



***In vitro* Antibacterial Activity of Organic Extracts from North-West Moroccan Medicinal Plant *Myrtus communis* (L.)**

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was designed to evaluate the antibacterial activity of methanol, ethanol, ethyl acetate and *n*-hexane extract from the leaves of *Myrtus communis* (L.) growing in North-West of Morocco.

Study Design: *In vitro* evaluating of antibacterial activity and determination of total phenols and flavonoids content of extracts.

Place and Duration of Study: Department of Biology (Faculty of Sciences), between July 2015 and November 2015.

Methodology: The total phenol content and flavonoids content of methanol, ethanol, ethyl-acetate and *n*-hexane extracts were determined using Folin-Ciocalteu method and Aluminum chloride (AlCl₃) colorimetric method respectively. The antibacterial activity of extracts was tested against

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five reference strains, *Escherichia coli* K12 MBLA, *Staphylococcus aureus* CECT 976, *Listeria monocytogenes* serovar 4b CECT 4032 and *Pseudomonas aeruginosa* IH using the agar well diffusion method and the micro-dilution assays.

Results: The amount of the total phenolic content and total flavonoid content ranged between 86,31-137,46 gallic acid equivalents (GAE mg/g) and 14,31±1,09–31,24 quercetin equivalents (QE mg/g) of dry weight of extract, respectively. All extracts had a significant antibacterial activity against the tested bacterial strains. The biggest zones of inhibition were observed against two positive bacteria (*L. monocytogenes*, *S. aureus*). While, Gram negative bacteria (*E. coli* K12, *P. aeruginosa*) had showed to be resistant toward extracts.

Conclusion: Considering these results, *M. communis* leaves can be used as a source of novel antimicrobial and antioxidant compounds.

Keywords: Antibacterial activity; phenol content; flavonoid content; *Myrtus communis*.

1. INTRODUCTION

Infectious diseases pose a serious threat worldwide. The development of drug resistant pathogens due to indiscriminate use of antibiotics has compounded the need for new sources of antimicrobial agent. Plants are good source of natural products that may have potential antimicrobial properties. In developing countries, like Morocco, medicinal plants have been used as an alternative in the management of infectious diseases where treatment and medicine may be too expensive or are unavailable.

Myrtaceae family includes 100 genera and 3000 species. *Myrtus* genus belongs to this family of evergreen shrubs or small trees, which grow up to 5-m tall spontaneously [1]. It is native to Southern Europe, North Africa and Western Asia and also distributed in South America, North western Himalaya and Australia [2].

Province of Ouezzane is rich in aromatic and medicinal plants which some of them showed pharmacological properties such as antioxidant, anticancer, anti-inflammatory, antibacterial activities [3,4,5,6,7,8,9,10]. Among medicinal and aromatic plant of Ouezzane province we find *M. communis* L. (Myrtaceae). It is a perennial shrub, widely distributed in the Mediterranean area. Its leaves, fruits, flowers and roots are recommended in traditional medicine [11]. It is best known as a medicinal plant for its anti-hyperglycemic [12], analgesic [13], antigenotoxic [14], anticancer [7,8] and antibacterial activity [15,5]. Different parts of the plant find various uses in the food and cosmetic industries [16]. Myrtle is a very important plant because of the high essential oil content of its leaves, flowers and fruit glands [17]. The leaves contain tannins, flavonoids, such as quercetin, catechin

and myricetin derivatives, and essential oil [18]. Constituents of the fruits include essential oil, tannins, sugars, flavonoids and organic acids, such as citric and malic acids [18]. The plant is 2.4–3m high and branches forming a close full head, thickly covered with leaves [1]. Leaves are small and green and fruits are small and dark. The evergreen leaves are 2–5 cm long and aromatic after crushing such as in the case of myrrh or eucalyptus. The taste of it is bitter and intensive [19] which is mainly due to its astringency. The present study was undertaken to evaluate the antimicrobial activity of *M. communis* leaves extracts from Ouezzane province.

2. MATERIALS AND METHODS

2.1 Plant Material and Preparation of Extracts

The leaves of *M. communis* used in this research were collected in July 2015 from North-West of Morocco from Ouezzane province (34° 47' 50" N and 5° 34' 56" W). Plant was authenticated by Pr. ENNABILI Abdesalam (National Institute of Medicinal and Aromatic Plants, Taounate, Morocco). Samples were air dried under the shade and then milled into powder (using an electric grinder) for extraction.

