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Orginal Article

Combinatory *in vitro* effect of plant extracts with antibiotics on multi-resistant bacteria

Raoudha Dziri[1,2], Imen hammadi[1], Eya Agreby[1], Mayssa Trabelsi[1], Abderrazak Maaroufi[1]

[1] *Laboratory of Epidemiology and Veterinary Microbiology, Group of Bacteriology and Biotechnology, Institute Pasteur of Tunis, University of Tunis El Manar, Tunis, Tunisia*
[2] *Laboratory of Microorganisms and Active Biomolecules, Faculty of Sciences of Tunis, University of Tunis El Manar, Tunis, Tunisia*

Abstract

Introduction: Antimicrobial resistance is recognized as one of the major health challenges. Thus, urgent therapeutic solutions are needed. This study aims to test the activity of plant extracts against multi-resistant bacteria, as well as the synergistic effect of these extracts with some antibiotics.

Methodology: The evaluation of the antibacterial effect of eight medicinal extract plants (*G. alypum*, *R. graveolens*, *U. dioica*, *P. lentiscus*, *A. vulgaris*, *L. angustifolia*, *T. vulgaris,* and *J. phoenicea*) against 10 bacterial strains (*K. pneumoniae, E. coli*, *C. freundii*, *S. haemolyticus*, *S. epidermidis*, *S. saprophyticus*) has been performed using both wells and disks diffusion methods (DDM/WDM). The evaluation of the synergistic effect of some of the natural extracts with some antibiotics has been performed using the disk diffusion method (DDM).

Results: A significant difference resulting from the effect of various plant extracts on different bacterial species has been observed. Interestingly, an important inhibition zone related to the effect of the essential oil of *T. vulgaris* and *L. angustifolia* was observed in all bacterial strains. The combination of plants/antibiotics does not always give a more effective effect than the antibiotic /or the plant extract alone. The lavender oil seems to be able to enhance the activity of ertapenem on *C. freundii,* while the combination of *A. vulgaris*/ertapenem induced the reduction of the inhibition zone on the same species.

Conclusions: These results are of great importance; regarding the valorization of natural resources for the creation of solutions to urgent health problems while taking advantage of existing pharmaceutical resources.

Key words: Multi-resistant bacteria; natural plant extracts; combinatory effect; antibiotics.

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Introduction

Multi-resistant bacteria, particularly those resistant to last-resort antibiotics, pose a major health problem nowadays with very limited or even unavailable therapeutic options, thus constituting a real and alarming threat to human and animal health [1]. The overuse and misuse of antibiotics are the primary cause of the spread of many resistant pathogenic bacteria, which results in the reduction of the therapeutic arsenal, the delay in the implementation of an effective treatment, and the increased risks of complications and mortality [2-4]. According to a systematic analysis elaborated by Antimicrobial Resistance Collaborators (ARC), multidrug-resistant bacteria were directly responsible for 1.27 million global deaths in 2019 and contributed to 4.95 million deaths [5]. The situation is getting worse and worse, especially after the emergence and the dissemination worldwide of bacterial strains resistant to last-resort antibiotics, including carbapenem and colistin-resistant bacteria [6,7].

Current research is based on the recent boom in phytotherapy, which offers the opportunity to discover the curative virtues hidden in the natural environment, as well as the discovery of new biomolecules with extended antibacterial activity and which seem safer than the use of synthetic products [8,9]. Plants have been used in the past, indiscriminately, for the treatment of certain diseases, which confirms the richness of this natural treasure of beneficial and active substances [10]. Recent studies are focusing on the medicinal characteristics of these plants to resolve the actual health problems. These plants contain various metabolites or secondary components that are responsible for their pharmacological effectiveness and toxic effects on both humans and animals. These secondary metabolites are also potential sources of new medications [11]. Likewise, some combination strategies are adopted against bacterial infections [12], but they are still rarely used in treatment protocols due to the lack of information and research on this topic.

