Phytochemical study and antioxidant activity of two Moroccan Lamiaceae species: *Nepeta nepetella* subsp. *amethystina* and *Sideritis arborescens* Salzm. ex Benth.

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ABSTRACT

Medicinal plants are widely used in the treatment of numerous diseases. These plants synthesize a wide variety of bioactive metabolites that exhibit countless biological and pharmacological activities. *Nepeta nepetella* and *Sideritis arborescens* are aromatic and medicinal plants belonging to the Lamiaceae family. These species are used in folk medicine in the treatment of several diseases. The present investigation aims to carry out a phytochemical screening of the aqueous extracts of *N. nepetella* and *S. arborescens* to reveal the different groups of phytochemicals present in the polar extract of these species, to estimate the amounts of phenolic compounds including total polyphenols, total flavonoids, tannins, anthocyanins, and hydroxycinnamic acids, and to evaluate their antioxidant capacity using radical scavenging activity, molybdenum and ferric reduction potentials, and lipid peroxidation inhibition effects. The results of this study showed that these two Lamiaceae species contain a wide variety of metabolites including terpenoids, cardiac glycosides, phenols, proteins, flavonoids, tannins, quinons, coumarins, saponins, essential oils, and reducing sugars. Moreover, the estimation of the amounts of different phenolic compounds showed that both species have important phenolic compounds contents including flavonoids, tannins, and hydroxycinnamic acids. The antioxidant tests showed that both species display good antioxidant activity in which *S. arborescens* exhibit the highest antioxidant potential. In conclusion, the findings of this study suggest that *Nepeta nepetella* and *Sideritis arborescens* have important amounts of antioxidant phenolic compounds that can be used in the treatment of several diseases.

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1. Introduction:

Plants have been used as remedies for many years. The constituents of medicinal plants are being used in the times past as medication to treat several diseases [1]. They are a natural source of producing a wide variety of bioactive compounds in the most efficient way and with precise selectivity [2]. According to the biosynthetic origin of those bioactive phytochemicals, they can be divided into several categories such as alkaloids, phenolics, flavonoids, steroids, terpenes, saponins, etc. [3]. Phenolic compounds are the most abundant secondary metabolites in plants [4]. These bioactive

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compounds can act as antioxidants in several ways. Antioxidants can prevent or decelerate the oxidation of other molecules. Their protective action is exerted either by stopping the formation of free radicals or by scavenging these free radicals [5]. Moreover, the use of natural phenolic compounds as antioxidants is a field of growing interest, mainly in complementary medicines and food science, because of the harmful effects of some synthetic antioxidants on human health [6].

Lamiaceae incorporates a wide variety of plants with biological and medical applications and it is one of the most important families in the production of antioxidant phytochemical compounds [7]. This botanical family comprises about 250 genera and over 7,000 species. Moreover, several species of this family are widely used as culinary herbs and reported as medicinal plants in several folk traditions [8]. *Nepeta nepetella* (*N. nepetella*) and *Sideritis arborescens* (*S. arborescens*) are aromatic and medicinal plants belonging to the Lamiaceae family. The use of *Sideritis* species in folk medicine was reported in several investigations in the treatment of inflammatory, gastrointestinal, and respiratory diseases [9-10]. In addition, *Nepeta* species were reported to treat several ailments such as tuberculosis, malaria, pneumonia, influenza, stomach disorders, respiratory disorders, asthma, colds, coughs, etc. [11].

The present study aims to carry out a phytochemical screening of the aqueous extracts of *N. nepetella* and *S. arborescens* to reveal the different groups of phytochemicals present in the polar extracts of these species, to estimate the amounts of phenolic compounds including total polyphenols, total flavonoids, flavonols, tannins, anthocyanins, and hydroxycinnamic acids, and to evaluate their antioxidant capacity using radical scavenging activity, molybdenium and ferric reduction potentials, and lipid peroxidation inhibition effects.

2. Material and methods:

2.1. Plant material:
The aerial parts of *N. nepetella* were collected in the Middle Atlas of Morocco (Aït-Oumghar region). The aerial parts of *S. arborescens* were collected in Goulmima (Errachidia region). The plant material of the two species was air-dried for a week before extraction.

