



Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology

ISSN: 1019-763X (Print), 2305-0284 (Online)

<http://www.pakps.com>



CHEMICAL COMPOSITION, ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS AND LEAF EXTRACTS OF *MENTHA PULEGIUM* (L) AND *GLYCYRRHIZA FOETIDA* (DESF) AGAINST THE PHYTOPATHOGENIC BACTERIA

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ABSTRACT

This research was conducted to evaluate the components of the *Mentha pulegium* (L) and *Glycyrrhiza foetida* (Desf) that were extracted by hydro distillation and analyzed using GC-MS methods. They are spontaneous plants widespread in Gharb of Morocco. The aim of the study is to investigate the antibacterial activity of the essential oils and leaf methanolic extracts of these two medicinal plants against five phytopathogenic bacteria. The evaluation of the antimicrobial activity of the two species by estimating the diameter of the inhibition zone has shown that the essential oil of *M. pulegium* exhibited a higher antimicrobial activity than *G. foetida* which varied according to the sensitivity of the phytopathogenic strains. The results obtained revealed different degrees of sensitivity toward methanolic extract. However, the methanolic extract and the essential oil of *M. pulegium* are more active based on the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC). The antimicrobial activities of leaf extracts from the two plants highlighted in this study could justify their therapeutic use. These results should be strongly recommended as an alternative to a chemical compound that still represents the problem of toxic residues.

Keywords: Essential oils; Medicinal plant; Antibacterial activity; Leaves extracts; Phytopathogenic bacteria.

INTRODUCTION

Plant diseases are caused by living organisms (fungi, bacteria, viruses and nematodes) (Messiaen *et al.*, 1991). Phytopathogenic bacteria can live and proliferate. Although they are close to pathogenic germs of humans and animals, they are not able to cause disease on other hosts than plants. However, some of these diseases can have dreadful social and economic consequences; without exaggerating the risks, they must be carefully considered and developed appropriate parries to maintain their impact at an acceptable level (Paulin *et*

al., 2001). Facing the toxicity problem and taking into account the development and the promotion of organic agriculture by the major importers of Africa in this new world concerned about the health of consumers and to the preservation of the balance of the ecosystems, the ideal would be that the pesticides of the future are natural products biodegradable and able to interfere directly or indirectly with the metabolism of pathogens (Valnet, 1975). The therapeutic virtues of aromatic essences have been known since antiquity, but the interest in the scientific study of the power of aromatic and medicinal plants has increased only in recent years with the aim of seeking alternatives to chemical substances pose risks to human health and the environment (Maisuthisakul *et al.*, 2007).

Morocco, like other Mediterranean countries, is rich in medicinal and aromatic plants that are mostly used in

Submitted: March 14, 2019

Revised: May 17, 2019

Accepted for Publication: May 23, 2019

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traditional medicine to fight against several diseases (Ghourri *et al.*, 2013; Jamila *et al.*, 2014; Youbi *et al.*, 2016). The particular geographical situation of the zone of Gharb offers rich and diversified vegetation. Aromatic plants grow there spontaneously on isolated land and uncultivated plots. *Mentha pulegium* is a medicinal plant belonging to the family Lamiaceae. Plants of this family include about 160 genera and over 3000 species grown almost all over the world especially in the Mediterranean region (Jafarpour *et al.*, 2013). This species is known as a medicinal plant for its pharmacological and biological properties, it is used in the treatment of colds, sinusitis, cholera, food poisoning, bronchitis and tuberculosis (Shirazi *et al.*, 2004; Díaz-Maroto *et al.*, 2007). Several studies have examined the antimicrobial activity of this aromatic plant (Mahboubi and Haghi, 2008; Kanakis *et al.*, 2012). Indeed, the pulegone in essential oil has antibacterial activity (Hmiri *et al.*, 2011; Bouyahya *et al.*, 2017).