The investigated dried powdered plant materials were extracted by maceration. The powder (25 g) of leaves was placed in an Erlenmeyer flask in 100 ml ethanol, methanol, and n-hexane for 72 h. The plant extracts were filtered by Whatman No. 1 filter paper and the combined filtrate was then dried under vacuum using a rotary evaporator (Heidolph Collegiate, LV28798826, New Jersey, USA) at a temperature not exceeding 45°C. The methanol concentrated extract was dissolved in

distilled water and extracted with ethyl acetate to obtain ethyl acetate fraction. All extracts were stored in a dark bottle for investigation at 2 - 8°C.

2.2 Determination of Total Phenolic Content (TPC)

The concentration of the phenolic compounds in the plants extracts was determined using the Folin Ciocalteu assay [20], with some modifications [3,4]. In brief, the extract was diluted to the concentration of 1mg mL⁻¹, and aliquots of 100 µl or a standard solution of gallic acid (20, 40, 60, 80 and 100 mg L⁻¹) were mixed with 500 µl of Folin Ciocalteu reagent (previously diluted 10-fold with distilled water) and 400 µl of Na₂CO₃ (7%). After 40 min of incubation at room temperature (23±2°C), the absorbance was measured at 760 nm using a Spectro-photometer against a blank sample. The total phenolic content was calculated using a calibration curve for gallic acid (R²= 0.998). The results were expressed as the gallic acid equivalent per gram of dry weight of extract (mg of GAE/g of extract). All samples were analyzed in triplicate.

2.3 Determination of Total Flavonoid Content (TFC)

The total flavonoid content of the extracts was determined using the aluminum chloride (AlCl₃) colorimetric method described by Brighente et al. [21] with minor modifications [3,4]. Briefly, 1 ml of the extract (1 mg mL⁻¹ in methanol) or a standard solution of quercetin (20, 40, 60, 80 and 100 mg/L) were mixed with 1 mL of 2% AlCl₃ in methanol. After 40 min of staying at room temperature (23 ± 2°C), the absorbance against blank was measured at 430 nm using a Spectrophotometer. The total flavonoid content was calculated using a calibration curve for quercetin (R²= 0.985). The results were expressed as the quercetin equivalent per gram of dry weight of extract (mg of QE/g of extract). All samples were analyzed in triplicate.

2.4 Antibacterial Activity

2.4.1 Bacteria strains

For evaluating the antibacterial activities M. communis leaves extracts, we have used the flowing bacteria: *Escherichia coli* K12 (Laboratory of Food Microbiology, UCL, Belgium: MBLA), *Staphylococcus aureus* CECT 976, and *Listeria monocytogenes* serovar 4b CECT 4032

(Spanish Type Culture Collection: CECT), and *Pseudomonas aeruginosa* (Institute of hygiene, Rabat, Morocco: IH). Strains are maintained on an inclined agar medium at 4°C. Before use, the bacteria were revived by two subcultures in an appropriate culture medium: Luria-Bertoni (LB) broth at 37°C for 18 to 24 hours. For the test, final inoculums concentrations of 10⁶ CFU mL⁻¹ bacteria were used according to the National Committee for Clinical Laboratory Standards, USA (NCCLS 1999).

2.4.2 Agar-well diffusion assay

For determining the diameter inhibition of our extracts against bacterial strains tested we have used Agar-well diffusion method. Briefly, a basal layer was prepared by Muller-Hinton agar. After the agar plates were solidified, sterile 8 mm diameter cylinders were deposited. Six ml of LB medium in superfusion containing 0.8% agar were inoculated by a fresh culture of indicator bacterial strain (a final concentration was 10⁶ CFU mL⁻¹). After solidification, the wells were filled with 50 µl of diluted extracts at 25 mg mL⁻¹. After incubation at appropriate temperature for 24 h, all plates were examined for any zone of growth inhibition, and the diameter of these zones was measured in millimeters. All the tests were performed in triplicate.

2.4.3 Determination of minimum inhibitory concentration (MIC)

MICs were determined using the broth micro-dilution assay, as previously described by Amensour et al. [22], with a slight modification [3]. In brief, 50 µl of Bacteriological Agar (0.15% w/v) was distributed from the 2nd to the 8th well of a 96-well polypropylene microtitre plate. A dilution of the each extract was prepared in DMSO (10%), to reach a final concentration of 32 mg mL⁻¹; 100 µl of these suspensions was added to the first test well of each microtitre line, and then 50 µl of scalar dilution was transferred from the 2nd to the 7th well. The 8th well was considered as growth control, because no extract was added. Then, we added 50 µl of a bacterial suspension to each well at a final concentration of approximately 10⁶ CFU mL⁻¹. The final concentration of the extract was between 16 and 0.25 mg/l. After incubation at 37°C for 18 h, 10 µl of resazurin was added to each well to assess bacterial growth, as proposed by Mann and Markham [23]. After further incubation at 37°C for 2 h, the MIC was determined as the lowest essential oil concentration that prevented a

change in resazurin color. Bacterial growth was detected by reduction in blue dye resazurin to pink resorufin. A control was carried out to ensure that, at the concentrations tested, the extract did not cause a color change in the resazurin. Experiments were performed in triplicate.