2.2. Extraction:
The aqueous extracts were obtained by the decoction method. For that, 10 g of crushed and powdered plant material was extracted with 500 ml of deionized water for 30 minutes. After cooling, the mixture was filtered and the obtained filtrate was concentrated to dryness in a ventilated oven (45 °C), then the dried extracts were collected and stored at 4 °C until use.

2.3. Phytochemical screening:
Chemical tests for the screening and the identification of alkaloids, terpenoids, cardiac glycosides, phenols, proteins, flavonoids, tannins, saponins, iridoids, essential oils, reducing sugars, and coumarins were carried with extracts prepared using the standard procedures [12].

2.4. Determination of different families of phenolic compounds:

2.4.1. Total polyphenolic content (TPC):
The total polyphenolic content was determined by the Folin – Ciocalteu method, described by Singleton and Rossi (1965) [13], with slight modifications. In brief, Aqueous extracts (100 µL), Folin-Ciocalteau reagent (500 µL), and 10% sodium carbonate (400 µL) were introduced into test tubes. After 30 minutes of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid was used as a standard for the calibration curve and results were expressed as Gallic acid equivalents (GAE mg/ g extract, mean ± STD of three replicates).

2.4.2. Total flavonoid content (TFC):
The total flavonoid content of each extract was determined using the aluminum trichloride colorimetric method, described by Bouhlali et al. (2016) [14], with slight modifications. Briefly, 0.5 mL of test samples were mixed with 100 µL of sodium nitrate (5%). After 6 min, 100 µL of aluminum chloride (10%) and 1mL of sodium hydroxide (1M) were added and left at room temperature for 15 min. The absorbance of the mixtures was measured at 510 nm and total flavonoid content was calculated as rutin equivalents. The calibration curve was prepared in the same conditions using 0.0125-1 mg/mL of rutin. The results were expressed in mg of rutin equivalent per g of extract (RE mg/ g E, mean ± STD of three replicates).

2.4.3. Flavonols content (FC):
The total flavonol content in extracts was estimated using the method reported by Formagio et al. (2014) [15] with slight modifications. To 1 mL of sample, 1 mL AlCl₃ (2%) prepared in ethanol and 3 mL (5%) sodium acetate were added. The
mixture was vortexed and incubated for 2 hours at ambient temperature. Afterward, absorbance was read at 440 nm. The calibration curve was prepared in the same conditions using quercetin. Total flavonols were expressed as mg of quercetin equivalents per gram of extract (mg QE/g E, mean ± STD of three replicates).

2.4.4. Total condensed tannins content (TCT):
Determination of condensed tannins was carried out according to the method of Heimler et al. (2006) [16]. Briefly, 200 µL of each extract was mixed with 1,5 mL of 4% methanolic solution of vanillin and 0,75 mL of concentrated hydrochloric acid. The absorbance of mixtures was determined at 500 nm after 15 minutes of incubation at room temperature. The calibration curve was prepared in the same conditions using catechin. Results were expressed in mg of catechin equivalent per g of extract (CE mg/ g E, mean ± STD of three determinations).

2.4.5. Total hydroxycinnamic acids assay (THA):
Estimation of total hydroxycinnamic acids content was carried out using the method described by Štefan et al. (2014) [17]. To 0.2 g of the powdered plant material of each plant, 80 mL of ethanol (50 %) was added, and the mixture was boiled in a water bath under a reflux condenser for 30 min. After cooling, the extract was filtered and the filter was rinsed with 10 mL of ethanol (50 %). The combined filtrate and the rinsings were made up to 100 mL with alcohol (50 %) and served as the stock solution. One mL of the stock solution was mixed with 2 mL of 0.5 M hydrochloric acid, 2 mL of a solution containing 10 % sodium nitrite and 10 % sodium molybdate, then 2 mL of sodium hydroxide (8.5 %) was added, and distilled water was added to complete 10 mL of the reaction mixture. Distilled water was used instead of stock solutions for blank. The absorbance of the test solutions was measured immediately at 505 nm. The content of THA expressed as gram of THA per 100 g of plant material, which was calculated from the following expression: THA (g/100g PM) =Ax2.5/m, where (A) is the absorbance of the test solution at 505 nm and (m) is the mass of the used plant material powder in grams (THA g/ 100g PM, mean ± STD of two determinations).

