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(RESEARCH ARTICLE)

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Phytochemical and biological investigation of leaf extracts of *Phlomis crinita* Cav. from northern Algeria

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Abstract

Phlomis crinita Cav., is a Lamiaceae very widespread in the North-West of Algeria (Chlef), but very little studied on the chemical and pharmacological levels; for this, a phytochemical screening, then a dosage of total phenolic compounds (TPC), total flavonoids (TFL) and condensed tannins (TCN) obtained by (02) methods (reflux and ultrasonic bath), followed by an evaluation of antibacterial activities and antioxidants, were carried out on (03) fractions: chloroform, ethyl acetate and n-butanol.

The n-butanol extract (reflux) gives the best contents of total phenolic compounds (T_{TPC}= 15.96 ± 0.09 ug Eq. Catechin / mg of extract), The acetate ethyl extract (reflux) gives the best contents of total flavonoids (T_{TFL} = 33.98 ± 4.98 ug Eq. Rutin / mg of extract), and the chloroform extract (reflux) is richer on condensed tannins (T_{TCN}= 757.67 ± 14.32 ug Eq. Catechin / mg of extract).

The antibacterial activity of the extracts was slightly positive against Pseudomonas aeruginosa and Staphylococcus aureus. On the other hand, no antibacterial activity was observed against strains of Escherichia coli.

The chloroform and n-butanol (reflux) extracts showed a strong ability to scavenge the DPPH radical comparable to that of ascorbic acid ($IC50_{\text{Chloroform Ext.}}$ = 79.99 ± 1.48 ug/ml; $IC50_{\text{n-Butanol Ext.}}$ = 94.96 ± 1.70 ug/ml and $IC50_{\text{Ethyl Acetate Ext.}}$ = 588.68 ± 10.88 ug/ml versus $IC50_{\text{vit C}}$ = 55.26 ± 1.01 ug/ml).

Keywords: Phlomis; Reflux; Ultrasound; Polyphenols; Antioxidants; Antimicrobials

1. Introduction

The genus *Phlomis* is widely used in the Mediterranean basin for its many therapeutic virtues. The objectives of this study are a phytochemical study and an evaluation of the antioxidant and antibacterial activities of leaf extracts of a species widespread in the region of Chlef in western Algeria: Phlomis crinita Cav. (1).

2. Material and methods

2.1. Plant material

The leaves are harvested in January 2018 in the Oued El Fodda region of the municipality of Beni Rached in the wilaya of Chlef at an altitude of 830 meters (Latitude: 36° 16' 50.19"N - Longitude: 1 ° 30' 57.70"E). The Algerian flora of Quezel and Santa (1963) was used to confirm the identity (2). The leaves (500 grams) are dried for a few weeks in a thin layer,

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in the shade, at room temperature, in a dry and ventilated place until completely dry. They are stored in paper bags protected from light, humidity and dust until use (3).

2.2. Methods

The experimental part was carried out in (05) stages:

2.2.1. Botanical study

Macroscopic study of the drug: The drug was identified jointly at the Normal Institute of Agronomy (INA) and at the Pharmacognosy Laboratory of the Algiers Pharmacy Department.

Microscopic study of the drug (The powder): The dried leaves are ground in a grinding machine followed by a mortar. A tiny quantity of the powder obtained is mounted between slide and coverslip with a drop of Gazet du Chatelier's reagent which colors the elements of the powder. The powder is then observed under a light microscope at different magnifications (Grx100 and Grx400) (4).

2.2.2. Phytochemical screening:

It is carried out on aqueous or organic phases by usual reactions with reference to, Bruneton 2016, Faugeras G 1965 as well as the work of Ahmed T et al 2013, Bhandary SK et al 2012, Dialla D 2000 and Tiwari P et al 2011.

Each reaction was compared to a control, and the results are expressed as follows: Frankly positive reaction: (++), Positive reaction: (+), Reaction difficult to interpret: (±), Negative reaction: (-) (5,6,7).

Extraction of polyphenols

The leaves of Phlomis crinita were dried for a few weeks in a thin layer, in the shade, at room temperature, in a dry and ventilated place until complete desiccation, then crushed using a mortar until obtaining of a powder, which was extracted by two extraction methods, one traditional by reflux and the other modern by ultrasound (5,7).

Five (05) grams of plant material are extracted by the mixture: methanol - water (70/30, V/V) for (01) hour. After filtration and evaporation to dryness, the solution is left to macerate in distilled water for 24 hours (5.7).

