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Phytochemical Screening, Antiradical and Antibacterial Activities of *Cistus crispus* from Morocco

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Abstract

The aim of this study is to investigate the antioxidant and antibacterial activities of *Cistus crispus* L. aerial part, and to estimate the total phenols and flavonoids content of its extracts. 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 colorimetric method respectively. The antibacterial activity was assessed by well diffusion assay and the minimum inhibitory concentration by microplaque dilution. The antiradical activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method. The amount of the total phenolics content and total flavonoid content ranged from 53.27±0.68 to 19.22±0.96 gallic acid equivalents (GAE mg/g extract) and 11.72±0.72to 02.31±0.69 quercetin equivalents (QE mg/g extract) respectively. The methanolic extract showed a high antiradical activity (IC_{50}=53.95±7.55 µg/mL) comparable to standard antioxidants (ascorbic acid, IC_{50}=27.20±4.3 µg/mL and Trolox: IC_{50}= 43.72±7.53 µg/mL). In addition, all extracts had a significant antibacterial activity against the tested bacterial strains. Gram-positive bacteria (*L. monocytogenes, S. aureus*) were the most sensitive toward tested extracts than Gram-negative bacteria (*E. coli K12, P. aeruginosa*).

1. Introduction

Medicinal plants have been used since ancient times to treat against diseases. With the emergence of antibiotic resistance and side effects shown by several synthetic antioxidants, we have to search natural products that are derived from medicinal plants.

By its bio-geographical location, Morocco is very rich in vegetation fromwhich some species are medicinal. Several Ethnomedical studies have shown the powerful effectiveness of medicinal plants in disease control through Morocco [1-7]. These plants are rich in bioactive molecules (essential oils, polyphenols, flavonoids, tannins, ...) which have various pharmacological activities such as antibacterial [8-16], antioxidant [10,13, 14;17-19**]**, antiviral [20], antileishmanial [13, 21]and antitumor [12,22-26]. However, the evaluation of Moroccan medicinal plants has not been generally done and some areas are not yet studied. The region of Ouezzane (North-Western of Morocco) is rich in medicinal plants with an endemism rate.

Cistaceae family is among the most representative botanical families of medicinal species in this region. This family comprises generally eight genera and the genus Cistus comprises 21 species [27]. This family is presented in Ouezzane province by various species as *Cistus crispus*, *C. albidus*, C. *monspeliensis* and *C. ladaniferus*. A study carried out by our laboratory has demonstrated the antibacterial activities of *C. albidus* and *C. monspeliensis* organic extracts [8]. These results are correlated with the phenolic and flavonoids contents of each extract. In the current study, our objective was to evaluate the total flavonoid and phenol contents, the antibacterial and antiradical activities of *Cistus crispus* aerial part extracts.

2. Experimental

2.1. *Collection of plant material*

The leaves of *C. crispus* used in this research were collected in July 2015 from Ouezzane Province (North-West of Morocco) (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 further transported to the laboratory. Samples were air dried under the shade and then milled into powder (using an electric grinder) for extraction.

2.2. Preparation of plant extracts

The powder (25 g) of aerial part was placed in an Erlenmeyer flask in 100 ml ethanol (EECC), methanol (MECC), and *n*-hexane (HECC) 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 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 (EACC). All extracts were stored in a dark bottle for investigation at 2 - 8°C.

2.3. Determination of Total Phenolic Content (TPC)

The concentration of the phenolic in the plants extracts was determined using the Folin Ciocalteu assay [28], with some modifications. In brief, the extract was diluted to the concentration of 1mg/ml, and aliquots of 100 µL or a standard solution of gallic acid (20, 40, 60, 80 and 100 µg/mL) 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 (23±2), 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 (R^2= 0.998)**(Fig. 1)**. 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.