2.4.6. Determination of total anthocyanin content (TA):
The total anthocyanin content of the extracts was measured using the pH differential method [18] with slight modifications. In brief, 200 µL of each extract was separately dissolved in potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate (0.4 M, pH 4.5) with a pre-determined dilution factor. Sample measurement absorbencies were read at 510 and 700 nm against blank containing distilled water. The absorbance (A) of the diluted sample was then calculated as follows:

\[ A = (A_{\text{vis-max}} - A_{700 \text{ nm}})_{\text{pH 1.0}} - (A_{\text{vis-max}} - A_{700 \text{ nm}})_{\text{pH 4.5}} \]

The monomeric anthocyanin pigment concentration in the original sample was calculated according to the following formula:

Total monomeric anthocyanins (mg/L) = \( A \times MW \times 1000/(e \times C) \)

With “MW” is cyanidin-3-glucoside molecular weight (449.2 g/mole); “e” : Molar absorptivity ( 26900); “C” is the concentration of extract in the buffer in milligrams per milliliter. Results were expressed as the mean of three determinations ± STD.

2.5. Antioxidant activity:
2.5.1. Ferric reducing antioxidant power assay (FRAP):
This technique was developed to measure the ability of antioxidants to reduce ferric iron (Fe³⁺) to ferrous iron (Fe²⁺) at low pH, based on the method of Benzie & Strain (1999) [19]. The FRAP reagent was made by mixing 100 mL of acetate buffer (0.3 M : pH=3.6), 10 mL tripyridyltriazine (TPTZ) solution (10 mM) prepared in HCl (40 mM) and 10 mL of Ferric chloride solution (20 mM). Fifty microliters of each sample were added to 2 mL of the freshly prepared FRAP reagent. After 10 min of incubation at room temperature, the absorbance was measured at 593 nm against a blank containing distilled water instead of extract solution. Known concentrations of ferrous sulfate (FeSO₄, 0,1-1 mM ) were used as a reference to generate the calibration curve. The results were expressed as Fe²⁺ equivalent (mmol) per gram of extract (Fe²⁺ E mmol/gE mean ± STD of three replicates).

2.5.2. DPPH radical scavenging activity assay (DPPH):
DPPH’ (1,1-diphenyl-2-picrylhydrazil) free radical scavenging activity was carried out according to the method described by Bouhlali et al. [16], with slight modifications. One hundred microliters of each concentration (0,1-0,5 mg/ml) of extracts were added to 1 mL of freshly prepared methanolic solution of DPPH (0,1 mM). The mixture was allowed to stand for 20 minutes. The absorbance was measured at 517nm versus methanol. A positive control solution
was made by mixing 0.1 mL of distilled water with 1 mL of DPPH solution and BHT (Butylated hydroxytoluene) was used as a standard antioxidant. The percentage of inhibition of scavenging the DPPH radical was calculated using the following formula:

\[ I\% = \frac{A(\text{control}) - A(\text{test})}{A(\text{control})} \times 100 \]

Where, \( A(\text{test}) \) : absorbance of test sample, and \( A(\text{control}) \) : absorbance of control. The results were expressed as IC\(_{50}\) concentration (half-maximal inhibitory concentration) in mg/ml which was determined from the linear regression equation of the inhibition percentage values.

2.5.3. Total antioxidant capacity (TAC):
The total antioxidant capacity of the extracts was carried out using the procedure described by Prieto et al. (1999). Two hundred microliters of each extract was combined with 2 ml of the reagent solution (0.6M sulfuric acid, 4 mM ammonium molybdate, and 28 mM sodium phosphate). The reaction mixtures were incubated at 95°C for 90 minutes. After cooling their absorbance was measured at 695 nm against a blank. A standard curve of ascorbic acid was used to determine the antioxidant activity of extracts. The results were expressed as mg of ascorbic acid equivalent per g of extract (AAE mg/gE mean ± STD of three replicates).

