising. Nevertheless, we suggest that additional research should be conducted to carry out *in vivo* studies of its active components of these plant extracts, and to elucidate their mode of action.

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