Journal of Plant Pathology & Microbiology
ISSN: 2157-7471

Research Article Open Access

Determination of Phenol Content and Antibacterial Activity of Five Medicinal Plants Ethanolic Extracts from North-West of Morocco

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Abstract

The aim of this study is to screening the phytochemical content of five medicinal plants of Ouezzane province for their antibacterial activity. Ethanolic extracts were prepared using solid-liquid extraction. The total phenolic content was assessed by the Folin-Ciocalteau assay, total flavonoid content was assessed by aliminuim chloride ($AlCl_3$) colorimetric assay. The antibacterial activity of extracts was tested against two reference strains, *Escherichia coli* K12 MBLA and *Staphylococus aureus* CECT 976 using the agar well diffusion method. The total phenol content of five ethanolic extracts ranged between 34,64 ± 1,16 and 112,48 ± 1,75 mg GAE per g extract, and the flavonoid content ranged between 9,28 ± 1,37 and 24,55 ± 0,58 mg QE/g extract. In the determination of the *in vitro* antimicrobial activity, flowering extracts prevented the growth of the tested strain by forming significant inhibition zones. The inhibitory activity of *Ajuva Iva* ethanolic extracts was especially remarkable (17,5 mm against *E. coli* and 21 mm against *S. aureus*). These species could be considered as potential sources of antibacterial compounds. Further studies are necessary for chemical characterization of the active principles and more extensive biological evaluations.

Keywords: Medicinal plant; Phenols content; Flavonoids content; Antibacterial activity

Introduction

Medicinal plants have been used in folk medicine in Morocco areas at relatively cheaper expenses than modern medicine. They have been widely used as diuretics and topical anti-inflammants [1]. Plants generally produce several secondary metabolites like Phenols, flavonoids, quinones, tannins, alkaloids, saponins, and sterols, which are important sources of biocides, and many other pharmaceutical drugs [2,3]. Medicinal plants are important in pharmacological research and drug development [4]. The most important secondary plant metabolites are phenols and flavonoids [5,6] they have distinctive biological activity as natural antibacterials which exceed many other synthetic ones [7].

Infectious diseases pose a serious health concern worldwide. The development of drug resistance pathogens due to indiscriminate use of antibiotics has compounded the need for new source of antimicrobial agents. These factors have inspired the widespread screening of new plant species for possible medicinal and antioxidant properties, while the isolation and characterization of diverse phytochemicals [8,9].

Medicinal Plants are a good source of natural products that may have potential antimicrobial activity. In developing countries like Morocco, these medicinal plants have been used as alternative in the management of infectious diseases where treatment and medicine may be too expensive or are unavailable.

Province of Ouezzane (North of Morocco) characterized by a Mediterranean climate is rich in medicinal plant. A few studies have been conducted in this region for screening and discovering news bioactive molecules. Therefore, the aim of this study is to screening the phytochemical content of five medicinal plants of Ouezzane province for their antibacterial activity.

Materials and Methods

Collection of plant material

The selected plants were collected in different areas of Ouezzane province in July 2015 and were authenticated by Pr. Ennabili Abdesalam (National Institute of Medicinal and Aromatic Plants, Taounate, Morocco). Samples were further transported to the laboratory. The Table 1 show collected plants, their families, their trivial names, place of collection and part plant collected.

Preparation of ethanolic extracts

Samples were air dried under the shade and milled into powder using an electric grinder, the investigated dried powdered plant materials were extracted by maceration. The powder (25 g) of flowering aerial parts were placed in an Erlenmeyer flask containing 100 ml ethanol for 72 h. The plant extracts were filtered with a filter paper (Whatman. No. 1) 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. All extracts were stored in a dark bottle at 2-8°C until analysis.