2.5.4. Thiobarbituric acid reactive substances (TBARS):
The lipid peroxidation inhibition effect of extracts was performed according to the method described by Salman & Qadeer (2021) [20] with minor modifications. In brief, fifty microliters of each concentration of extracts and ascorbic acid (antioxidant standard) were individually added to 200 µL of egg yolk (10%) prepared in phosphate buffered saline (PBS), then 25 µL of 0.07 M AAPH and 200 µL of PBS were added. The mixture was shaken vigorously and incubated at 37°C for 30 min. Next, 750 µL of acetic acid (20%), 750 µL of 0.8% (w/v) thiobarbituric acid prepared in sodium dodecyl sulfate, and 25 µL of trichloroacetic acid were added to the mixture. After boiling at 95°C and cooling for 60 min, 3 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation (%) by the sample was calculated according to the following formula:

\[ \text{percentage of inhibition} (\%) = \left( 1 - \frac{Ae}{Ac} \right) \times 100 \]

Where \( Ac \) is the absorbance value of the fully oxidized control and \( Ae \) is the absorbance of samples with extracts. The results were expressed as IC\(_{50}\) value (mg/ml) which was determined from the linear regression equation of the inhibition percentage values.

2.6. Statistical analysis:
Data with two groups were analyzed using unpaired t-test and data with three groups were analyzed using one way Anova test. Graphpad prism 8 software was used to carry out the analysis. \( P \) value less than 0.05 was considered statistically significant. Results are expressed as means± standard deviation (SD).

3. Results and discussion:
3.1. Phytochemical screening:
The results of the phytochemical screening are represented in table 1. Preliminary phytochemical screening is an important procedure that allows identifying new sources of therapeutically and industrially useful compounds. The phytochemical screening of *N. nepetella* and *S. arborescens* showed that these two species contain a wide variety of phytochemicals. Both species contain terpenoids, cardiac glycosides, phenols, proteins, flavonoids, tannins, quinons, coumarins, saponins, essential oils, and reducing sugars. Moreover, alkaloids were absent in the two extracts, whereas iridoids were detected only in *S. arborescens*. All these constituents have been reported to exert several biological activities and medicinal properties [21]. Therefore, the isolation of these bioactive phytochemicals can help in the development of new drugs. Furthermore, several studies investigated the chemical constituents of *Nepeta* and *Sideritis* species. The study of González-Burgos et al. (2011) [22] reviewed the chemical components found in *Sideritis* genus. This study revealed that *Sideritis* plants contain terpenes, phenolic compounds, flavonoids, iridoids, essential oils, coumarins, lignans, and sterols, where diterpenes, flavonoids, and essential oils occur in almost every species. Moreover, the study of Sharma et al. (2021) [23] investigated the chemical composition of *Nepeta* species and revealed that the species of this genus contain a variety of bioactive metabolites that belong to different classes of secondary metabolites, such as phenolic acids and their glycosides, flavonoids and their glycosides, iridoids, terpenoids,
steroids, lignans, amino acids, carbohydrates, volatile oils, etc.. These investigations showed that the species of these two genera are an important source of bioactive phytochemicals.

**Table 1. Phytochemical screening of extracts.**

<table>
<thead>
<tr>
<th>Compounds</th>
<th><em>N. nepetella</em></th>
<th><em>S. arborescens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quinones</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Iridoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Essential oils</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* +: present; -: absent

**3.2. Determination of the amounts of different families of phenolic compounds:**

The results of the estimation of different phenolic compounds in *S. arborescens* and *N. nepetella* are represented in table 2. These results showed that both species have important amounts of these phenolic compounds. Flavonoids, tannins, and hydroxycinnamic acids seemed to have the greatest amounts, whereas anthocyanins are present in a very low quantity. Moreover, *S. arborescens* showed the highest polyphenol composition content in which its TPC was 53% higher than that of *N. nepetella*. Flavonoids including flavonols, tannins, and hydroxycinnamic acids showed also the highest amounts in *S. arborescens*. Polyphenols including flavonoids, tannins, and phenolic acids are important metabolites. Several studies reported the amounts of these phenolic compounds in *Nepeta* and *Sideritis* species. There is a wide variety of *Nepeta* species that revealed similar TPC amounts including *N. humilis* (66.20 GAE mg/gE) [24], *N. cadmea* (53.45 GAE mg/gE) [25], *N. binaludensis* (41.23 GAE mg/gE). However, these amounts are generally lower than those reported for *Sideritis* plants including *S. scardica* (188.45 GAE mg/gE) [26], *S. amasiaca* (389.9 GAE mg/gE), *S. argyrea* (262.8 GAE mg/gE), etc. [27]. These results showed that *Sideritis* plants including *S. arborescens* generally have more phenolic compounds contents than *Nepeta* plants including *N. nepetella*.