2.4.4 Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) corresponded to the lowest concentration of the essential oil yielding negative subcultures after incubation at appropriate temperature for 24 h. It is determined in broth dilution tests by sub-culturing 10 µl from negative wells on PCA medium. All the tests were performed in triplicate.

2.5 Statistical Analysis

All assays were carried out in triplicates and results were reported as mean ± standard error. The statistical significance between phenolic content, antioxidant activity and antibacterial values of the extracts was evaluated with one-way ANOVA followed by LSD test. Values of P less than 0.05 were considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1 Total Phenol Content

Total phenol content was estimated by the Folin-Ciocalteu colorimetric method in comparison with standard gallic acid and the results were expressed in terms of mg gallic acid equivalents /g extract using an equation obtained from a calibration curve (Fig. 1). The equation is given below:

$$\text{Absorbance} = 0.01 \times \text{mg (gallic acid)} + 0.002, (R^2 = 0.998).$$

Among the extracts investigated, total phenolic content ranged from 86.31±0.89 to 137.46±0.35 mg GAE/g extract (Table 1). The amounts of phenolic compounds in all extracts were highest and no significant difference between the solvents extracts is detected ($p > 0.05$). Others studies are reported that leaves extracts of *M. communis* are rich in phenol components. Amensour et al. [24] are found that the total phenol components of ethanol, methanol, ethyl-acetate and water extract ranged between 7.81 to 35.56 mg GAE/g extract. This difference in total phenol components could be explained by the geographical situation of collected plant. Indeed, Tawaha et al. [25] estimated that a total phenolic content higher than 20 mg GAE/g extract could be considered as very high. On the basis of this result, all our extracts of leaves of *M. communis* must be considered as highest sources of phenolic compounds.

3.2 Total Flavonoid Content

The determination of levels of flavonoids in five extracts of *M. communis* was made by using trichloride aluminum (AlCl₃) colorimetric assay. Results were expressed in terms of mg QE/g extract using an equation obtained from a calibration curve (Fig. 2). The equation is given below:

$$\text{Absorbance} = 0.022 \times \text{mg (gallic acid)} + 0.006, (R^2 = 0.999).$$

Their values ranging from 18.41±0.43 to 31.24±1.23 quercetin equivalents of dry weight of extract (QE mg/g) (Table 1). The n-hexane and methanol extracts showed higher flavonoids content than ethanol and ethyl acetate extracts ($p < 0.05$).

Table 1. Total phenolic content (TPC) and total flavonoid content (TFC) of extracts of *M. communis*

Extract	TPC (mg GAE^a/g) of extract	TFC (mg QE^b/g) of extract
EtOH	126,41±1,03 ^d	18,41±0,43 ^e
MtOH	137,46±0,35 ^d	27,24±0,83 ^f
n-hexane	122,72±1,16 ^d	31,24±1,23 ^f
EtOAc	86,31±0,89 ^c	14,31±1,09 ^e

TPC and TFC values are mean ± standard deviation of three separate experiments. ^a gallic acid equivalents;

^b Quercetin equivalents; MeOH, methanol extract; EtOH, ethanol extract; EtOAc, ethyl acetate fraction nd (not determined), values in the same row not sharing a common letter