The genus *Glycyrrhiza foetida* (Desf) is a plant known as "Guenfdo" in western Morocco (Gharb). According to our survey carried out in 2012, this plant is widely used in traditional medicine by farmers in case of hepatic disorders in cattle, by the constitution of a bed of the fresh plant to which the calf must install 3 days with the change of these leaves every 24 hours.

Therefore, the chemical composition and *in vitro* bactericidal properties of *Mentha pulegium* (L) and *Glycyrrhiza foetida* (Desf) essential oils taken from this area have not yet been valorized; particularly for its antibacterial activity against pathogens agents. The objective of the present work is to study the chemical composition and to investigate, *in vitro*, the antibacterial activity of essential oils and extracts of *Mentha pulegium* (L) and *Glycyrrhiza foetida* (Desf) against phytopathogenic bacteria.

MATERIAL AND METHODS

Plant material and essential oil extraction: Harvesting of the two plants *Mentha pulegium* (L) and *Glycyrrhiza foetida* (Desf) was carried out from plant population of the Gharb zone (located in the northwestern part of Morocco) between April and July 2014. The two plants were identified at the Biodiversity and Natural Substances Laboratory of the Ibn Tofail University of Kenitra-Morocco, on the basis of the document "Practical Flora of Morocco Volume 1, 2 and 3". The plant material was then cut and then dried in the shade at room temperature for four weeks. Leaves were

subjected to steam distillation using a Clevenger-type apparatus. Briefly, the plant leaves were completely immersed in water and heated to boiling, after which the essential oil was evaporated together with water vapor and finally collected after decantation. The distillate was isolated and dried in a Rota-vapor to giving an oil. The oil was stored at 4 °C until the antimicrobial screening (Song *et al.*, 2009; Ait-Ouazzou *et al.*, 2011).

The yield of essential oils: The yield in OE is the ratio between the weight of the oil extracted and the weight of the plant material used, the yield is expressed as a percentage (%) is calculated by the following formula (Akrouit *et al.*, 2004):

$$R = (Ph/Pv) \times 100$$

R: Oil yield in %

Ph: The weight of oil in g

Pv: The weight of plant material in g.

Gas chromatography-mass spectrometry analysis:

The GC-MS unit consisted on a Shimadzu GC-2010 gas chromatograph, equipped with BP-5 capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm; SGE, Ltd.), and interfaced with a Shimadzu QP2010 Plus mass spectrometer (software version 2.50 SU1). Oven temperature was programmed, 60-200 °C, at 3 °C.min⁻¹, and then held isothermal for 5 min ; transfer line temperature, 300 °C; ion source temperature, 200 °C; carrier gas, helium, adjusted to a linear velocity of 36.5 cm.s⁻¹; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-400 u; scan time, 1 s. Components identification was carried out by comparison of their retention indices relative to C₉-C₂₀ n-alkanes on the BP-5 column confirmed by comparison of recorded mass spectra with those of a computer library (Shimadzu corporation library and NIST05 database/ ChemStation data system) and by comparing with authentic reference compounds whenever possible.

Preparation of methanolic extracts: A sample of 2.5g powder leaves was put in 25 ml flask and macerated with absolute methanol under magnetic agitation for 30 min. The extract was then stored at 4 °C during 24 h, filtered and the solvent was evaporated to dryness under reduced pressure at 50 °C using a rotary evaporator (Falleh *et al.*, 2008).

Preparation of aqueous extracts: Ten gram of powder was dissolved in 150 ml of distilled water and heated to reflux for 2h, after cold filtration; the filtrate was then evaporated to dryness under reduced pressure at 65 °C using a rotary evaporator (Majhenic *et al.*, 2007).

The bacterial strains tested: Five pathogenic strains *Pseudomonas savastanoi* pv. *savastanoi* (PSS 2066-1), *Clavibacter michiganensis* subsp. *michiganensis* (CMM 1616-3), *Pectobacterium carotovorum* subsp. *carotovorum* (PCC 2657-1)

Allorhizobium vitis (*A.vitis* s4) and *Agrobacterium tumefaciens* (*A. tumefaciens* c58) from the pathogenic collection of the laboratory of "Plant Bacteriology and Biological control "RUPP CRRA-Meknes" (Morocco) (Table 1).

Table 1. Description of *Bacterial* strains used in this study

Bacterial species	Strain	Plant of isolation	Origin	References
<i>Allorhizobium vitis</i>	S4	Black raspberry,	Hungary	Popoff <i>et al.</i> , 1984 .
<i>Agrobacterium tumefaciens</i>	C58	Cherry	USA	Kerstens <i>et al.</i> , 1973 .
<i>Pseudomonas savastanoi</i> pv <i>savastanoi</i>	2066-1	Olive plant	Morocco	Bouaichi <i>et al.</i> , 2015 .
<i>Clavibacter michiganensis</i> subsp. <i>Michiganensis</i>	1616-3	Tomato	Morocco	Laboratory of Phytobacteriology and Biocontrol- National Institute of Agronomic Research- Meknes.
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	2657-1	Potato	Morocco	Laboratory of Phytobacteriology and Biocontrol- National Institute of Agronomic Research- Meknes

Five pathogenic strains *Pseudomonas savastanoi* pv *savastanoi* (PSS 2066-1), *Clavibacter michiganensis* subsp. *michiganensis* (CMM 1616-3), *Pectobacterium carotovorum* subsp. *carotovorum* (PCC 2657-1) *Allorhizobium vitis* (*A.vitis* s4) and *Agrobacterium tumefaciens* (*A. tumefaciens* c58) from the pathogenic collection of the laboratory of "Plant Bacteriology and Biological control "RUPP CRRA-Meknes" (Morocco) (Table 1).

Evaluation of antibacterial activity by disk diffusion method: The antibacterial activity of the essential oils was determined by using the paper disk diffusion technique (Popoff *et al.*, 1984). From fresh colonies (18 to 24h old), a bacterial suspension was performed in sterile distilled water. The turbidity of this suspension is adjusted to 0.5 Mc Farland and then diluted to the serial dilution. Petri dishes containing YPGA medium (5g yeast extracts; 5 g bacto peptone; 10g glucose; 20g agar; distilled water to 1.0 l) were inoculated by Sterile disk (6 mm diameter) was soaked by 2 µl of essential oil and placed in the center of the medium. The Petri dishes are first left for 1 hour under-flow laminar cabinet for diffusion before to be incubated at 26 °C in the oven during of 24h to 48h. The negative control was a sterile disk soaked with 2 µl of sterile distilled water.

Antibacterial activity is determined by measuring the diameter of the inhibition zone around each disc. All tests were performed three repetitions.

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC): The microdilution method was developed to determine the minimum inhibitory

concentration (MIC) of the oil-based compounds (Ismaili *et al.*, 2004). The redox dye resazurin was used to determine the MIC of the samples of the essential oils of *M. pulegium* and *G. foetida* for a decreasing range of essential oils (0.5; 1; 2.5; 5; 10; 20 µl/ml), methanolic and aqueous extracts (1.67; 3.33; 6.66; 10; 13.33; 16.67 mg/ml). The use of a 0.2% (v/v) concentration of agar as a stabilizer overcame the solubilization problem between the oil and the medium while avoiding chemical emulsifiers (Remmal *et al.*, 1993). After incubation at 26°C, observation of the range provides access to the Minimum Inhibitory Concentration (MIC), which corresponds to the lowest concentration of extract or oil capable of inhibiting bacterial growth.

STATISTICAL ANALYSIS

The comparison of the results concerning the antibacterial activities of essential oils and extracts was carried out by SPSS.10 software. For this, we performed a one-way analysis of the variance ANOVA. Differences between means are considered significant at (P= 0.05).