Methodology

Plant Sampling

Eight different medicinal plants are included in our study, notably *Globularia alypum*, *Ruta graveolens*, *Urtica dioica*, *Artemisia vulgaris*, *Lavandula angustifolia*, *Thymus vulgaris*, *Juniperus phoenicea*, and *Pistacia lentiscus*. The plant leaves were collected from two different regions in Tunisia, Gafsa in the southwest of the country and Zaghouan in the northeast of the country, in January and March 2023, respectively. Some samples were provided in March 2023 in the form of natural hydrolate and essential oil from a team of Tunisian women from the Zaghouan region working on the extraction of medicinal and aromatic plant derivatives. All information, including the origin and the nature of the samples, is included in Table 1.

Bacterial strains

The bacterial strains included in this study belong to different species, including *Escherichia coli* (C7288, C7293), *Klebsiella pneumoniae* (SM13, SM16, SM25), *Citrobacter freundii*(C197), *Staphylococcus haemolyticus (*C8202, C8206), *Staphylococcus epidermidis* (C8217), and *Staphylococcus saprophyticus* (C8207), and are previously characterized as multidrug-resistant strains [13-16].

Evaluation of the effect of various plant samples on multi-resistant bacteria using agar well diffusion method (WDM)

This method consists of preparing a bacterial suspension of 0.5 McFarland, which is done by taking a few perfectly identical colonies of bacteria from a pure 24-hour culture and inoculating them in 5 mL of sterile physiological water, followed by a step of homogenization of the bacterial suspension by vortex and a step of inoculation on Müller Hinton agar. This step is carried out by swabbing, which consists of soaking a sterile cotton swab with the bacterial suspension, then rubbing it on the surface of the Mueller-Hinton agar so as to form very tight streaks by rotating the plate and finally passing the swab over the periphery to obtain equal distribution of the inoculum. Then, the antibacterial activity of plant extracts is carried out by creating wells on the medium on which the bacteria were cultivated and then filling them with plant extracts.

Evaluation of the effect of various plant samples on multi-resistant bacteria using the disk diffusion method (DDM)

After the preparation and inoculation of the bacterial suspension of 0.5 McFarland, the sterilized white disks are placed on the culture Muller Hinton plates, then pressed using sterile bacteriological tweezers and impregnated with the plant extracts.

Effect of plant extract/antibiotic synergistic activity on multi-resistant bacteria

The tests of the synergistic effect of plants/antibiotics are carried out using the disk diffusion method on Muller-Hinton agar. The antibiotic disks are placed on the culture plates, pressed using sterile bacteriological tweezers, and then impregnated with plant extracts. The antibiotics, as well as the plant extracts included for the evaluation of the plant/antibiotic synergistic effect, are shown in Table 2.

Antibacterial activity interpretation

The reading of antibacterial activity was evaluated by measuring the diameters of the inhibition halos around the discs/wells using a ruler with precision in millimetres; then, we classified plant extracts as having either high activity, medium activity, low activity, or no antibacterial activity. The reading and interpretation of the results of the antibiotic/plant synergy test were done by measuring the zone of inhibition of the antibiotic alone and comparing it to the zone of inhibition of the antibiotic impregnated with the extracts of the plants.

Results

The current study focuses on the evaluation of the

Table 1. The origin and the nature of the collected natural plant samples.

Name of the plant sample	Origin	Nature of the sample
Globularia alypum	The mountains of Mthilla (Gafsa)	Plant leaves
Ruta graveolens	The Forest of Zaghouan	Natural hydrolate
Urtica dioica	The Forest of Zaghouan	Natural hydrolate
Artemisia vulgaris	The Forest of Zaghouan	Plant leaves/Natural hydrolate/Essential oil
Lavandula angustifolia	The Forest of Zaghouan	Plant leaves/Natural hydrolate/Essential oil
Thymus vulgaris	The mountains of Zaghouan	Plant leaves/Natural hydrolate/Essential oil
Juniperus phoenicea	The mountains of Zaghouan	Plant leaves/Natural hydrolate/Essential oil
Pistacia lentiscus	The Forest of Zaghouan	Essential oil

Table 2. Antibiotics and plant extracts included for the evaluation of the synergistic effect on multi-resistant bacteria.

Plates/Strains	**Antibiotics**	**Plant Extracts**
Escherichia coli (B9/ C7288; B10/ C7293)	Cefotaxime (CTX)	Essential oil of *Lavandula angustifolia,* Aqueous extract of *Juniperus phoenicea,* Natural hydrolate of *Artemisia vulgaris,* Natural hydrolate of *Urtica dioica*
Klebsiella pneumoniae (B6/SM25; B7/SM16; B8/SM13)	Ertapenem (ETP)	Essential oil of *Lavandula angustifolia,* Aqueous extract of *Juniperus phoenicea,* Natural hydrolate of *Artemisia vulgaris,* Natural hydrolate of *Urtica dioica*
Citrobacter freundii (B16/C197)	Ertapenem (ETP)	Essential oil of *Lavandula angustifolia,* Natural hydrolate of *Artemisia vulgaris*
Staphylococcus haemolyticus (B17/ C8202; B18/ C8206)	Oxacillin (OXC), Cefoxitin (FOX)	Natural hydrolate of *Urtica dioica*
Staphylococcus epidermidis (B20/ C8217)	Oxacillin (OXC), Cefoxitin (FOX)	Natural hydrolate of *Urtica dioica*
Staphylococcus saprophyticus (B21/ C8207)	Oxacillin (OXC), Cefoxitin (FOX)	Natural hydrolate of *Urtica dioica*

antibacterial effect of various extracts of eight medicinal plants (*Globularia alypum*, *Ruta graveolens*, *Urtica dioica*, *Pistacia lentiscus*, *Artemisia vulgaris*, *Lavandula angustifolia*, *Thymus vulgaris* and *Juniperus phoenicea*) against 10 bacterial strains belonging to various bacterial species, the effect of each plant extract by the two methods (DDM/WDM) was reflected by the presence or absence of an inhibition halo around the disks/wells and were expressed in terms of diameter of inhibition zone in millimeters (mm) as shown in Table 3. The results obtained showed a significant difference between the zones of inhibition resulting from the effect of various plant extracts on different bacterial species. Results vary from one extract to another and from one species to another. Along the same lines, some differences were also related to the method used (Table 3). Interestingly, an important inhibition zone was observed in all bacterial strains and was mainly related to the effect of the essential oil of *Thymus vulgaris* and the essential oil of *Lavandula angustifolia*. The other plant extracts showed variable results depending on the species, the nature of the plant extract, and the method used in this study. The natural hydrolate of *Artemisia vulgaris* showed an appreciable activity mainly against *Klebsiella pneumoniae, Staphylococcus haemolyticus, Staphylococcus epidermidis*, *and Staphylococcus saprophyticus.* Whereas, the natural hydrolate of *Urtica dioica* showed less activity with lower inhibition diameters (18mm; 20mm/DDM) against only *Staphylococcus haemolyticus* and *Citrobacter freundii,* and seems not to be effective against the other tested bacterial species.

The natural hydrolate of *Ruta graveolens* showed an inhibition zone with WDM against *Citrobacter freundii* and *Staphylococcus saprophyticus*. Additionally, an appreciable activity of the aqueous extract of *Globularia alypum* was observed by the DDM on all bacterial species included in this study. It should be noted that relatively significant differences in the results obtained by the two techniques used (discs and wells) for the evaluation of the effect of plant extracts on the strains were observed. Some extracts showed a difference in the importance of the inhibition diameters between those obtained by the disc method compared to the well method. We suggest, that these differences could be related to several factors such as the diffusion of the substances tested, and their action on the bacterial species involved, nevertheless, there is

Table 3. Diameter (mm) of the inhibition zones of plant extracts by the disc diffusion method (DDM) and by the well diffusion method (WDM).