**Table 2. The results of TPC, TFC, FC, TTC, THA, and TA assays.**

<table>
<thead>
<tr>
<th>Plant</th>
<th>TPC (GAE mg/gE)</th>
<th>TFC (RE mg/gE)</th>
<th>FC (QE µg/gE)</th>
<th>TCT (CEmg/gE)</th>
<th>THA (g/100g PM)</th>
<th>TA (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. arborescens</em></td>
<td>116.71±2.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.78±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>130.72±6.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.46±0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.30±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>N. nepetella</em></td>
<td>54.60±0.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.83±1.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.99±6.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.41±1.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43±0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in the same column with the same letter group are not significantly different (P<0.05). Data are represented as the mean of 3 replicates ± SD.

**3.3. Antioxidant activity:**

The results of antioxidant tests are shown in table 3. Both plants showed antioxidant effects in the four assays. This antioxidant activity is generally attributed to the polyphenols which are abundant metabolites in the extracts of these plants including flavonoids, tannins, and phenolic acids. *S. arborescens* showed the highest radical scavenging activity, molybdenum, and ferric reduction potentials, and lipid peroxidation inhibition effects, whereas *N. nepetella* showed a low antioxidant effect (P<0.05). This can be explained by the richness of *S. arborescens* extract in the different phenolic compounds.

**Table 3. The results of antioxidant activity.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH (IC&lt;sub&gt;50&lt;/sub&gt; mg/ml)</th>
<th>TAC (AAE mg/gE)</th>
<th>FRAP (Fe&lt;sup&gt;2+&lt;/sup&gt;E mmol/gE)</th>
<th>TBARS (IC&lt;sub&gt;50&lt;/sub&gt; mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. arborescens</em></td>
<td>0.24 (±0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30 (±0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.16 (±0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.48 (±0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>N. nepetella</em></td>
<td>0.56 (±0.01)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22 (±0.01)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.53 (±0.01)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.79 (±0.02)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trolox</td>
<td>0.1 (±0.004)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Values in the same column with different letter group are significantly different by Tukey’s multiple comparison tests (P<0.05). Values in the same column with different symbol (*,#) are significantly different by unpaired t-test. Data are represented as the mean of 3 replicates ± (SD).
Seladji et al. (2014) [28] studied the antioxidant effect of the extracts of *N. nepetella* and found that the radical scavenging activity of the aqueous extract of this plant was very lower (IC$_{50}$ = 10.43 mg/ml) than that reported in our study (IC$_{50}$ = 0.56 mg/ml). Nevertheless, there are several *Nepeta* plants that are reported to exhibit better radical scavenging activity such as *N. fessa* (IC$_{50}$ = 0.19 mg/ml) [29], *N. nuda* (IC$_{50}$ = 0.03 mg/ml) etc. [30]. Furthermore, *S. arborescens* showed the highest antioxidant potential. The radical scavenging activity of this plant can be considered as a moderate activity (IC$_{50}$ = 0.24 mg/ml) compared to some *Sideritis* plants with higher radical scavenging activity such as *S. stricta* (IC$_{50}$ = 0.03 mg/ml) [31] and others with lower radical scavenging activity such as *S. syriaca* (IC$_{50}$= 0.46 mg/ml) [32].

Polyphenols are important plant metabolites with diverse health-promoting effects. They are powerful antioxidants with numerous biological and pharmacological effects such as anti-inflammatory, immunomodulatory, and anti-mutagenic effects, etc. [33]. Furthermore, countless scientific evidences support the association between polyphenol intake and reduced risk for chronic diseases [34]. Therefore, the traditional intake of medicinal plants with high polyphenol contents can help in the treatment and the prevention of several diseases.

### 4. Conclusion:

In this present study, two traditional medicinal plants namely *N. nepetella* and *S. arborescens* were investigated by carrying out a phytochemical screening, estimation of different phenolic compounds contents, and antioxidant capacity evaluation. The results of these tests showed that these two Lamiaceae species contain a wide variety of metabolites, and important phenolic compounds contents including flavonoids, tannins, and hydroxy-cinnamic acids. The antioxidant tests showed that both species display good antioxidant activity in which *S. arborescens* exhibit the highest antioxidant potential.

### References: