Evaluation of Antimicrobial Properties of Moroccan Plant-Derived Essential Oils

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Abstract

The aim of this work is the *in vitro* evaluation of the antibacterial activities of essential oils extracted from three aromatic and Moroccan plants: *Thymus vulgaris*, *Mentha spicata* and *Citrus limonum*, on four bacterial strains: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aerations* and *Klebsiella pneumoniae*. These strains have been chosen for their pathological impact due to their pathogen city in relation to problems of resistance to common antibiotics. The results obtained showed that for the strains of *Escherichia coli* and *Pseudomonas aerations*, the essential oil of *Thymus vulgaris*, showed the highest antibacterial activity, while for the strains of *Staphylococcus aureus* and *Klebsiella pneumonia*, the essential oil of *Menthe spicata* which proved to be the most active.

Introduction

The history of aromatic and medicinal plants is associated with the evolution of civilizations. In all regions of the world, plants have always occupied an important place in medicine. Medicinal plants contain one or more active ingredients capable of preventing, relieving or curing diseases (Schoenberg and Paris, 2006). Currently, the use of plant essences from aromatic and medicinal plants can affects several fields such as cosmetics, perfumery and the pharmaceutical industry. These essential oils (EO) are mixtures of aromatic substances produced by aromatic plants and appear as tiny droplets in leaves, fruit skin, branches and wood (Padrini and Lucheroni, 2006). These are mixtures of lipophilic compounds and they are distinguished from vegetable fixed oils by their chemical volatility (Regnault-Roger and *et al*., 2012).

The aromatic and medicinal plants sector represents an important commercial activity in Morocco, which exports the equivalent of 250 million dirhams of these plants to the USA and the European Union. Essential oils alone account for about 165 million dirhams and are estimated to be in full growth (Bencheqroun, 2012). The antimicrobial properties of essential oils in aromatic and medicinal plants have been recognized for a long time but have been confirmed scientifically only with the evolution of technical and analytical means. Among these plants are *T*. *vulgaris*, *C*. *limonum* and *M. spicata*, which have considerable advantages thanks to the progressive discovery of their applications in health and their uses in other areas of economic interest by the World Health Organization. They have important therapeutic potential for curing several infectious diseases (Janovska *et al.*, 2003).

The aim of this work is to study the antibacterial activity of the essential oils extracted from the three aromatic species of Moroccan origin: *Thymus vulgaris*, *Menthe spicata* and *Citrus limonum* on four strains: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aerations* and *Kalispell pneumonia*.

Materials and methods

Plant material

T. vulgaris samples were collected from Tafilelt, *C. limonum* in the province of Agadir and *M*. *spicata* from the province of Settat. The pickings were carried out during the month of April. The

leaves of *T. vulgaris*, *M. spicata* and the fruits of *C. limonum* were cut by hand and placed in bags, transported immediately to the laboratory and stored at room temperature until use.

Biological model

Four bacterial strains: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiell apneumoniae* were used to carry out this work. These strains were taken from the Averroes laboratory of medical analysis in Settat, Morocco.

Preparation of essential oils

The oils were prepared by hydro distillation using a Clevenger apparatus containing 2 liters of distilled water and surmounted by a column (60cm in length and 2cm in diameter) connected to a refrigerant. 500g of the leaves of each plant was used to extract the essential oils of *T. vulgaris* and *M. spicata*. On the other hand, 700g of *C. limonum* bark were used for the preparation of essence. After removing traces of water with anhydrous sodium sulfate, the EO obtained were stored in small, opaque bottles and placed in a refrigerator at 4°C until used.

Bacterial Culture Medium

The culture mediums used in this work were supplied by the Averroes laboratory of medical analyzes at Settat, Morocco.

• *The Muller-Hinton medium*

The Muller-Hinton medium is the most widely used for susceptibility testing to antibacterial agents (Mueller and Hinton, 1941).

• *The Chapman medium*

The Chapman agar is the selective medium of halophilic bacteria and more particularly ferments mannitol (Chapman, 1945).

• *The CLED medium*

The CLED medium (Cystine Lactose Electrolyte Deficient) is a non-selective medium, widely used in the study of bacteria. Being a nonselective medium of many bacteria, both Gram (+) and Gram (-), will be able to develop (Sandys, 1960).

Confirmation of bacterial strains

For each of the strains, a pre-enrichment was carried out on the selective medium, then a fresh examination and a Gram staining were made:

• *Fresh examination of colonies*

One colony was removed by a loop and one observation was made in a drop of water between theslide and coverslipunder a microscope. These observations concern the mobility and shape of bacteria.

• *Examination after Gram staining* A bacteria smear was prepared, then subjected to air drying and flame fixation. Then, the smear was covered with the purple crystal and then rinsed after 1 min with distilled water. The blade is covered with Lugol and then rinsed after 1 min with distilled water. It is covered with ethanol and then rinsed after 30 seconds with distilled water and covered with fuchsin and rinsed after 1 min with distilled water. Finally, the bacterial smear was dried and observed under an optical microscope (x 100). Some bacteria will appear in dark purple and will be said to Gram (+), others will appear in pale pink and will be said to Gram (-) (Morgan and Huttenhower, 2012).

Aromatogram technique

It is a method of in vitro measurement of the antibacterial activity of essential oils. Different types of aromatograms, in solid, liquid, are exploitable. However, the solid medium is the simplest and most easily reproducible (El Amri *et al*., 2014). This technique is identical of the antibiogram, the only difference is the replacement of antibiotics by essential oils.

• *Preparation of the bacterial suspension*

A colony outcome from a 24 hour culture and was introduced by a handle into 5 ml of sterile physiological water (0.9%) contained in a test

tube. After the homogenization of the bacterial suspension, the opacity must be equivalent to 0.5 McFarland.

• *Seeding*

Seeding was carried out 15min after preparation of the inoculum in a Muller-Hinton medium, dipping a sterile swab into the bacterial suspension and rubbing it over the entire agar surface, in tight striations and the operation was repeated twice, turning the Petri dish 60° each time.

• *Impregnation of the discs*

The discs required for the production of the aromatogram technique are made from Waxman paper with a diameter of 6 mm and then placed in a test tube and sterilized in an autoclave. These disks were impregnated with 5 µl of each dilution: 1/5, 1/10, 1/20, 1/25, 1/30, 1/50, 1/75, 1/100, 1/125, as well as control. In order to obtain these different dilutions, DMSO

(dimethylsulfoxide) was used, since this solvent is preferable for the majority of authors, in particular Gachkar *et al.* (2007) that proved that DMSO has no antibacterial activity.

• *Incubation of the Petri dishes*

Once the Muller-Hinton medium is inoculated, the impregnated disks of each EO are placed on the surface of the agar using a bunsen spout sterilized forceps. Finally, the Petri dishes were incubated for 24 hours at 37°C and the inhibition diameters surrounding the disks were measured. The results are expressed by the diameters of the zones of inhibition, the threshold of which is 15mm. If the diameter of the zone is below this threshold, the strain is considered resistant and beyond that the strain is called sensitive (Zhiri, 2006).

Results and discussion

Fresh examination of colonies, gram staining and microscopic observation

The results of the fresh examination and microscopic observations after the gram staining of the bacterial strains studied are shown in Table 1.

Antibacterial activities of the essential oils studied We tested the antibacterial activities of essential oils of *T. vulgaris*, *M. spicata* and *C. limonum* using the aromatogram method. The results obtained are presented in tables 2, 3, 4 and 5 and in Fig.s 1, 2, 3 and 4.

Table 1. Results of microscopic observations and gram staining of the bacterial strains studied.

Bacterial strains	Culture medium	Gram	Microscopic morphology
Staphylococus aureus	Chapman	+	Coccies
Escherichia coli	CLED	-	Bacilles
Pseudomonas aureginosa	CLED	-	Bacilles
Klebsiella pneumoniae	CLED	-	Bacilles

Table 2. Means of inhibition diameters (mm) ± standard deviation.

	(v(µl)/v(µl))	*T. vulgaris*	*M. spicata*	*C. limonum*
E. coli	1/125	15.50 ± 0.33	9.57 ± 0.38	0
	1/100	17.17 ± 0.22	11.67 ± 0.22	0
	1/75	19.77 ± 0.18	13.50 ± 0.33	6.70 ± 0.22
	1/50	20.80 ± 0.53	20.47 ± 0.36	9.17 ± 0.56
	1/30	23.13 ± 0.18	21.47 ± 0.36	12.70 ± 0.20
	1/25	30.20 ± 0.27	24.87 ± 0.42	13.67 ± 0.44
	1/20	31.17±0.22	25.83 ± 0.56	15.73 ± 0.22

1/10	32.53 ± 0.36	30.20 ± 0.80	17.87 ± 0.18
1/5	41.50 ± 0.33	36.17 ± 0.22	19.43 ± 0.38

Dilutions Means of inhibition diameters (mm) ± standard deviation Bacterial strains

The diameter of the discs is included (6mm).

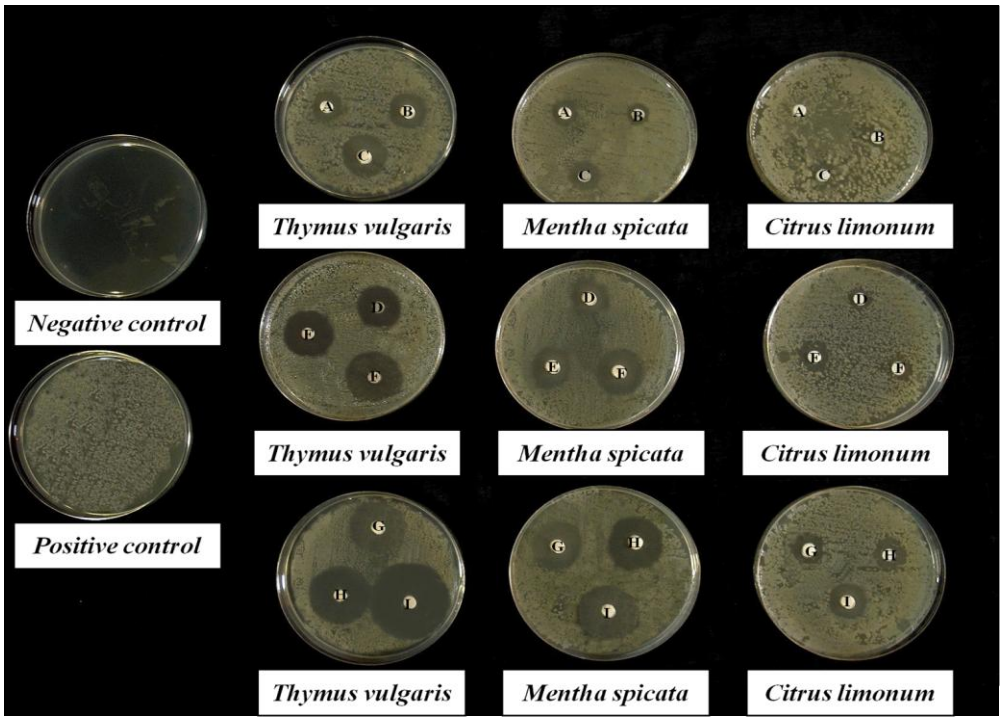

Fig. 1. Activities of various essential oils on the growth of *Escherichia coli*. (A: 1/125; B: 1/100; C: 1/75; D: 1/50; E: 1/30; F: 1/25; G: 1/20; H: 1/10; I: 1/5).

Case of *Staphylococcies aureus*

Table 3. Means of inhibition diameters (mm) ± standard deviation.

Bacterial strains	(v(µl) /v(µl))	*T. vulgaris*	*M. spicata*	*C. limonum*
S. aureus	1/125	15.13±0.20	15.20±0.36	13.17±0.88
	1/100	17.20±0.20	18.17±0.42	15.15±0.33
	1/75	20.17±0.10	21.53±0.33	16.67±0.10
	1/50	22.87±0.44	22.20±0.88	17.87±0.10
	1/30	23.83±0.22	23.43±0.33	18.83±0.67
	1/25	25.20±0.33	25.67±0.22	20.73±0.56

1/20	25.67±0.56	26.13±0.10	18.67±0.88
1/10	28.73±0.88	29.70±0.44	19.20±0.44
1/5	29.43±0.80	32.17±0.20	20.13±0.22

Dilutions	Means of inhibition diameters (mm) ± standard deviation

The diameter of the discs is included (6mm).

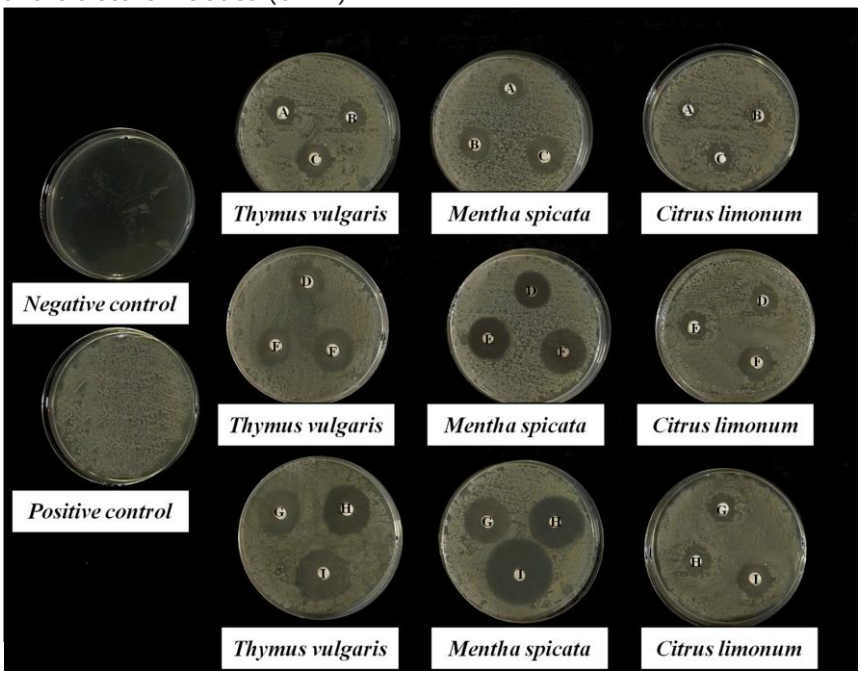

Fig. 2. Activities of various essential oils on the growth of *Staphylococcus aureus.* (A: 1/125; B: 1/100; C: 1/75; D: 1/50; E: 1/30; F: 1/25; G: 1/20; H: 1/10; I: 1/5).

Case of Pseudonymes aeruginosa

Table 4. Means of inhibition diameters (mm) ± standard deviation.

Bacterial strains	Dilutions (v(µl) /v(µl))	Means of inhibition diameters (mm) ± standard deviation		
		T. vulgaris	*M. spicata*	*C. limonum*

P. aeruginosa	1/125	20.20±0.88	15.20±0.10	12.87±0.56
	1/100	22.13±0.24	17.67±0.33	13.73±0.20
	1/75	23.83±0.22	20.67±0.42	15.13±0.67
	1/50	25.13±0.44	21.20±0.56	16.43±0.44
	1/30	26.20±0.10	25.73±0.88	17.20±0.36
	1/25	27.43±0.36	26.83±0.56	19.67±0.42
	1/20	41.73±0.67	27.44±0.44	20.17±0.22
	1/10	45.17±0.20	29.13±0.20	22.20±0.33
	1/5	50.67±0.56	30.20±0.67	23.13±0.10

The diameter of the discs is included (6mm).

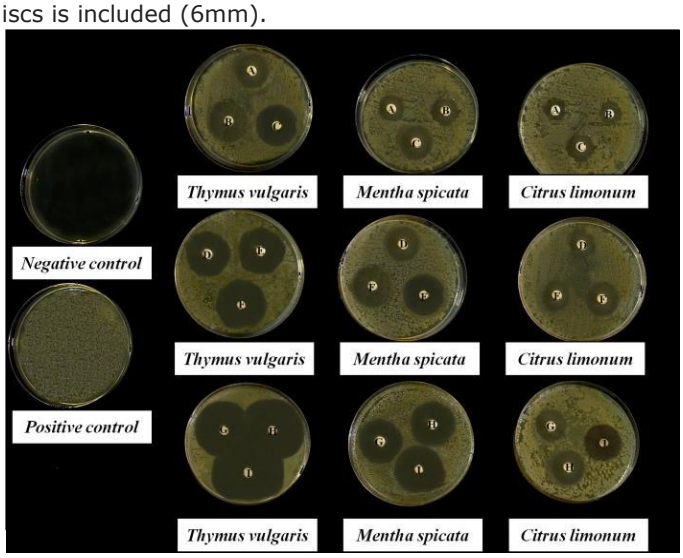

Fig. 3. Activities of various essential oils on the growth of *Pseudomonas aeruginosa.* (A: 1/125; B: 1/100; C: 1/75; D: 1/50; E: 1/30; F: 1/25; G: 1/20; H: 1/10; I: 1/5).

Case of Klebsiella pneumoniae

Table 5. Means of inhibition diameters (mm) ± standard deviation.

Bacterial strains	Dilutions (µl) /v(µl))	Means of inhibition diameters (mm) ± standard deviation		
		T. vulgaris	*M. spicata*	*C. limonum*
K. pneumoniae.	1/125	13.20±0.44	16.13±0.10	11.43±0.67
	1/100	14.73±0.88	19.20±0.33	14.17±0.24
	1/75	16.67±0.33	20.20±0.36	15.20±0.56
	1/50	17.13±0.56	21.83±0.56	16.13±0.22
	1/30	19.43±0.22	25.20±0.44	17.73±0.20
	1/25	20.43±0.36	30.17±0.22	18.43±0.33
	1/20	22.83±0.20	32.13±0.24	20.83±0.88
	1/10	32.20±0.24	35.73±0.67	21.67±0.36
	1/5	38.17±0.10	40.43±0.88	25.20±0.44

The diameter of the discs is included (6mm).

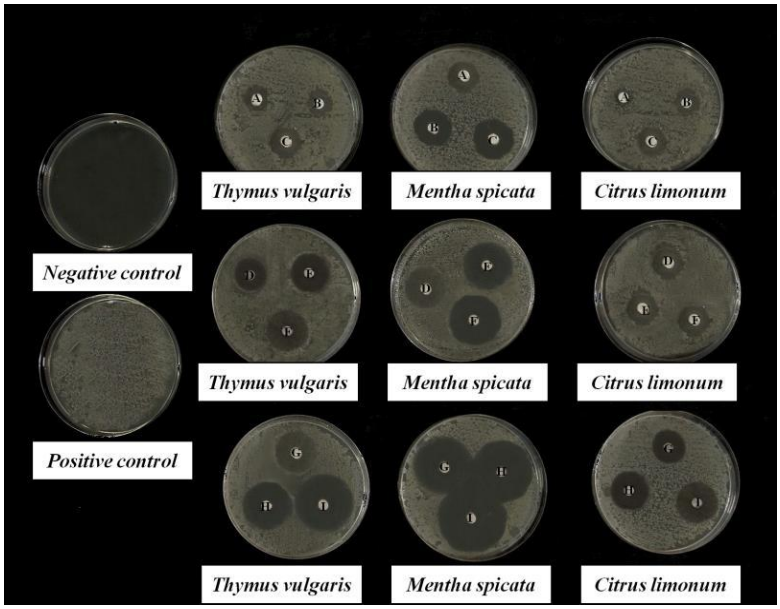

Fig. 4. Activities of various essential oils on the growth of *Klebsiella pneumoniae.* (A: 1/125; B: 1/100; C: 1/75; D: 1/50; E: 1/30; F: 1/25; G: 1/20; H: 1/10; I: 1/5).

The results obtained during this work showed that the three essential oils extracted from aromatic plants: *T. vulgaris*, *M. spicata* and *C. limonum*, possess variable antibacterial activities on the strains studied: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

The means of the inhibition diameters obtained during the three replicates of each test and obtained at the 1/5 dilution showed that:

Escherichia coli

The essential oil of the *T. vulgaris* gave a strong antibacterial activity with an inhibition diameter of 41.50 ± 0.33 mm, followed by the essence of *M. spicata* which also showed a strong inhibition on the growth of this bacterial strain with an inhibition diameter of 36.17±0.22mm and finally the *C. limonum* oil gave an inhibition diameter of 19.43±0.38mm.