Plate name	**Plant Extracts**																
	***Artemisia vulgaris* (Natural hydrolate)**		***Thymus vulgaris* (Essential oil)**		***Lavandula angustifolia* (Essential oil)**		***Pistacia lentiscus* (Essential oil)**		***Globularia alypum* (Aqueous extract)**		***Juniperus phoenicea* (Aqueous extract)**		***Urtica dioica* (Natural hydrolate)**		***Ruta graveolens* (Natural hydrolate)**		**T-**
	DDM	**WDM**	**DDM**	**WDM**	**DDM**	**WDM**	**DDM**	**WDM**	**DDM**	**WDM**	**DDM**	**WDM**	**DDM**	**WDM**	**DDM**	**WDM**	
B6	TI[a]	TI	TI	TI	TI	34	TI	0	TI	0	0	22	TI	0	0	0	0
B7	18[b]	15	TI	TI	TI	32	TI	0	TI	0	0	14	12	0	0	0	0
B8	0	0	TI	TI	18	17	0	0	TI	0	0	0	0	0	0	0	0
B9	TI	0	TI	TI	TI	0	TI	0	18	0	0	0	0	0	0	0	0
B10	16	0	TI	TI	TI	34	TI	0	TI	0	18	0	20	0	0	0	0
B16	17	25	TI	TI	26	26	TI	0	TI	0	0	0	0	0	0	TI	0
B17	TI	20	TI	TI	TI	24	TI	0	TI	18	0	14	18	0	0	0	0
B18	TI	TI	TI	TI	TI	35	TI	0	TI	0	0	TI	20	0	0	0	0
B20	TI	26	TI	TI	TI	30	TI	0	TI	0	12	0	0	0	0	0	0
B21	TI	30	TI	TI	TI	30	TI	TI	TI	15	0	0	12	0	0	20	0

B6: *Klebsiella pneumoniae* (SM25); B7: *Klebsiella pneumoniae* (SM16); B8: *Klebsiella pneumoniae* (SM13); B9: *Escherichia coli* (C7288); B10: *Escherichia coli* (C7293); B16: *Citrobacter freundii* (C197); B17: *Staphylococcus haemolyticus* (C8202); B18: *Staphylococcus haemolyticus* (C8206); B20: *Staphylococcus epidermidis* (C8217); B21: *Staphylococcus saprophyticus* (C8207). a Total inhibition; b Diameter in millimeters; DDM: Disc Diffusion Method; WDM: Well Diffusion Method.

no clear and concise scientific explanation especially for differences of the results related to the diffusion method used for the natural substances because of their variable composition influenced, often, by external conditions related to the cultivation conditions of these plants.

Combining current treatment strategies (antibiotics) with previously used strategies (natural substances) could be of great importance as one of the possible solutions in the treatment of infections with multidrug-resistant bacteria. So that, for the evaluation of synergistic effect (plants/antibiotics), the antibiotics used in this study were selected for strains that have proven to be resistant (Table 4). The combination tests carried out for carbapenem-resistant *Klebsiella pneumoniae* showed that the extract of the natural hydrolate of *Artemisia vulgaris* (An) induced a synergistic effect with ertapenem (ETP) and successfully inhibited the growth of the ertapenem-resistant *Klebsiella pneumoniae* strain, with a great diameter zone observed (44 mm). Indeed, this combination increased the inhibition zone by 26 mm compared to the inhibition diameter resulting from the activity of the plant extract alone (18 mm). Nevertheless, the combination of *Lavandula angustifolia* essential oil and ertapenem induced a 4 mm decrease in the inhibition diameter compared to that resulting from the essential plant oil activity alone (Table 4). The natural hydrolate of *Artemisia vulgaris* (An) appears to have a synergistic effect with cefotaxime on third-generation-cephalosporin-resistant *Escherichia coli,* with a slight increase of the inhibition diameter compared to that resulting from the effect of the plant extract alone, as shown in Table 4. Nevertheless, the combined effect of cefotaxime with aqueous extract of *Juniperus phoenicea* (J) and the natural hydrolate of *Urtica dioica* (Un) induced a reduction in the inhibition diameter, showing an inhibitory effect on the activity observed by the two plant extracts alone. The synergistic effect of ertapenem and essential oil of *Lavandula angustifolia* appears to be important with an increase in the inhibition diameter, resulting from the combination of this antibiotic and this plant extract, in contrast to the effect of the combination of ertapenem with the natural hydrolate of *Artemisia vulgaris*, which led to a significant reduction in the diameter of inhibition.

For methicillin-resistant Staphylococci species, combining the natural hydrolate of *Urtica dioica* (Un) with oxacillin showed that the zones of inhibition became narrower than those obtained by the plant extract alone, and this could be explained by the fact that Oxacillin abolished the effect of *Urtica dioica* (Un). While such a combination between *Urtica dioica* (Un) and cefoxitin showed an increase in the diameters of inhibition compared to those obtained by the plant extract alone.

Discussion

In response to the strong demand to return to nature, herbal medicine has received great interest in the world of biomedical research to enable the extensive use of medicinal plants in modern medicine. It is within this framework that this scientific research takes place while

Table 4. Results of the combination ATB/PE on various multi-resistant bacterial species by disk diffusion method.

	Klebsiella pneumoniae **(Carbapenem-resistant strains)**	***Citrobacter freundii*** **(Carbapenem-resistant strain)**	***Escherichia coli*** **(3GC resistant strains)**	***Staphylococcus spp*** **(Methicillin resistant strains)**
ATB	ETP [d = 10 ± 01 (Plates B6/B7/B8)]	ETP [d = 12 (Plate B16)]	CTX [d = 0 (Plates B9/ B10)]	OXC [d = 0 (Plates B17/ B18/B20/B21)] FOX [d = 16/11/11/15 Plates B17/ B18/B20/B21)]
PE	Lh [d = TI (Plate B6) /d = 18(Plate B8)]	Lh [d = 26 (Plate B16)]	J [d = 18 (Plate B10)]	Un [d = 12 (Plate B17)]
	An [d = 18 (Plate B7)]	An [d = 18 (Plate B16)]	Un [d = 20 (Plate B10)]	Un [d = 20 (Plate B18)]
	J [d = 0 (Plates B6/B7)]		An [d = 16 (Plate B10)]	Un [d = 0 (Plate B20)]
	Un [d = 12 (Plate B7)]			Un [d = 12 (Plate B21)]
PE + ATB	Lh + ETP [d > 35 (Plate B6) / 12 (PlateB8)]	Lh + ETP [d = 35 (Plate B16)]	J + CTX [d = 0 (Plate B10)]	Un + OXC [d = 0 (Plates B17/ B18/B20/B21)]
	An + ETP [d = 44 (Plate B7)]	An + ETP [d = 12 (Plate B16)]	Un + CTX [d = 0 (Plate B10)]	Un + FOX [d = 16 (Plate B17)]
	J + ETP [d = 0 (Plates B6/B7)]		An + CTX [d = 19 (Plate B10)]	Un + FOX [d = 12 (Plate B18)]
	Un + ETP [d = 0 (Plate B7)]			Un + FOX [d = 10 (Plate B20)]
				Un + FOX [d = 15 (Plate B21)]

B6: *Klebsiella pneumoniae* (SM25); B7: *Klebsiella pneumoniae* (SM16); B8: *Klebsiella pneumoniae* (SM13); B9: *Escherichia coli* (C7288); B10: *Escherichia coli* (C7293); B16: *Citrobacter freundii* (C197); B17: *Staphylococcus haemolyticus* (C8202); B18: *Staphylococcus haemolyticus* (C8206); B20: *Staphylococcus epidermidis* (C8217); B21: *Staphylococcus saprophyticus* (C8207). ATB: Antibiotic; 3GC: Third generation cephalosporins; ETP: Ertapenem; CTX: Cefotaxime; OXC: Oxacillin; FOX: Cefoxitin; PE: Plant Extract; Lh: Essential oil of Lavandula angustifolia; An: Natural hydrolate of *Artemisia vulgaris*; J: Aqueous extract of *Juniperus phoenicea*; Un: Natural hydrolate of *Urtica dioica*.

