New Assay Method of the Atractyloside and Carboxyatractyloside in the Human Blood

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Abstract

Atractylis gummifera L. or thistle glue, is a plant of the Asteraceae family, known in Arabic as Addad or Choûk El-Eulk. It is a plant of the Mediterranean region, found in
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North Africa and southern Europe. In Morocco, the plant is frequently found in the wild life, and is characterized by the production of a highly toxic glycoside called atractyloside (ATR). The main purpose of this work is to develop a new plasma assay method of Atractyloside and carboxyatractyloside in human blood, with simple and practical materials to save lives in danger. Root samples were collected in the region of Fés boulmane, and extracts by the new dosing method, were analyzed by the system of ultra-violet spectrometry (JASCO-530). The validation of our method is based on the recommendations of ICH guidelines for the adaptation of new analytical techniques. The coefficient of determination is 0.99, the limit of detection, limit of quantification, replicability and repetability are respectively 93 µg/ml, 310 µg/ml, 6.97 % and 7.71 %. While the sensitivity is 0.29, which reduces the risk of interference in the dosage of the active ingredient of the thistle glue plant. Many methods for the determination of atractyloside and carboxyatractyloside are published, often based on the use of liquid chromatography, gas or techniques cumbersome, complex and lacking in sensitivity and specificity. The results of our study show that this new method is practical, simple and applicable to all types of laboratories.

Keywords: Atractylis gummifera, Atractyloside, Carboxyatractyloside, Assay, Plasmatique determination

1. Introduction

Atractylis gummifera is a thistle of the Asteraceae family, known in Arabic as Addâd or choûk el-eulk (Daniele et al., 2005). It is a herbaceous plant, prickly, fragrant, perennial by its underground part consisting of a flexuous rhizome and taproots. distributed worldwide but especially abundant in the Mediterranean regions: in Northern Africa (Algeria, Morocco and Tunisia) and in Southern Europe (Italy, Greece, Spain and Portugal) (David et al., 1996).

Atractylis gummifera L, is an acaulescent perennial medicinal herb with a long woody root up to 30–40cm long and 7–8cm diameter. It has deeply divided prickly long leaves in a rosette 30–70mm wide; pink florets in 3–7cm wide capitula with middle bracts with three long apical spines. It grows in Northern Africa and Southern Europe in moist soils of cultured grounds and waste places. (Vallejoa and Perala; 2009, Errai and al 2018).

In traditional medicine, Atractylis gummifera is still widely used for therapeutic purposes such as fumigation against colds, dizziness and headache, infusion against bleeding during childbirth, antisyphilis, gangrene, hemorrhoids, skin cancer, poultice dries, heal wounds, as well as against boils, as a purgative, emetic and worming (Otero, 1983). Interestingly, the effectiveness of the remedy as an anti-inflammatory and anticancer agent is supported by pharmacological studies (Gaudeau and Gouthiere, 1991; El-Hilaly, 2003; Fakchich, 2014).

This plant is the source of several cases of severe poisoning, often accidental (confusion with wild artichoke). According to the Poison Control Centre of Morocco, The Atractylis gummifera ranks first plants responsible for poisoning, of 487 cases reported between 1980 and 2013 (CAPM, 2013).

Despite the plant's well-known toxicity, its ingestion continues to be a common cause of poisoning. The toxicity of Atractylis gummifera resides in atractyloside and carboxyatractyloside, two diterpenoid glucosides capable of inhibiting mitochondrial oxidative phosphorylation. Both constituents interact with a mitochondrial protein, the adenine nucleotide translocator, responsible for the ATP/ADP antiport and involved in mitochondrial membrane permeabilization (Daniele C et al., 2005).

All the underground parts of this plant contain two toxic diterpenoid glucosides: atractyloside (ATR) and carboxyatractyloside (CATR) (Fig. 1).
However, ATR was isolated from the roots of *Atractylis gummifera* for the first time by Lefranc (1868). The corresponding aglycone (attractyligenine) is a non-volatile diterpene of the (−) kaurene family, with a perhydrophenanthrenic structure (Piozzi et al., 1978).

CATR was isolated for the first time in 1964 and called gummiferin and subsequently identified as 4-carboxyatractyloside (Danieli et al., 1971). CATR differs from ATR owing to the presence of a second carboxylic group in position C-4 of the diterpene ring (Luciani et al., 1978).

The poisoned patients manifest characteristic symptoms such as nausea, vomiting, epigastric and abdominal pain, diarrhoea, anxiety, headache and convulsions, often followed by coma. No specific pharmacological treatment for *Atractylis gummifera* intoxication is yet available and all the current therapeutic approaches are only symptomatic (David et al., 1996).