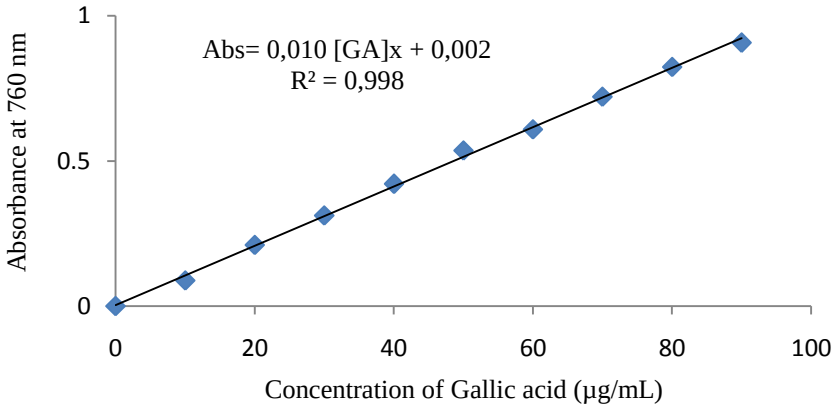

Figure 1: Calibration curve of Gallic Acid

2.4. Determination of Total Flavonoids Contents (TFC)

The total flavonoid content of the extracts was determined using the aluminium chloride ($AlCl_3$) colorimetric method described by Brighente et al. with minor modifications [29]. Briefly, 1 mL of the extract (1 mg/mL in methanol) or a standard solution of quercetin (20, 40, 60, 80 and 100 µg/mL) 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) **(Fig. 2)**. 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.

2.5. Evaluation of the antioxidant Activity

The antioxidant activity of *M. communis* leaves extracts is measured by using the test 2,2-diphenyl-1-picrylhydrazyle (DPPH). It is reduced to the form of hydrazine (no radicular) by accepting a hydrogen atom.

2.5.1. DPPH radical scavenging capacity assay

The ability of the plant extracts to scavenge DPPH free radicals was assessed using the standard method with some modifications [30]. In brief, Aliquots (0.2 mL) of various concentrations (30.125-1000 µg/ml) of the plant extracts samples were added to 1.8 mL of a 0.004% methanolic solution of DPPH. After an incubation period of 30 min in darkness at room temperature (23 ± 2◦C), the absorbance was recorded against a blank at 517 nm with

a spectrophotometer. Absorption of a blank sample containing the same amount of methanol and DPPH solution acted as the control. Samples were analyzed in triplicate.

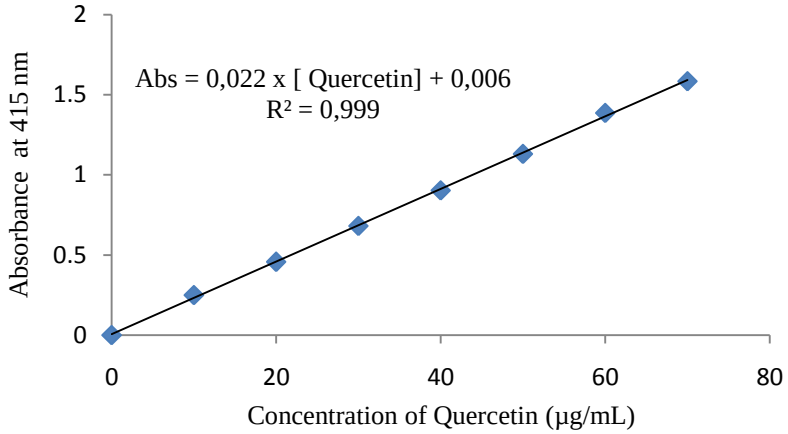

Figure 2: Calibration curve of Quercetin

DPPH free radical-scavenging activity in percentage (%) was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_{blanc}-A_{sample})/A_{blanc}] \times 100$$

Where A_{blanc} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Trolox and ascorbic acid was used as positive control and extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted of inhibition capacity (AA in %) against extracts and standards concentrations, by using linear regression equations.Relationships between antioxidant capacity (IC_{50}) and total phenol content or flavonoid content of extract were evaluated by correlation coefficients. This correlation was determined by modeling the antioxidant capacity versus total phenol content or flavonoids content.