focusing on the possibility of combining the virtues of nature with existing drugs in the treatment of bacterial infections, especially after the dissemination of multi-resistant bacteria with therapeutic impasses [17]. According to the results obtained in this research, the extracts of *Artemisia vulgaris*, *Lavandula angustifolia*, *Globularia alypum*, *Thymus vulgaris* and *Pistacia lentiscus* are the most active products that have shown an appreciable activity on most of the species included in this study, but with variable inhibition zones from one species to another, from one extract to another and also from one method to another (Discs/Wells), as mentioned by a previous research study showing that several factors could influence the results of in *vitro* tests regarding the activities of plant extracts [18]. The essential oil of *Thymus vulgaris* (Th), the aqueous extract of *Globularia alypum* (G), and the essential oil of *Lavandula angustifolia* exhibit strong antibacterial activity with significant inhibition zones on *Klebsiella pneumoniae* species. In the same lines, several studies have reported the efficacy of *Lavandula* oil extract on *Klebsiella pneumoniae* [19-21] and it has been also reported that this antibacterial activity of the essential oil of *Lavandula* was related to the fact that the most of its components are lipophilic, which favours the penetration and accumulation of hydrophobic *Lavandula* essential oil in the phospholipid bilayer of the cell membrane of microbes [22,23].On the other hand, the natural hydrolate extract of *Urtica dioica* and *Ruta graveolens* showed no effect against this species. According to a study conducted by a Romanian team, the oil extract of *Ruta graveolens* showed an antimicrobial activity classified as moderate, with an inhibition zone ranging from 15.21 ± 0.14 mm to 20.61 ± 0.21 mm [24].

Regarding *Escherichia coli* species, a significant inhibitory effect was observed for *Lavandula angustifolia* (Lh) and *Thymus vulgaris* (Th) oils; this does not exclude the effect of the other plant extracts, with inhibition zones ranging from 16 to 20 mm. These results are in agreement with a study published in 2006, which showed that the essential oil of *Thymus vulgaris* extracted from flowering tops in France had an antibacterial activity (inhibition diameter = 20 mm) on this species [25]. This same study also showed a high inhibition activity of *Pistacia lentiscus oil* with a complete inhibition zone against this species. This significant activity could be explained by the richness of this plant in polyphenols [26]. Our results also demonstrated an inhibitory activity of the aqueous extract of *Juniperus phoenicea* (J) on *E. coli*. The antimicrobial effect of *Juniperus phoenicea* has also been reported by a Moroccan team, showing a significant effect with an inhibition zone of 34 mm on *Escherichia coli* [27].

Regarding the effect of our plant extracts on *Citrobacter freundii*, the essential oil of *Thymus vulgaris* seems to be the most effective on *Citrobacter freundii* species, with a large inhibition zone. On the same lines, a very recent study conducted in Italy showed a bactericidal in *vitro* effect of the Essential oil of *Thymus* on several bacterial species, including *Citrobacter freundii* [28].

For *Staphylococci* species, all plant extracts seem to be effective except *Juniperus phoenicea*, *Urtica dioica*, and *Ruta graveolens*. This was at variance with a recent study showing a strong antimicrobial activity of *Ruta graveolens* on methicillin-resistant *Staphylococcus aureus* strains [29].

Although few studies have reported testing the combination of antibiotics and plant extracts, this may be of paramount importance in providing solutions for the treatment of extremely serious bacterial infections [30]. It has been reported that combinations of plant extracts together with antibiotics not only contribute and enhance the overall antimicrobial effect but may also act as a resistance-modifying/modulating agent [30].

Conclusions

These results are encouraging for expanding the therapeutic arsenal of medicinal plants with antibacterial activities, which could be used as alternatives to conventional antibiotics that have become ineffective. Moreover, the study of plant/antibiotic synergy remains a necessity in order to avoid any risk of cancelling the desired effect. Following these results, it would be interesting to deepen this study by identifying the adjuvants responsible for the antibacterial activity for each plant and determining the mechanisms of action of the latter to manufacture antibiotics based on plant for the treatment of infections caused by multi-resistant bacteria as well as the study of the toxicity of plants to adjust the exact therapeutic doses to avoid the damage associated with the use of bio-antibiotics and in particular an expanded study of synergies with other classes of antibiotics.

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Authors contributions

Raoudha DZIRI (Project administration, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft), Imen HAMMADI (Data curation, Formal analysis, Investigation, Methodology), Eya AGREBI (Formal analysis, Investigation, Methodology), Mayssa TRABELSI (Formal analysis, Investigation, Methodology), and Abderrazak MAAROUFI (Investigation, Visualization, Validation)

Corresponding author

Raoudha Dziri PhD
University of Tunis El Manar,
Farhat Hached University Campus
Postal code 1068 Tunis, Tunisia.
Tel: +21699880420
E-mail: rawdha_dziri@hotmail.fr

Conflict of interest

No conflict of interest is declared.

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