Aim of the present work is the development of a new and simple method for determining of the plasma levels of atractyloside and Carboatractyloside in the roots of *Atractylis gummifera* using blood from healthy patients to the Fez University Hospital Center Morocco. In addition, the emergence of the number of Deaths and poisoning by this plant starts to become a major public health problem. To the best of our knowledge, the Determining the plasma levels of atractyloside and carboxyatractyloside contained in the roots of thistle glue of Morocco Has not-been the carried out before..

**II. Materials and Methods**

**II.1. Plant Material**

With the aim of meeting the objectives of our work and carrying out the necessary tests, we gathered the fresh roots of *Atractylis gummifera* from one of its natural habitats in Morocco between May and June 2014 in Moulay Yaakoub in the region of Fez-Meknes. The area where we have chosen our study to be carried out in is at an altitude of about 560 m above sea level, and is characterized by a rugged ground, with different altitudes ranging from 350 m to 500 m, and fertile soils. The climate of this area is a continental one characterized by a minimum temperature of 10 ° and a maximum of more than 30 °, but on the day of taking the samples, the temperature was around 37 °c (figure 2).
Once we have located the plants of Atractylis gummifera, thanks to its aerial part that was well described in the literature and after having confirmed it by one of the most recognized botanists, Professor Maatouï of the Department of Science and Production Techniques of the National School of Agriculture in Meknes, we harvested a good amount of roots from the latter, using a sappe (see figure 3).

The roots were quiet large (between 40 and 60 cm long and 20 to 30 cm wide), fleshy, with fine roots and having creeping rhizomes, hard, fibrous, yellowish sections. (Figure 3)

**Figure 2:** Sampling area of *Atractylis gummifera* roots

![Sampling area of Atractylis gummifera roots](image)

**Figure 3:** Collection of the roots of *Atractylis gummifera*

![Collection of the roots of Atractylis gummifera](image)

Afterwards, the harvested roots were cleaned and broken up into small pieces of 1 to 3 cm in diameter; these fragments were then dried at room temperature away from sunlight, with the purpose of preserving the integrity of the molecules. The plant material was then coarsely grounded in a blade mill to get a fine powder and get the extracts to be studied prepared.

**II.2. Blood Samples**

The samples of human blood were collected at the Hassan II Hospital Center (CHU) in Fes, in glass tubes. After that, they were centrifuged to get the plasma separated with the aim of analyzing on the same day of receiving the blood.
II.3. Extraction Method

On a daily basis, a six-concentration standard range was prepared (0.03, 0.06, 0.12, 0.24, 0.5, 1 mg / ml) from a stock solution of 1 mg of glutton with 1 ml of distilled water.

To a volume of 1 ml of the calibration range in 6 ml glass tubes, 1 ml of plasma, 2 ml of TCA are successively added. After centrifugation, 1 ml of the liquid phase is recovered in glass tubes. 1 ml of sulfuric acid was added giving a violet color (Figure 6).

**Figure 5:** Blood samples

**Figure 6:** Method of preparation of samples assayed spectrometer

- Immersion of dried root *Atactylis gummifera* (1g) in 100 ml of hot water for 1 hour
- Preparation of 7 levels of dilutions in a final volume of 1 ml
- Filtration
- Adding 1ml of plasma in each tube
- Adding 2 ml TCA (in each tube)
- Centrifugation 4min
- Agitation
- Organic phase
- Recovery of the aqueous phase
- Adding 1 ml of sulfuric acid
- Adding 1ml of sampling each tube
- Take sample of each dilution
- Spectrometer Jasco 530
  
  *(The waven length: 410 nm)*
II.4. Dosage and Analysis by UV Spectrometer

The spectrometric instrument used (Jasco-V-530) is made up of a light source, double beam. The data acquisition system associated to the spectrophotometer makes it possible to draw the curve giving the absorbance of the mixture as a function of concentration.

With the aim of testing and validating our new assay method, we looked for the maximum absorption of our sample and which was of the order of $\lambda = 410$ nm (figure 7).