2.6. Antibacterial activity

2.6.1. Bacteria strains

In order to evaluate the antibacterial activity of the various extract of *C. crispus*, the flowing bacteria were used: *Escherichia coli* K12 MBLA (Laboratory of Food Microbiology, UCL, Belgium: MBLA), *Staphylococcus aureus* CECT 976, *Listeria monocytogenes serovar*4b CECT 4032 (Spanish Type Culture Collection: CECT), and *Pseudomonas aeruginosa* IH (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: Lysogeny broth (LB) (Biokar Diagnostics, Beauvais, France) at 37°C for 18 to 24h. 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).

2.6.2. Antibacterial susceptibility test: Agar-well diffusion assay

For the determination of quantitative antibacterial activity we have employed the 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 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.

2.6.3. Determination of minimal inhibitory concentration (MIC)

MICs were determined using the broth micro-dilution assay, as previously described by Ismaili et al. [31], with a slight modification: agar at 0.15% (w⁄v) was used as stabilizer of the extract–water mixture and resazurin as bacterial growth indicator. 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; 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 without extract added was considered as growth control. Then, we added 50 µl of a bacterial suspension to each well at a

final concentration of approximately 10^6CFU/mL. The final concentration of the extract was between 16 and 0.25 mg/mL. After incubation at 37°C for 18 h, 10 µl of resazurin was added to each well to assess bacterial growth. 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.

2.6.4. Determination of minimal bactericidal concentration (MBC)

MBCs 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 subculturing 10 µL from negative wells on plate count agar (PCA) medium. All the tests were performed in triplicate.

2.7. Data analysis

The statistical analysis was performed by a one-way ANOVA analysis of variance followed by Duncan's test, and results were considered to be statistically significant with a 95% confidence level ($p< 0.05$). The measurements of total phenolic compounds, total flavonoids, DPPH radical scavenging activity, and antibacterial activity were carried out for three replicates. The results are expressed as mean ± SD.

3. Results

3.1. Percentage yield, total phenolic content and total flavonoid content

The estimation of Percentage yield, total phenolic content (TPCs) and total flavonoids content of *C. crispus* extracts is presented in **table 1**. The extract yields of the aerial part of *C. crispus were as* follows: 34.42 % (w/w) for MECC, 24.61% (w/w) for EECC, 21.16 % (w/w) for EACC and 9.72 % (w/w) for HECC. The amount of the total phenolic content and total flavonoid content ranged 53.27±0.68-19.22±0.96gallic acid equivalents (GAE mg/g extract) and 11.72±0.72–02.31±0.69 quercetin equivalents (QE mg/g extract) respectively.

Table 1: Yield of extract, total phenolic content (TPC) and total flavonoid content (TFC) of extracts of *C. crispus*

Extract	% Yield	TPC (mg GAE/g extract)	TFC (mg QE/g extract)
EECC	24.61	37.60±1.12	07.60±0.32
MECC	34.42	53.27±0.68	11.72±0.72
EACC	21.16	19.22±0.96	02.31±0.69
HECC	09.72	35.54±0.35	9.64±0.85

TPC and TFC values are mean ± standard deviation of three separate experiments ($p < 0.05$). GAE, Gallic acidequivalents; QE Quercetin equivalents; MECC: methanol extract; EECC: ethanol extract; EACC: ethyl acetate and HECC*: n*-hexane extract.

3.2. Antiradical activity

The reduction capability of DPPH radicals was determined by the antioxidant-induced decrease in its absorbance at 517 nm, which is visually noticeable as a discoloration from purple to yellow. The result is showed in **figure 3**. The IC_{50} value was defined as the concentration of sample that scavenged 50% of the DPPH. It was calculated from the percentage inhibition of DPPH versus the concentrations of the extracts and standards using the exponential equation. The result is presented in **figure 4.**