(c-d-e-f) differ significantly at $p < 0.05$

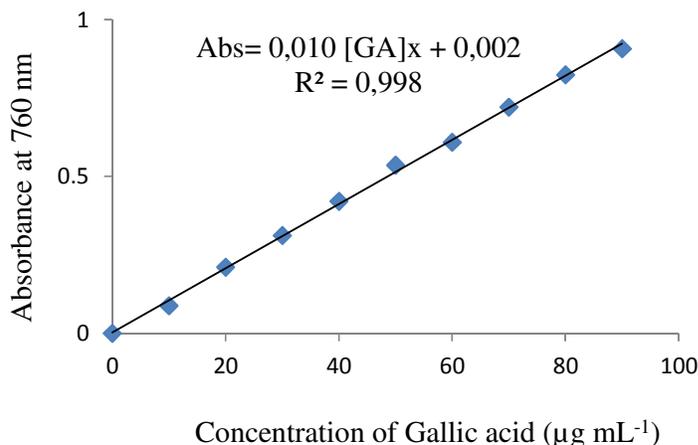


Fig. 1. Calibration curve of Gallic acid

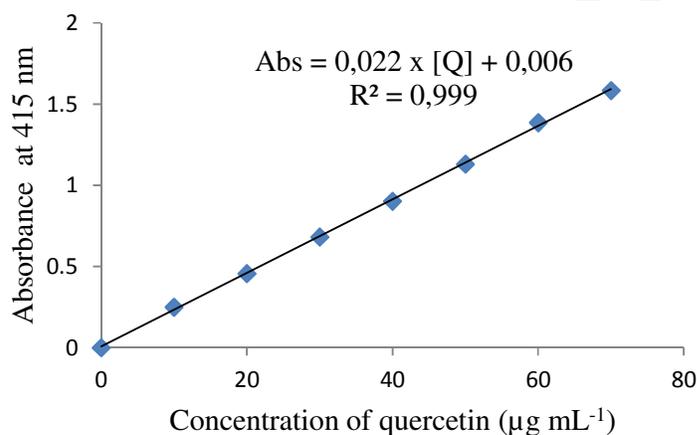


Fig. 2. Calibration curve of Quercetin

The chemical compositions of *M. communis* leaves extract have been identified by several studies [26,27,28,29]. Extracts composition may significantly vary, depending on plant organ used for extraction and showed that leaf extracts contain significantly higher amount of total phenolic compounds than berry extracts [14,24,26,27,28,29].

The contents of total phenols, tannins, flavonoids and proanthocyanidins vary among myrtle parts. According to Aidi Wannas et al. [26], leaf and flower are particularly rich in total tannins. Since the proanthocyanidins are weakly presented and authors suggested that leaf and flower tannins belong to hydrolyzed tannin class. However, they estimated that the myrtle stem is poor in tannins and moderately rich in flavonoids (catechin). Flavanols in *M. communis* leaves extract are

detected in relatively large amounts [26], with the exception of quercetin derivatives and phenolic acids, which were found only in small amounts [29].

Except myrtle plant parts, final extract composition depends also on extraction solvent used for extract preparation, mainly because of its polarity [30]. The most commonly used solvents for myrtle extract preparation are water, alcohol (methanol or ethanol), ethyl acetate, diethyl ether and chloroform. Tuberoso et al. [27] proved that ethanol and water extracts showed higher amount of extracted compounds in comparison to ethyl acetate extracts, but the highest antioxidant activity was found in ethanol and ethyl acetate extracts. According to them, ethanol extracts has the highest content of phenolic compounds. In addition, their results

showed a highly significant correlation between the amount of total phenols and antiradical or antioxidant activities in myrtle leaf extracts.

3.3 Antibacterial Activity

The results of the antimicrobial activity of *n*-Hexane, MeOH, EtOH and EtOAc extracts of *M. communis* leaves extract examined against different pathogenic microorganisms are presented in Table 2 (inhibition zones in the Agar-well diffusion assay). All the tested extracts revealed antimicrobial activity showing different selectivity for each microorganism. *n*-hexane extract showed significant activity against *P. aeruginosa* (21±0.77 mm), *L. monocytogenes* (33±0.61mm), *S. aureus* (29±0.31 mm), and *E. coli* (17±0.12 mm). EtOH and MtOH extracts showed a significant activity, especially against *S. aureus* and *L. monocytogenes* (Gram negative bacteria). The EtOAc have showed a moderate antibacterial activity. From our results, it appears that Gram positive bacteria were the most sensitive than Gram negative bacteria. In general, the Gram-negative bacteria show less sensitivity to plant extracts possibly as a result of their extra lipopolysaccharides and protein cell wall that provides a permeability barrier to the antibacterial agent. Furthermore, the Gram-positive bacteria are more sensitive to the extracts because of the single layer of their cell wall, while the double membrane of Gram-negative bacteria should make them less sensitive [31].

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts were determined by using microtitration assay. The Tables 3 and 4 illustrate the results obtained. The MIC of extracts ranged from 0.25

to >8 (mg mL⁻¹) and the MBC values are ranging between 0.5 and >8 (mg mL⁻¹). The *n*-hexane extract showed antibacterial activity against all bacteria with MIC values of 0.25–4 mg mL⁻¹ and MBC values of 0.5–4 mg/ml, while MeOH and EtOH extracts showed moderate antibacterial activity. In some cases, the MIC was equivalent to the MBC indicating a bactericidal action of extracts.

High antibacterial activity of methanol, ethanol, and ethyl acetate leaf and berry myrtle extracts was observed when it was tested against food borne pathogens. Methanol leaf extract of *M. communis* showed antibacterial activity even against *L. monocytogenes* and *P. aeruginosa*, but it was not active against *E. coli*K12 [22].

Methanol extract activity was also confirmed in other studies, such as Gortzi et al. [20]. While, hydroalcoholic extracts of myrtle leaves inhibited *S. aureus* with a low MIC (0.2 mg/ml); it was less effective against *E. coli* and *V. cholera* (8 and 2 mg mL⁻¹, respectively) and ineffective against *P. aeruginosa* [32]. In this study, detected MBC was 5–10 times greater than MIC. On other hand, *M. communis* infusion, prepared from dry leaves, showed lower activity against examined Gram negative bacteria, with MIC varied from 12.5 to 50 mg mL⁻¹ [33].

Different modes of action are involved in the antimicrobial activity of extracts. Because of the variability of quantity and chemical profiles of the extract components, it is likely that their antimicrobial activity is not due to a single mechanism. It is considered that these components have several sites of action at the cellular level.

Table 2. In vitro antibacterial activity of *M. communis* leaves extract

Extract	Inhibition zones diameter ^a (mm) (mean values ± SD)			
	Bacteria ^b			
	<i>E. coli</i> K12	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>P. aeruginosa</i>
MtOH	13±1,2 ^c	25±0,29 ^e	39±0,57^f	17±0,72 ^d
EtOH	12±0,8 ^c	28±0,46 ^e	27±0,54 ^e	15±0,60 ^c
EtOAc	nd	14±0,20 ^c	23±0,35 ^e	nd
<i>n</i> -hexane	17±0,12^d	29±0,31^e	33±0,61 ^f	21±0,77^d

^a The diameter of the well (Ø=8 mm) was included

^b Final bacterial density was around 10⁶ UFC mL⁻¹.

nd (not determined), values in the same row not sharing a common letter (c-d-e-f) differ significantly at *p* < 0.05.

Values in bold represent the most potent extract for each strain

Table 3. Minimum inhibitory concentration (MIC) of extracts (mg mL⁻¹) against tested bacteria

Extract	MIC ^a (mg mL ⁻¹)			
	Bacteria ^b			
	<i>E. coli</i> K12	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>P. aeruginosa</i>
MtOH	>8	0,5	0,5	0,5
EtOH	>8	0,5	4	4
<i>n</i> -hexane	4	0,25	0,25	0,25

^a MIC: Minimum inhibitory concentration (as mg mL⁻¹).^b Final bacterial density was around 10⁶ UFC mL⁻¹**Table 4. Minimum bactericidal concentrations (MBC) of extracts (mg mL⁻¹) against tested bacteria**

Extract	MBC ^a (mg mL ⁻¹)			
	Bacteria ^b			
	<i>E. coli</i> K12	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>P. aeruginosa</i>
MtOH	-	2	0,5	4
EtOH	-	4	>8	>8
<i>n</i> -Hexane	4	0,5	0,5	0,5

^a MIC: minimum bactericidal concentration (as mg mL⁻¹).^b Final bacterial density was around 10⁶ UFC mL⁻¹

Generally, there are six possible mechanisms of antimicrobial action, which include: (1) disintegration of cytoplasmic membrane, (2) interaction with membrane proteins (ATPases and others), (3) disturbance of the outer membrane of gram negative bacteria with the release of lipopolysaccharides, (4) destabilization of the proton motive force with leakage of ions, (5) coagulation of the cell content, and (6) inhibition of enzyme synthesis [22,34]. The mode of myrtle extract and essential oil activity affect mainly cell wall and membrane structures. It was reported that the permeability of bacterial cell wall and cell membrane are affected by these extracts, leading to the release of intracellular contents outside of cell. This can be accompanied with the disruption in the membrane function such as electron transfer, enzyme activity or nutrient absorption [22].

4. CONCLUSION

Myrtus communis leaves extract revealed strong antibacterial activity against tested bacterial strains which justify the traditional use of *M. communis* for food preservatives and diseases treatment. In further studies, bioactivity guided isolation from these extracts may provide fractions or constituents with high antimicrobial effect, which can substitute synthetics of equal efficacies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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