In a separatory funnel, delipidation is carried out with n-hexane and successive extractions with solvents of increasing polarity with chloroform, ethyl acetate and n-butanol, with volumes of 3 x 50 ml for each solvent. The (03) extracts are evaporated to dryness at 44° then weighed and taken up in methanol. (5.7).

Yield calculation: it is determined by the following ratio: R (%) = P1 - P2 / P 3 × 100; (R: Yield; P1: weight of the flask after evaporation; P2: weight of the flask before evaporation; P3: weight of the starting plant material).

2.2.3. Assay of total phenolic compounds (TPC):

The method for assaying the TPCs is that of Folin-Ciocalteu developed by Singleton et al (9,10).

Principle

The Folin-Ciocalteu reagent consists of a mixture of phosphotungstic acid (H3PW12O40) and phosphomolybdic acid (H3PMo12O40). It is reduced by phenols to a mixture of blue oxides of tungsten (W8O23) and molybdenum (Mo8O23). This blue coloration, the intensity of which is proportional to the levels of phenolic compounds present in the medium, gives an absorption maximum at 765 nm (9.10).

Procedure

It consists in taking a volume of 200 µl of the extract, and adding a volume of 1 ml of the Folin-Ciocalteu reagent (diluted ten times). After 4 minutes, pour a volume of 800 µl of sodium carbonate (Na2CO3) (7.5%) onto the solution. Place the tubes in the dark. After two hours, read the results on a spectrophotometer at 765 nm (Catalano et al, 1999) (9.10).

Expression of results

The CPT concentration is calculated from the regression equation of the calibration range, established with the catechin standard (0-100µg/ml) and expressed in micrograms of catechin equivalents per microgram of extract (µg EC/mg) (9.10).

2.2.4. Assay of total flavonoids (TLF):

Total flavonoids were quantified by the aluminum trichloride (AlCl3) method (9,11).

Principle

Principle the technique is based on the formation of a flavonoid-aluminum complex which has a maximum absorption at 430 nm (9,11).

Procedure

1ml of each extract is added to 1ml of a 2% AlCl3 solution. After 10 minutes of reaction in the dark, the absorbance is read at 430 nm. (Lamaison and Carnat (1991) cited by Bahorun (1997) (9.11).

Expression of results

The concentration of total flavonoids is calculated from the regression equation of the calibration range, established with the rutin reference standard (0-40µg/ml) and expressed in micrograms of rutin equivalents per microgram of extract (µg RE/mg) (9.11).

2.2.5. Assay of condensed tannins (TNC):

The assay of condensed tannins is carried out using the vanillin method (8,11).

Principle

This method is based on the ability of vanillin to react with condensed tannin units in an acid medium to produce a colored complex (carbonium ion colored red) measured at 500 nm (9.12).

Procedure

400 µl of each extract are added to 3 ml of a 4% methanol solution of vanillin, then 1.5 ml of concentrated hydrochloric acid is added. After 15 min of reaction in the dark, the absorbance is read at 550 nm (Heimler et al. 2006) (9,12).

Expression of the results

The concentration of condensed tannins is calculated from the regression equation of the calibration range, established with the catechin reference standard (0-300µg/ml) and expressed in micrograms of catechin equivalents per microgram of extract (µg EC/mg) (9.12).

2.3. Pharmacological screening:

2.3.1. Study of antioxidant activity:

The antioxidant activity obtained with the solvent (methanol-water) (by reflux and was tested by the method of DPPH (2,2-diphenyl-1-picrylhydrazyl). (Molyneux P, 2006) (13,14).

Principle

DPPH is a stable free radical with a dark purple color that turns pale yellow when reduced (in the presence of an antioxidant) (12,13).

Procedure

Preparation of the DPPH solution: DPPH 2,2-diphenyl-1-picrylhydrazyl ($C_{15}H_{12}N_5O_6$; Mr 394.33) is dissolved in absolute methanol to obtain a 0.004% solution (stable for 48 hours in the dark and at +4°C) (13.14).

Preparation of the extracts to be tested

For each of the 2 extracts, a range of dilutions is prepared from the stock solution (obtained after evaporation) (13,14).

DPPH test

1 ml of each dilution is introduced into dry tubes, plus 1 ml of 0.004% DPPH methanolic solution, followed by vortexing. The tubes are placed in the dark, at room temperature for 30 min.

The reading is carried out by measuring the absorbance at 517 nm by a spectrophotometer. A blank (negative control) is prepared; it is composed of 1 ml of methanol and 1 ml of DPPH (0.004%).

For each dilution, the operation is carried out three times (13,14).