Determination of total phenolic content (TPC)

The concentration of the phenolic compounds in the plants extracts was determined using the Folin Ciocalteu assay [10], with some modifications. In brief, the extract was diluted to the concentration of 1 mg /ml, and aliquots of 100 µl or a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l) were mixed with 500 µl of Folin Ciocalteu reagent (previously diluted 10-fold with distilled water) and 400 µl of Na_2CO_3 (7%). After 40 min of incubation at room temperature

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Received March 21, 2016; **Accepted** April 04, 2016; **Published** April 08, 2016

Citation: Bouyahya A, Abrini J, El-Baabou A, Bakri Y, Dakka N (2016) Determination of Phenol Content and Antibacterial Activity of Five Medicinal Plants Ethanolic Extracts from North-West of Morocco. J Plant Pathol Microbiol 7: 342. doi:10.4172/2157-7471.1000342

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Plants species	Family	Trivial name	Place of collection	Part plant collected
Daphne gnidium (L.)	Thymelaeaceae	Metnan	Zoumi	Leaf
Ajuga iva (L.) *Schreb*	Lamiaceae	Chandgora	Zoumi	Leaf
Lavandula stoechas (L.)	Lamiaceae	Halhal	Kalaat Boukorra	Flowering top
Cistus albidus (L.)	Cistaceae	Chteppa	Kalaat Boukorra	Aerial part
Cistus monspeliensis (L.)	Cistaceae	Lossik	Bni kolla	Leaf

Table 1: Collection of plants species used in this study.

(23 ± 2°C), the absorbance was measured at 760 nm using a Spectrophotometer against a blank sample. The total phenolic content was calculated using a calibration curve for gallic acid (R^2 = 0.998). The results were expressed as the gallic acid equivalent per gram of dry weight of extract (mg of GAE/g of extract). Alls samples were analyzed in triplicate.

Determination of total flavonoid content (TFC)

The total flavonoid content of the extracts was determined using the aluminum chloride ($AlCl_3$) colorimetric method described by Brighente et al. [11] with minor modifications. Briefly, 1 ml of the extract (1 mg/ml 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 (R^2 = 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.

Bacteria strains

In order to evaluate the antibacterial activity of ethanolic extract of five species, two bacteria strains were used: *Escherichia coli* K12 (Laboratory of Food Microbiology, UCL, Belgium: MBLA) and *Staphylococus aureus* CECT 976 (Spanish Type Culture Collection: CECT).

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 (Biokar Diagnostics, Beauvais, France) at 37°C for 18 to 24 hours. For the test, final inoculums concentrations of 10^6 CFU/ml bacteria were used according to the National Committee for Clinical Laboratory Standards, USA (NCCLS 1999).

Antibacterial activity tests

Agar-well diffusion assay: 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 10^6 CFU/ml). After solidification, the wells were filled with 50 µl of diluted extracts at 25 mg/ml. 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.

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.

Results and Discussion

Percentage yield, total phenol content and flavonoid content

The extract yields of ethanolic extract are significantly different ($\alpha < 0.05$) between the plant extract (Table 2). The highest value of yield ethanolic extract is obtained with *C. monspeliensis* (25,18%) while the lowest value was obtained with L. stoechas (11,61%). Various factors modulate the synthetic pathway of the secondary metabolite in plant including climate, sol type, geographical location, genetic pool and epigenetic modification.

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 GAE/g dry extract. using an equation obtained from a calibration curve (Figure 1). The equation is given below:

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

The ethanolic extracts of plants studied had an important charge of phenols and their values varied widely for plant to another (Table 2). Among the ethanolic plant extract investigated, total phenolic content ranged from 23112,48 ± 1,75 to 34,64 ± 1,16 gallic acid equivalents (GAE mg/g) of dry weight of extract (Table 1).

The amounts of phenolic compounds in the *Cistus albidus, Cistus monspeliensis and Daphne gnidium ethanolic* extracts were highest and lowest in the *Lavandula stoechas and Ajuga iva* ethanolic extracts ($\alpha < 0.05$). According to Tawaha et al. [12], who have reported that a total phenolic content higher than 20 mg GAE/g dry weight could be considered as very high. Ethanolic extract of all our plants must be considered as a very good source of phenolic compounds. Phenolic compounds are secondary metabolites that can act as antibacterial

Table 2: Percentage yield, total phenolic content (TPC) and total flavonoid content (TFC) of ethanolic extract of fives species studied.

Extract	% Yield	TPC (mg GAE/g extract)	TFC (mg QE[b]/g extract)
Daphne gnidium (L.)	21,51	83,46 ± 1,12	17,68 ± 1,23
Ajuga iva (L.) *Schreb*	14,62	49,75 ± 2,08	11,97 ± 0,42
Lavandula stoechas (L.)	11,61	34,64 ± 1,16	9,28 ± 1,37
Cistus albidus (L.)	19,38	112,48 ± 1,75	24,55 ± 0,58
Cistus monspeliensis (L.)	25,18	79,19 ± 2,42	19,43 ± 1,34

[a]Gallic acid equivalent
[b]Quercetin equivalent

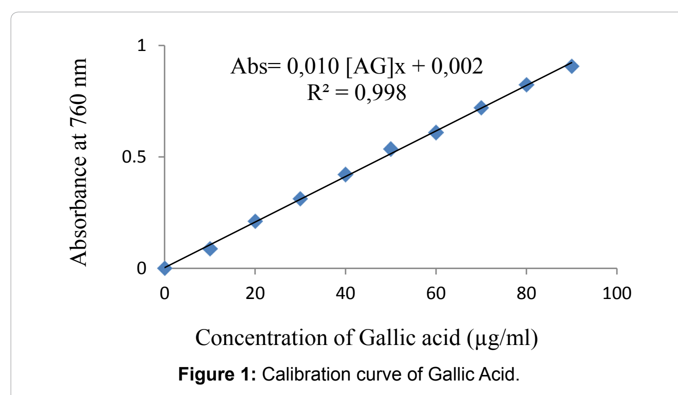

Figure 1: Calibration curve of Gallic Acid.

agents that have been widely investigated in many medicinal plants, fruits, and vegetables [13]. The antibacterial activity could be attributed to the hydrophobic character of phenolic content.

The total flavonoids content was estimated by a colorimetric method using quercetin as standard flavonoid. Results were expressed in terms of mg GAE/g dry extract using an equation obtained from a calibration curve (Figure 2). The equation is given below:

Absorbance = 0.022 × mg (gallic acid) + 0.006, (R^2 = 0.999).

As in the case of total phenolic content, the concentration of flavonoids in the extracts was dependent on plant extract. Their values ranging from 24,55 ± 0,58 9,28 ± 1,37 quercetin equivalents of dry weight of extract (QE mg/g) (Table 2). As in the case of total phenolic, the extracts from *Daphne gnidium, Cistus albidus, Cistus monspeliensis* showed higher flavonoids content (α < 0.05) than the extracts from *Ajuga iva and Lavandula stoechas.* Flavonoids, a large group of polyphenolic compounds, have a benzo-γ-pyrone structure and are found to be very useful as an antimicrobial agent [14].

Antibacterial activity

Antimicrobial activity was determined by using agar well diffusion assay. Our results indicated a potent antimicrobial activity of ethanolic extract of *D. gnidium*, *A. iva, L. stoechas, C. albidus* (L.) *and C. monspeliensis*. In the present study ethanolic extracts of plants have been tested against two resistant bacteria. The antimicrobial activity of the extracts and their potency was quantitatively assessed by the presence or absence of inhibition zone and zone diameter (Figure 3).

The antibacterial activity of extracts can be classified into three levels [15]: (i) weak activity (inhibition zone≤12 mm), (ii) moderate activity (12 mm<inhibition zone<20 mm) and (iii) strong activity (inhibition zone ≥ 20 mm).

Significant difference of activities of the investigated ethanolic plant extract against the tested bacterial strains was observed (*P<0,05*). The ethanolic extract of ajuva iva has showed the highest diameter inhibition two tested strains (17,5 mm and 21 mm against *E. coli* and *S. aureus* respectively). While, lavender extract has demonstrated a low zone of inhibition (8 mm and 11 mm against *E. coli* and *S. aureus* respectively).