Staphylococcus aureus

The essence of *M. spicata* gave the highest antibacterial activity with an inhibition diameter of 32.17 ± 0.20 mm, followed by *T. vulgaris* oil with 29.43±0.80mm and finally *C. limonum* gave a low inhibition with 20.13±0.22mm.

Pseudomonas aeruginosa

The EO of *T. vulgaris* showed a very strong inhibitory activity on the growth of this strain with an inhibition diameter of 50.67±0.56mm, followed by *M. spicata* EO with a diameter of 30.20 ± 0.67 mm. While *C. limonum* oil gave the lowest antibacterial activity with a diameter of 23.13±0.10mm.

Klebsiella pneumoniae

The *M. spicata* oil gave the highest inhibitory activity on the growth of this bacterium with an inhibition diameter of 40.43 ± 0.88 mm followed by the EO of *T. vulgaris* with 38.17±0.10mm. While the essence of the *C. limonum* gave an inhibition diameter of 25.20±0.44mm.

The mechanism of action of essential oils depends primarily on the type and characteristics of the active components, in particular their hydrophobic property which allows them to penetrate the phospholipid bilayer of the bacterial cell membrane, causing a change of K^+, an action on energy metabolism (Gill and Holley, 2004), an interruption of the motor proton force of the cell membrane, a non-specific denaturation of the cytoplasm membrane of the cell membrane (Manou and *et al*., 1998, Nanasombat and

Wimuttigosol, 2011) and denaturation of proteins by phenolic compounds (Madigan and*al*., 1997). Other authors mention a relationship between the structure and the antibacterial activity of molecules that make up essential oils by the presence of certain functional groups: aldehyde and phenolic compounds are very active

(Friedman and *et al*., 2002). Phenolic structures are the most active, and because of the presence of the hydroxyl group and its position, aldehydes also have an antimicrobial activity, the presence of oxygen in ketones increases the antimicrobial properties of terpenes (Dorman and Deans, 2000). Certain phenolic compounds of essential oils interfere with membrane proteins of microorganisms such as the ATP ase enzyme either by direct action on the hydrophobic part of the protein or by interfering in the translocation of protons in the membrane preventing the phosphorylation of ADP. Essential oils can also inhibit the synthesis of DNA, RNA, proteins and polysaccharides (El Amri and *et al*., 2014).

In our work, the inhibitory activity of *T. vulgaris* EO on the growth of the bacterial strains studied could be due to the presence of thymol, which is known for its antibacterial activity. Guarda and *et al*. (2011) demonstrated that the antimicrobial nature of EO is related to their high phenolic content, particularly in thymol and carvacrol. Pinto and *et al*. (2006) reported that species of the Thymus genus, which are rich in phenols, exhibit a broad spectrum of activity on bacterial and fungal germs.

The phenolic compounds of this oil penetrate into the lipid bilayer and are positioned between the fatty acid chains. This deformation of the structure increases the membrane fluidity, resulting in a change in the passive permeability. The bacteria exposed to carvacrol, there is a decrease in intracellular ATP, but also a decrease in membrane potential.

As regards the botanical species *M. spicata*, several works have proved the richness of the *Mentha*genus in essential oils. In the literature, it is recognized that essential oils of several *Mentha* species including *M. spicata*, *M. piperita* and *M. pulegium* possess antimicrobial properties (Kaur and*al*., 2002; Daferera and *et al*., 2003). The essential oil of *M. spicata* is rich in carvone which constitutes a major component of this essence and is known for its antibacterial activities.

Finally, the essential oil of *C. limonum* is rich in limonene. This chemical component is recognized in the literature for its antibacterial properties (Mansouri and *et al*., 2011).

Conclusion

From the results obtained,the essential oils of *T. vulgaris*, *M. spicata* and *C. limonum* showed important inhibitory activities on the growth of the strains studied. These activities can be attributed to the richness of the chemical composition of these plant species in compounds known in the literature for their bactericidal properties. The detection of the performance of these three essential oils on the inhibition of the growth of bacterial strains: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aureginosa* and *Klebsiella pneumoniae* can lead to in-depth studies and prospects for their application as agents phytomedication and preservation of food.

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