Expression of the results

To obtain the effective concentration which reduces the initial concentration of DPPH by 50%, the results are expressed as antioxidant activity. &The antioxidant activity, which expresses the ability to trap free radicals, is estimated by the percentage of discoloration of DPPH in solution in methanol: AA (%) = (A0-A /A0) x 100; (AA=The antioxidant activity (%); A0= The optical density of the blank; A= The optical density of the diluted extract). The results are then plotted on the curve AA = f (concentration of the extract) (13,14).

The IC50 value is defined as the concentration of the substrate which causes the loss of 50% of the activity of DPPH and is determined graphically by the linear regressions of the plotted graphs; percentages of inhibition according to different dilutions of the extracts tested. This value is compared with that of ascorbic acid tested under the same conditions (range of 25-750ug/ml) (13,14).

2.3.2. Study of antibacterial activity

It is a qualitative in vitro evaluation of the action of leaf extracts of Phlomis crinita L; on strains selected according to their availability and their pathogenicity (nosocomial infections). These are young strains from the "American Type Culture Collection" (ATCC) [Staphylococcus aureus (ATCC 25923); Pseudomonas aeruginosa (ATCC 27853); Escherichia coli (ATCC 25922)] (15,16):

Re-isolation of the ATCC strains: by taking the most apparent colonies from the ATCC dishes using a Pasteur pipette previously sterilized by the blue flame of the Bunsen burner; then inoculation of the colonies taken from a nutrient agar, by deconcentration using the technique of the four cadrons. The boxes are placed in an oven at 37°C for 24 hours (15,16).

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3. Results

3.1. Botanical study

The results are represented in (figures.1 and .2)

Figure 1 General appearance of *Phlomis crinita* Cav. Lamiaceae; (A: Seedling; B: Flower; C: Adult plant) (16)

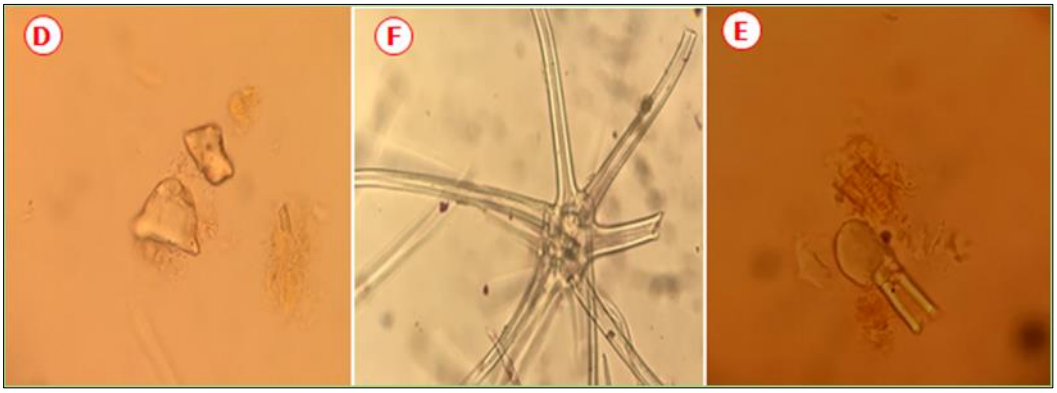

Figure 2 Microscopic study of leaf powder from *Phlomis crinita* Cav. Lamiaceae; Gr x 400 (D: Calcium oxalate crystals; E: Multicellular branched covering hair; F: Secretory hair with unicellular foot and unicellular spherical head) (22)

3.2. Chemical study (yield)

The results are represented in (table.1)

Table 1 Result of the yield of the extracts obtained by reflux and ultrasound (%)

Extraction	Mass of dry extract in (g)	Yield (%)
Reflux (R)	0.364 ± 0.001	7.27 ± 0.01
Ultrason (U)	0.153 ± 0.001	3.06 ± 0.01

Extraction by reflux gave a better yield than that obtained by ultrasound (21).