In this study, the Gram-positive bacteria Gram-negative bacteria *S. aureus* was found to be more susceptible to the tested extracts than the Gram-negative bacteria *E. coli L.* Indeed, the majority of the compounds extracts assayed for their antibacterial properties showed

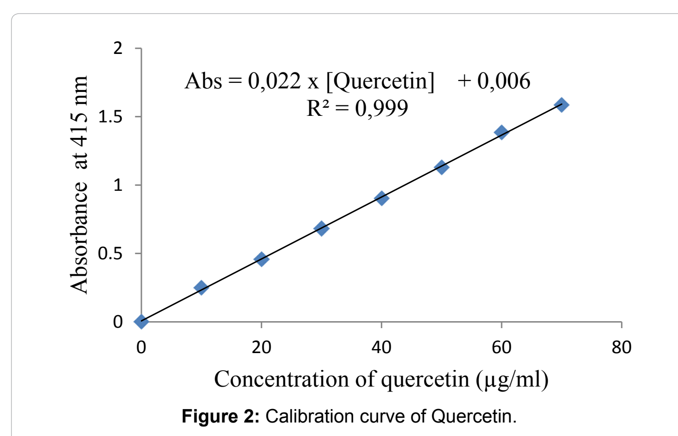

Figure 2: Calibration curve of Quercetin.

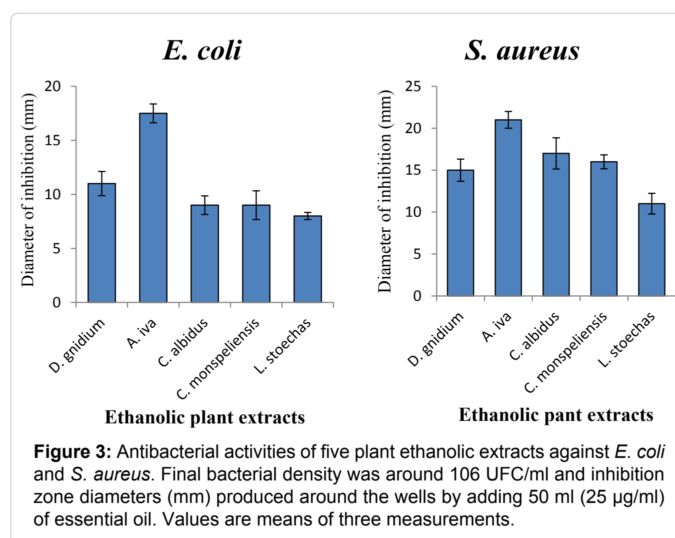

Figure 3: Antibacterial activities of five plant ethanolic extracts against *E. coli* and *S. aureus*. Final bacterial density was around 106 UFC/ml and inhibition zone diameters (mm) produced around the wells by adding 50 ml (25 µg/ml) of essential oil. Values are means of three measurements.

a more pronounced effect against the Gram +ve bacteria [16]. The resistance of Gram -ve bacteria to essential oils has been ascribed to their hydrophilic outer membrane, which can block the penetration of hydrophobic compounds into target cell membrane [17].

The wall of *Escherichia coli* (Gram -ve bacteria) is very rich in lipopolysaccharide (LPS) (lipid A, core oligosaccharide and antigen O) that prevent hydrophobic molecules such as terpenes to join him. Moreover, these microorganisms are movable, it is probably possible for these bacteria to be displaced deeper into the nutrient agar, and by consequence it could escape the action of the metabolites contained in plant extracts. In the case of Staphylococcus aureus (Gram +ve bacteria), this resistance to some plant extracts can be explained by the heterogeneous wall structure of the bacteria: the presence of the exopolysaccharid containing an outer layer (glycocalyx), the presence of certain components such as the teichoic acid and links between the various components highly cross linked polymer give the walls an unknown tertiary structure [18].

Conclusion

In light of the growing concern with antibiotic resistance these results show that the use of these residues (plant extracts) may represent a potential valuable source of bioactive compounds and an alternative for the treatment of infectious diseases caused *E. coli* and *S. aureus*. However, further studies need to be performed in order to identify and quantify the constituents present in these extracts that are involved in the synergistic effects.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Acknowledgements

The authors thank the Centre National pour la Recherche Scientifique et Technique (CNRST) from Morocco for support the doctoral grant of one of the authors.

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