RESULTS AND DISCUSSION

Yield and chemical composition: The duration of the extraction by steam distillation lasted three hours for *M. pulegium* and four hours for *G. foetida* in order to recover the maximum amount of essential oils. The yield of essential oils was 0.21% and 4.5% for *G. foetida* and *M. pulegium* respectively.

Qualitative characterization by using GC-MS of *M. pulegium* essential oil identified fifteen compounds (Table 2). The major compounds of essential oil of *M. pulegium* are Pulregone (35.21%), p-Menthone (19.49%), followed by Isopulegol (8.49%) and Menthol (6.73%). These results are

similar to numerous works already done in Morocco. The research presented by A Bouyahya *et al.*, (2017) showed that pulegone, 1,8-cineole and menthone are the major constituents of *M. pulegium* essential oil with 40.98%, 23.67% and 21.16% concentrations respectively. While, the study conducted by Hmiri *et al.*, (2011) revealed the abundance of pulegone (80.28%), 1,8-cineole (42.30%) and α -pinene (28.30%). This composition is relatively comparable to that of Sylvain Sutour in France, which characterized by the presence of the pulegone (87.7%),

pipériténone, (2.9%), menthone, (1.7%), limonene, (1.5%) and octan-3-ol, (1.1%) (Sutour, 2010). The *M. pulegium* essential oil studied in Iran by Zanjani *et al.*, (2015) showed very low levels of pulegone, cineole, and piperitonone. Their concentrations were 19.89%, 19.38% and 15.14% respectively. However, another chemotype in Iran exhibited a different composition compared with ours. The frequently found compound was piperitone (38.0%), piperitenone (33.0%), terpineol (4.7%), and pulegone (2.3%) (Mahboubi and Haghi, 2008).

Table 2. GC-MS data for essential oils components identified in *M. pulegium* and *G. feotid*

Compound	<i>M. pulegium</i> (%)	<i>G. feotida</i> (%)
α - Pinene	0.97	-
3-Octanol	3.18	-
Limonene	4.40	-
1-Terpinenol	1.12	-
p-Menthone	19.49	-
Menthol	6.73	-
Isopulegone	8.49	-
Pulegone	35.21	4,31
Piperitone	0.15	-
(+)-Carvone	0.15	-
Menthyl acetate	1.68	-
Chrysanthenone	3.39	-
Caryophyllene	0.22	-
Dihydrojasmane	0.13	-
Caryophyllene oxide	0.28	6,51
Myrcene	-	4,4
trans- β -damascenone	-	0,19
α -Himachalene	-	1,72
delta.-Guaiene	-	0,28
β -caryophyllene	-	24,65
α -Santalene	-	0,26
allo-aromadendrene	-	0,78
Geranyl acetone	-	0,38
α -caryophyllene	-	2,85
β -Ionone	-	0,43
Ledene	-	0,24
delta.-Cadinene	-	0,58
Palustrol	-	1,14
Ledol	-	0,29
Widdrol	-	0,56
α -Farnesene	-	1,76
Cembrene	-	2,05
γ -cadinene	-	0,16
β -Bisabolene	-	0,93
β -sesquiphellandrene	-	0,45
β -Farnesene	-	0,56
α -Isomethyl ionone	-	33,27
Phytol	-	0,4

(-): not determined

In this study, we have also analyzed the chemical composition of *G. foetida* essential oil in order to obtain important data on the structure of organic compounds. Thirty-nine compounds were separated and twenty-five of them were identified. Chemical identification of the components has shown that they had been characterized by its high rate of caryophyllene. Indeed, this analyses of essential oil exposed that the major products were α -Isomethyl ionone (33.27), β -Caryophyllene (24.65%), caryophyllene oxide (6.51%), Pulregone (4.31%), myrcene (4.4%). Our results were different from those previously reported. For example, essential oil of the of *Glycyrrhiza glabra* aerial parts from western Algeria was characterized by its high rate of Isoniazid and the chemical analysis showed that the major products were methacrylonitrile (9.69%), benzoic acid (5.37%), diethyltoluamide (6.56%), pyrazine, 1,4-carbon dioxide (2.20%) benzene (4.58%), linalool (2.25%) and bicyclo [4.1.0] hept-2-ene, 3,7,7-trimethyl- (2.80%) (Chouitah *et al.*, 2011). In Egypt, this same vegetable species (*G. foetida*) presented another chemotype. It contained geranylhexanolate (34%), isofenchon (16%), alpha-terpinene (12.5%), stragol (9.5%), beta-caryophyllene (7.7%), caryophyllene oxide (5.1%), and octanol (5.1%) (Ali, 2013). Recently another work conducted in Iran on *Glycyrrhiza triphylla* Fisch showed a predominance of b-caryophyllene (25.4%), limonene (16.7%), b-myrcene (16.0%) and a-humulene (4.4%) (Shakeri *et al.*, 2017).

Evaluation of antibacterial activity: The evaluation of the antimicrobial activity of the two species by estimating the diameter of the inhibition zone has shown that the essential oil of *M. pulegium* exhibited a greater antimicrobial activity than that of *G. foetida* which varies according to the bacterial strains.

The antagonism experiment showed the presence of antibacterial activity of *M. pulegium* aqueous extract against bacterial pathogens *A. vitis* S4, *A. tumefaciens* C58 and *PCC* 2657-1 which were more sensitive. Their corresponding inhibition diameters exceeded 20 mm. Similarly, *A. tumefaciens* C58 and *CMM* 1616-3 are the most sensitive to the aqueous extract of *G. foetida* with inhibition diameters of 23.6 and 22.6 mm respectively, unlike the *A. vitis* S4 and *PSS* 2066-1 which were resistant to treatment. *A. vitis* S4, *A. tumefaciens* C58, *CMM* 1616-3, and *PCC* 2657-1 presented inhibition zones greater than 20 mm. Therefore they were considered as sensitive to the essential oil of *M. pulegium*. However, *PSS* 2066-1 strain showed an inhibition zone of less than 20 mm. The maximum level of inhibition was observed with methanolic extracts of *M. pulegium* leaves (Figure 1). However, a weak inhibitory activity was recorded against *PSS* 2066-1 strain. Contrasting, other work revealed the *Lawsonia inermis* leaves extract had high antibacterial activity against *Pseudomonas savastanoi* pv. *savastanoi* and *Agrobacterium tumefaciens* (Trigui *et al.*, 2013). Thus, other works showed, the ability of the different essential oils of the oregano and thyme spice to retard and inhibit the growth of the different phytopathogenic bacteria; *Agrobacterium tumefaciens*, *Clavibacter michiganensis* subsp. *michiganensis*, *Erwinia amylovora*, *E. herbicola*, *Pseudomonas syringae*, *Pseudomonas viridiflava* and *Xanthomonas axonopodis* pv. *Vesicatoria*, (Scortichini and Rossi 1992, Yegen *et al.*, 1998, Daferera *et al.*, 2003). In general, several studies have shown the potential antimicrobial activity of *M. pulegium* essential oil (Mahboubi, and Haghi, 2008, Teixeira *et al.*, 2012). The study conducted by Zanjani *et al* (2015) showed a sensitivity of other kinds of strains *Bacillus subtilis*, *Proteus mirabilis* and *Zygosaccharomyces rouxii* with minimum inhibitory concentration values of 0.5%, 1.25%, and 1.5% respectively.

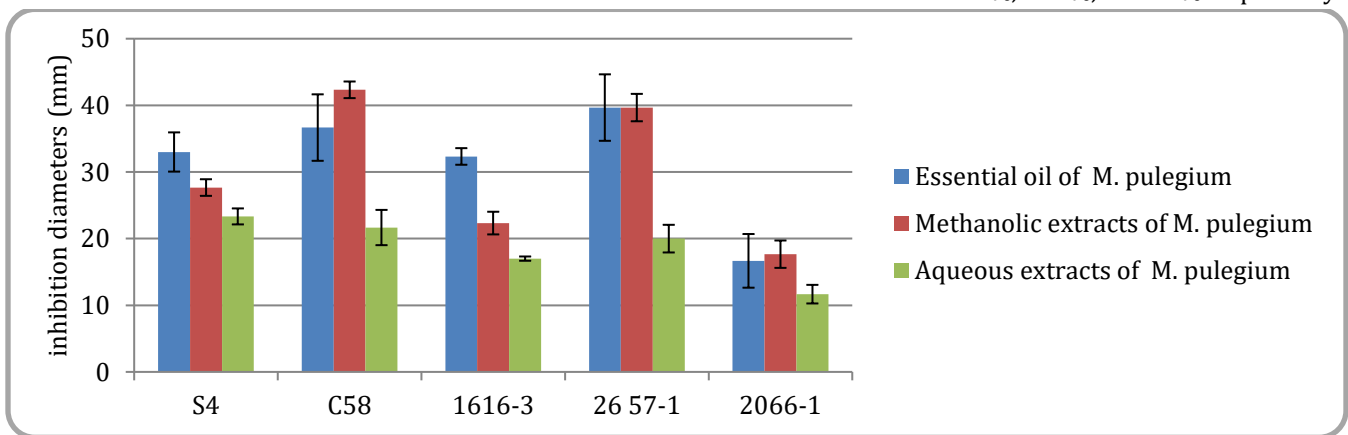


Figure 1. Growth inhibition of phytopathogenic strains (*A.vitis* s4), (*A. tumefaciens* C58), (*CMM* 1616-3), (*PCC* 2657-1), (*PSS* 2066-1) using different leaves extracts of *M. pulegium*.

The inhibitory effect of *G. foetida* extracts on the growth of phytopathogenic bacteria has shown that the methanolic extract is more effective than the essential oil (Figure 2). However, there was no inhibitory activity recorded by *G. foetida* oil against *A. vitis* S4, PSS 2066-1, PCC 2657-1, compared with *A. tumefaciens* C58 and CMM 1616-3 which were more sensitive to essential oil, methanolic and aqueous extracts (their inhibition zones exceed 20 mm). The results obtained revealed a varied sensitivity of strains to the different extracts. Generally,

the methanolic extract and the essential oil of *M. pulegium* are active with Minimal Inhibitory Concentration (MIC) of 10 mg/ml and 0.25% (v/v) respectively for methanolic extract and oil, and Minimal Bactericidal Concentration (MBC) values of 10 mg/ml and 0.5% respectively for the methanolic extract and oil toward *A. vitis* S4, *A. tumefaciens* C58), (CMM 1616-3). In addition, the same essential oil showed a bactericidal effect on (PSS 2066-1), (PCC 2657-1) at a concentration of 1%.

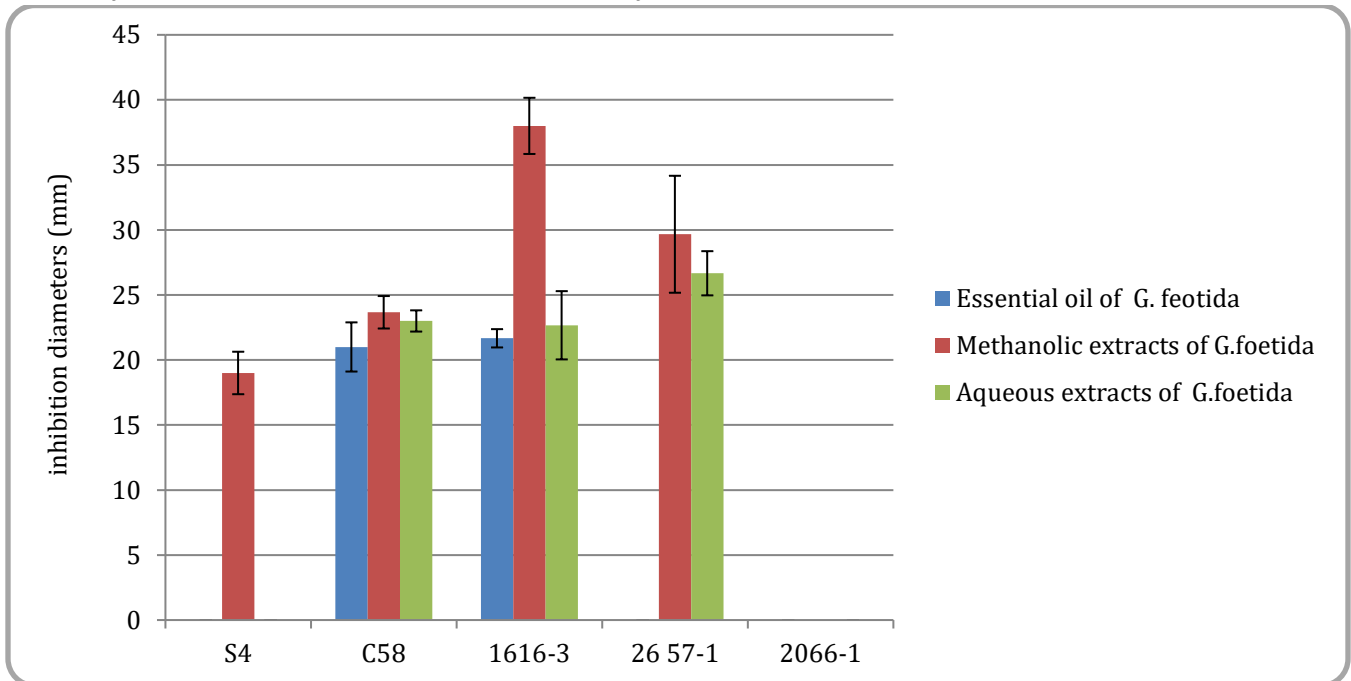


Figure 2. Growth inhibition of phytopathogenic strains (*A. vitis* S4), (*A. tumefaciens* C58), (CMM 1616-3), (PCC 2657-1), (PSS 2066-1) using different leaves extracts of *G. foetida*.

The results showed that the aqueous extract of *M. pulegium* has a weak inhibitory activity compared with methanolic extract at different concentrations tested on all bacterial strains. The MIC was 13.33 mg/ml. Nevertheless, the low sensitivity of all strains was observed with the aqueous extract of *G. foetida* with MIC value equal a mean of to 16.67 mg/ml.

Our results showed that the inhibitory and bactericidal activity of *G. foetida* essential oil depended on the bacterial species and strains. Indeed the MIC of 0.5% (v/v) was obtained against *A. vitis* S4), *A. tumefaciens* (C58), but it remained ineffective on the other strains. For the case of the extracts, the MIC is 10 mg/ml for the methanolic extract and 13, 33 mg/ml for the aqueous extract. Also, Chouitah *et al.* (2011) reported potent activity of *Glycyrrhiza glabra* oil against bacteria pathogens like *Staphylococcus aureus* (MIC 14.5 mg/ml),

Salmonella typhi (MIC 14.5 mg/ml) and *Escherichia coli* (MIC: 4, 2 mg/ml).

The antibacterial effect of our plant (*G. foetida*) may be due to the main constituents namely b-caryophyllene, limonene, b-myrcene and a-humulene already reported in other plant species (Jirovetz *et al.*, 2006; Qi *et al.*, 2014).

The antimicrobial activities of leaf extracts from both plants highlighted in this study could justify the therapeutic use in traditional medicine for the treatment of a large number of microbial infections.

ACKNOWLEDGEMENTS

This work was supported by the funds of National Institute of Agronomic Research Meknes, Morocco.

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