3.3. Chemical screening

The results are shown in (table.2) (5,6,7)

Table 2 Phytochemical screening results

Chemical group	Extract	Result
Alcaloïds	Macerat (acid)	(-)
Phenolic compounds	Infused (20 %)	(++)
Flavonoïds	Ethanolic	Alcalins (+)

		AlCl3 (++)
Tanins	Infused (20 %)	FeCl3 (+)
		Gelatin (++)
		Stiasny (+)
		Hydrolyzable (+)
Anthocyans	Infusé (20 %)	(-)
Coumarins	Ethanolic	(-)
Anthraquinones	Teinture (3%)	(-)
Saponosides	Decocted (1%)	(++)
Cardiac glycosides	Chloroformic	(-)
Amino acid/ Protein	Aqueous	Biuret (-)
Sucre (Molish)	Aqueous	(-)
Sucre réducteur	Aqueous	(±)
Desoxy sucre	Chloroformic	(-)

3.4. Total phenolic compounds content

The calibration curve obtained with the catechin standard is shown in (figure.3) (9,10):

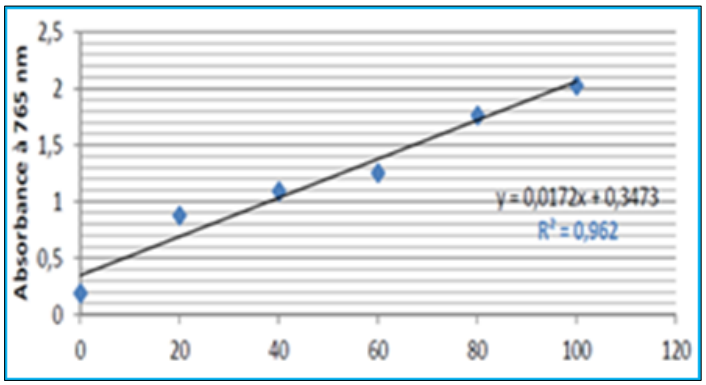

Figure 3 Catechin calibration curve (CPT)

The results are represented in (table.3)

The n-butanol extract (reflux) contains a higher content of CPT compared to the other extracts. The extracts obtained by ultrasound gave insignificant values.

Table 3 Content of total Phenolic Compounds (Folin – Ciocalteu, ug Eq. Catechin / mg of extract)

Extract	Concentration in ug of Eq. Catechin/mg of extract
chloroformic (R)	6.26 ± 1.04
acetate - ethyl (R)	0.19 ± 0.02
n-butanolic (R)	15.96 ± 0.09

3.5. Flavonoid content

The calibration curve obtained with the rutin standard is shown in (figure.4) (17,18)

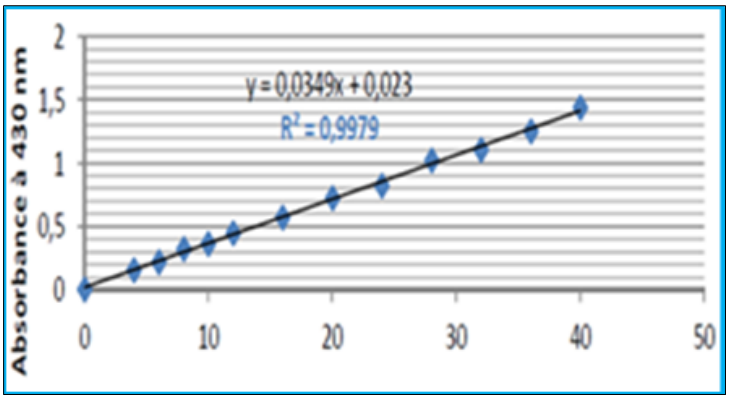

Figure 4 Rutin calibration curve (FLT).

The results are represented in (table.4)

Table 4 Flavonoid content (ug/ml) (AlCl3, ug Eq. Rutin / mg of extract)

Extract	Concentration in ug of Eq. Rutin/mg of extract
chloroformic (R)	3.92 ± 8.63
acetate - ethyl (R)	33.98 ± 4.98
n-butanolic (R)	2.89 ± 0.58
chloroformic (U)	26.03 ± 7.85
acetate - ethyl (U)	3.92 ± 8.63

Ethyl acetate (reflux) and Chloroformic (ultrasound) extracts contain the highest flavonoid contents.

3.6. Condensed tannin content:

The calibration curve obtained with the catechin standard is shown in (figure.5) (9.12):

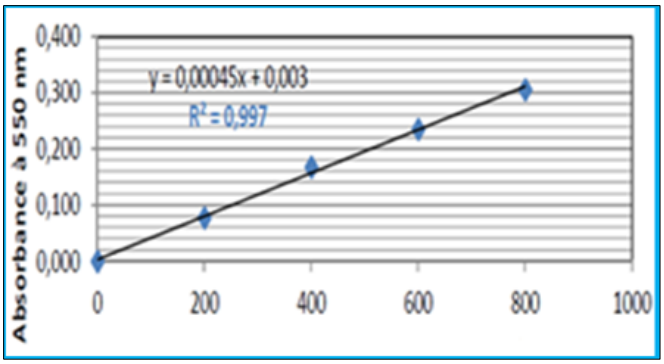

Figure 5 Calibration curve of catechin for the determination of condensed tannins

The results are represented in (table.5)

Table 5 Condensed tannin content (Vanillin, ug Eq. Catechin / mg of extract)

Extract	Concentration in ug of Eq. Catechin/mg of extract
chloroformic (R)	757.67 ± 14.32
acetate - ethyl (R)	28.61 ± 1.65
n-butanolic (R)	326.98 ± 12.39
chloroformic (U)	48.10 ± 12.40
acetate - ethyl (U)	291.85 ± 14.29

3.7. Pharmacological screening

3.7.1. Antioxidant activity

The results are shown in (figure.6)

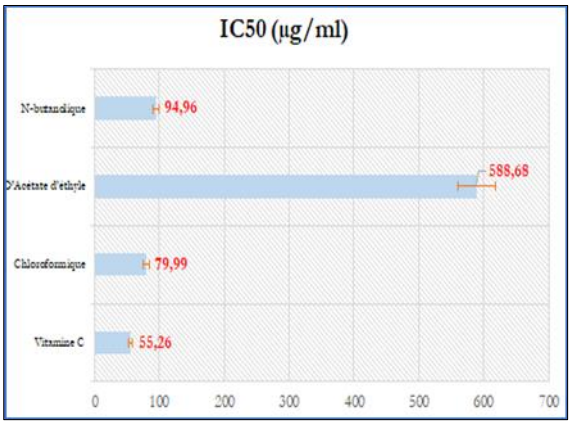

Figure 6 The IC 50 inhibitory concentrations of the different extracts (ug/ml)

The different extracts have a strong ability to trap the DPPH radical with IC50 values ranging from 79.99 ± 1.48 ug / ml to 588.68 ± 10.88 ug / ml.

The CPT content suggests that other compounds of a phenolic nature present in Phlomis are responsible for this activity (19,20).

3.7.2. Antimicrobial activity

Chloroform and n-butanol and ethyl acetate extracts slightly inhibited the growth of pseudomonas aeruginosa (ATCC 27853) and staphylococcus aureus (ATCC 25923) strains. On the other hand, no antibacterial activity was observed against strains of Escherichia coli (ATCC 25922) (20,24,25).

4. Conclusion

Microscopic analysis of leaf powder at magnification (x 400) shows the presence of branched covert hairs, unicellular head and foot secretory hairs, and calcium oxalate crystals; this could join the work of Fettah et al, 2011, but the lack of work on the histological level does not allow an objective comparison to be established.

Reflux extraction gave a better yield compared to ultrasound; this could be explained by the duration of contact between extractive solvent and plant material, which is longer in the first case.

Phytochemical screening demonstrated the presence of phenolic compounds (total, flavonoids, tannins) and saponosides, on the other hand alkaloids and cardiotonic glycosides are absent.

The extracts obtained from the leaves contain a significant quantity of phenolic compounds: flavonoids, tannins, saponins and reducing sugars with contents which vary according to the two extraction methods (reflux and ultrasound) and the choice of solvent, which corresponds the results of the work of Limem-Ben Amor I et al (2009).

The study of the antibacterial properties of the extracts revealed a slight activity on some pathogenic strains such as P. aeruginosa and S. aureus. On the other hand, it turns out to be completely negative on E. coli. This could be attributed to the presence of an outer membrane, which possessed hydrophilic polysaccharide chains that act as a barrier to polyphenolic compounds; whereas the cell membrane of Gram (+) bacteria contains mucopolysaccharides, proteins and phospholipids. Thus, the permeability, entry and reaction of most antibiotics and antimicrobial agents through the cell envelope (the outer and cytoplasmic membrane) are highly effective against Gram (+) bacteria depending on the reaction with the cell coat. protein (mucopolysaccharides or peptidoglycans)

The study of the antioxidant properties revealed a very significant activity of all the extracts (chloroformic, n-butanolic, acetate - ethyl) with IC50 values comparable to that of ascorbic acid; these results are similar to those of Limem-Ben Amor I. et al; 2009 and Ramili. I; 2013.

It would be interesting to complete this work with in vivo and in vitro toxicity studies, and to determine the chemical structure of the bioactive substances at the origin of the activities of the extracts.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors and all co-authors declare that they have no conflicts of interest in connection with this document, and the material described is not in the process of being published nor is it intended for publication elsewhere.

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