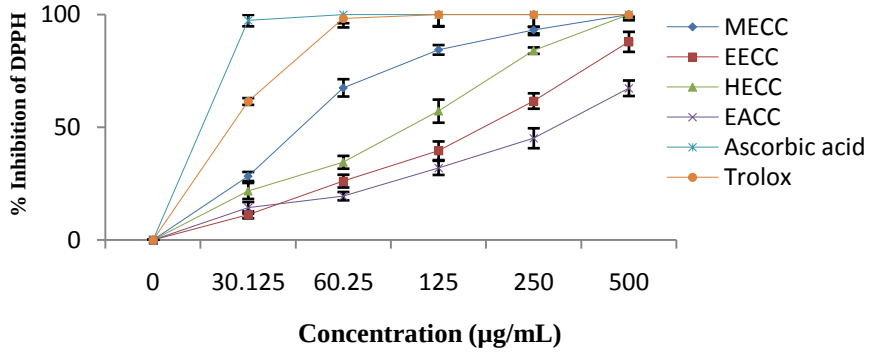

Figure 3: DPPH radical scavenging activities (%) of standard antioxidants (Trolox and ascorbic acid) and *Cistus crispus* organic extracts (MECC: Methanol; EECC: Ethanol; EACC: Ethyl-acetate and HECC: *n*-hexane). Values are means ± standard deviation of three determinations.

3.3. Antibacterial activity

The antibacterial activity of aerial parts cistus crispus extract was screened by wall diffusion method and the MIC was determined by micro-dilution assay. The results are showed in **table 2**. Gram-positive bacteria are more sensitive than Gram-negative bacteria. The highest diameter of inhibition is found with ethanolic extract against *S. aureus* and *L. monocytogenes.*

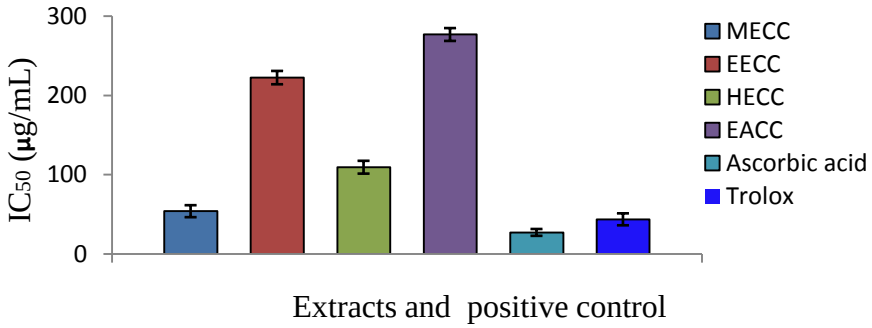

Figure 4: IC_{50} (µg/mL) values of MECC, EECC, HECC and EACCextracts of *C. crispus* and positive control (Trolox and ascorbic acid). Values are means ± standard deviation of three determinations.

Table 2: Antibacterial activity of extracts of *C. crispus* (inhibition zone diameters, MIC and MBC)

Bacteria[a]	Extracts	Inhibition zone[b]	MIC[c]	MBC[d]
E. coli K12	EECC	12±0.59	>8	NA
	MECC	10±0.65	>8	NA
	EACC	NA	NA	NA
	HECC	14±0.78	>8	NA
S. aureus	EECC	29±0.07	2	2
	MECC	25±0.81	1	4
	EACC	15±0.73	NA	NA
	HECC	22±42	3	>8
L. monocytogenes	EECC	29±1.24	8	>8
	MECC	21±0.52	1	4
	EACC	22±1.33	NA	NA
	HECC	31±0.92	1	2
P. aeruginosa	EECC	NA	>8	NA
	MECC	NA	1	4
	EACC	NA	NA	NA
	HECC	13±1.34	2	4

[a]Final bacterial density was around 10^6 CFU/mL.
[b]Inhibition zone diameters (mm) produced around the wells. Values are means of three measurements.
[c]MIC: minimum inhibitory concentration (as % mg/mL).
[d]MBC: minimum bactericidal concentration (mg/mL).
NA: no active

4. Discussion

Infectious diseases cause a real problem for public health because of the emergence of antibiotic resistance. In addition, oxidative stress is involved directly or indirectly in the pathogenesis of several diseases including cardiovascular diseases, diabetes and cancer [32]. For these problems, it is necessary to search alternatives molecules such as natural products that might participate in the resolution of these problems.Medicinal plants have constituted a reservoir for chemical molecules of various and very complex structures. These laters have been exploited for several pharmacological properties [24, 25, 26, 33]. In this context, our work aimed to valorize *C. crispus* organic extracts by evaluation of the antiradical and antibacterial effects. It appears that this medicinal plantis rich in organic extracts and the extraction efficiency varies depending on the solvent used. MECC is the solvent that has led more extract because it is the most polar solvent [34]. Indeed, most studies showed that the more polar solvents give higher extraction yields. Indeed, the most widely used solvent for

extracting phenolic substances from medicinal plants are methanol. This idea has confirmed by Cakir et al. in their study on phenolic compounds of *Teucrium orientale* L., who reported that methanol give the higher extraction yield than other solvents[35].In addition, the use of methanol, water, petroleum ether and chloroform by Sharififar et al. for the extraction of polyphenols from *Teucrium polium*, showed that methanol give the highest extraction yield [36].Phenolic compounds such as, flavonoids are considered to be the major contributors to the antioxidant capacity of plants [10]. Some of diverse biological activities of plants, such as antibacterial activity, may also be related to phenolic compounds [11].The TPC and TFC in the extracts are varied depending on extraction solvents. The MECC and HECC showed very high content in TPC and TFC. The high level of TPC was found in MECC, while the lowest was in MACC **(Table 1)**.

Given the complexity of molecules present in the plant extracts, it is necessary to focus on the most effective methods to test the extract for their biological activities. We have chosen the well diffusion method to access the preliminary results of antibacterial activity. These results are expressed in terms of diameter of the inhibition zones around the wells products **(Table 2)**. As regards, results obtained showed an important antibacterial activity that varies from a sample to another and from one strain to another. In addition, Gram-positive bacteria are more sensitive against extracts than Gram-negative bacteria. This result is previously cited in several studies [8,9,17,37]. The resistant of Gram-negative bacteria to the antibacterial agents may be attributed to an outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through the lipo-polysaccharide. Moreover, the periplasmic space contains enzymes, which are able to break down foreign molecules introduced from outside [38]. Strains exhibiting the largest inhibition zones were not always those which are most sensitive. These results are consistent with those reported with previous studies [17]. Indeed, the zones of inhibition do not reflect the antibacterial effectiveness of a product; it can be affected by the solubility, diffusion and evaporation of the extract [39]. The results of the antibacterial activity of *C. crispus* extracts obtained in this work are corroborated with those found in the literature ported on other species of *cistus* genus [8] and *C. crispus* [40].

The DPPH assay is a very common spectrophotometric method to determine the activity of any antioxidant. The advantage of this method is that the antioxidant activity is measured at ambient temperature, and thus, the risk of the thermal degradation of the molecule tested is eliminated [41].In our study, DPPH was used as a substrate to evaluate the antioxidant activity of MECC, EECC, HECC and EACC at various concentrations (0-500 µg/mL) compared to standards such as ascorbic acid and Trolox. **Figure 3** illustrates a highly significant decrease of the DPPH radical concentration ($p< 0.5$) due to the scavenging activity of each extract concentration and standards. Ascorbic acid and Trolox at 30.125 mg/L showed a high radical scavenging activity (97.32±1.5 and 79.47±2.54 %, respectively), while, the activity of the MECC, EECC, HECC and EACC ranged from 28.24±2.54 to 11.22±1.54 %. The various extracts activity was significantly lower than ascorbic acid and Trolox ($p< 0.05$). In all case, we have found that the activity to scavenge DPPH radical increases significantly with increasing extract concentration ($p< 0.05$). The antioxidant activity of *cistus genus* extracts and essential oils has previously reported by some authors [42,43]. They showed similar effects to our results with variability more or less significant amounts related to the origin of the plant and the experimental used method.The antiradical activity of four extracts does not correlate significantly with TPC ($R^2=0.744$) and TFC ($R^2=0.885$). This result is due to the fact that these compounds are not the only molecules implicated in the antibacterial properties. However, these activities could be attributed to several molecules present in plant drugs.

Conclusion

Organic extracts from *C. crispus* possess antiradical and antibacterial activities. The results revealed that MECC had significant antiradical scavenging effect and HECChad a significant antibacterial activity against tested strains. According to these results, *C. crispus* could be an excellent source for natural antibacterial and antiradical agents for biomedical applications. Therefore, other studies have to conduct for identifying and isolating bioactive molecules presented in MECC and HECC.

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