II.5. Validation

We based the validation of our method on the recommendations of the ICH guidelines for the adaptation of new chromatographic techniques. From the estimated detection limit (estimated MDL), proceed with the forthcoming steps:

From the obtained results, calculate:

- Arithmetic mean of replica
  \[
  \bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}
  \]

- Standard deviation of replica:
  \[
  s(n) = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}}
  \]

or

\[x: \text{arithmetic mean of a series of measurements;}
\]

\[xi: \text{individual measures;}
\]

\[n: \text{number of measurements;}
\]

\[s: \text{standard deviation of a series of measures.}
\]

II.5.1. Limit of Detection

The detection limit of a method refers to the lowest concentration for a compound analyzed in an actual matrix that, when it undergoes all the steps of a complete method, including chemical extractions and pretreatment, produces a detectable signal with a reliability defined statistically different from that produced by a "white" under the same conditions. This is the concentration equivalent to 3 times the standard deviation corresponding to the concentration indicated in II.5.

\[\text{LDM} = 3 \times s\]

Where, LDM: detection limit of the method; s: standard deviation of replica.

II.5.2. Limit of Quantification

The quantification limit of a method is the minimum concentration that can be quantified using an analytical method with a defined reliability. This is the equivalent concentration at 10 times the standard deviation obtained when establishing the MDL.

\[\text{LQM} = 10 \times s\]

II.5.3. Linearity

The linearity limit is the highest reliable level of measurement that can be used taking into consideration all the factors to be involved in a method. It was established through the study of the ability (within the dosing interval) to produce results directly proportional to the concentration of analyte in a sample.

II.5.4. Repeatability

Repeatability at a given level concordes with the narrowness of the agreement between the individual results obtained on the same sample tested for the same sample under the same conditions: same laboratory, same operator, same batches of reagents, even equipment and in a reduced time interval.
II.5.5. Replicability

Replicability at a given level corresponds to the close agreement between the successive individual results obtained on the same sample tested in the same laboratory and under the following conditions: same analyst, same device, and the same day. The value will be determined from the following equation:

\[ t_{(0.975,n-1)} \times S_1 \]

\[ \sqrt{n} \]

where s1: standard deviation of a series of measurements referring to replicability.

III. Results

The validation of our method was based on the recommendations of the ICH guidelines for the adaptation of new chromatographic techniques.

The results of the analytical validation obtained are outlined in Tables I and II. These displays the specificity, precision and efficiency that typifies this technique; with other key benefits that are essentially simplicity, speed and adaptation to the conditions of all types of laboratories.

Before proceeding to the validation of our new plasma Atractyloside and Carboxyatractyloside assay, contained in the roots of Atractylis gummifera, we began by looking for the maximum absorption of our sample and which was of the order of \( \lambda = 410 \) nm (see figure 7).

**Figure 7:** UV spectrum of the aqueous extract of Atractylis gummifera

**Figure 8:** UV spectrum of the resulting final preparation of the extraction process
Then, repeatability, linearity and fidelity were verified. The results of the present study demonstrate that the regression line, determined from 6 concentration levels (each point represents an average derived from a series of five determinations), has good linearity with the function $y = 0.2847x + 0.2639$, a regression coefficient $r = 0.9972$ and a coefficient of determination of about 1 (Fig 9).

**Figure 9: Linearity Test**

\[ y = 0.2847x + 0.2639 \]
\[ R^2 = 0.997 \]

Repeatability test counts and repeatability results are determined from intraday absorbance values (Table I). Data recorded on the same day demonstrated good linearity and a coefficient of determination close to 1 ($r = 0.99$).

Intermediate precision was evaluated by analyzing the results derived from within the same laboratory, changing one or more factors (day, operator, equipment, etc.) for a given time interval (Fig. 10).

**Figure 10: Repeatability Test**

The "LDD" limit of detection was estimated by reference to the lowest amount of analyte detectable by the method, whereas for the LOQ limit of quantification we determined the lowest concentration that could be measured with an acceptable degree of fidelity, and accuracy (precision $>80\%$ or bias $<20\%$).
Table I: Repeatability Test

<table>
<thead>
<tr>
<th>Concentration of ATR (mg/ml)</th>
<th>Repeat n°1</th>
<th>Repeat n°2</th>
<th>Repeat n°3</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>0.260</td>
<td>0.280</td>
<td>0.279</td>
<td>0.273</td>
<td>0.011</td>
<td>0.041</td>
</tr>
<tr>
<td>0.06</td>
<td>0.264</td>
<td>0.274</td>
<td>0.283</td>
<td>0.273</td>
<td>0.009</td>
<td>0.034</td>
</tr>
<tr>
<td>0.12</td>
<td>0.283</td>
<td>0.280</td>
<td>0.320</td>
<td>0.294</td>
<td>0.022</td>
<td>0.076</td>
</tr>
<tr>
<td>0.25</td>
<td>0.321</td>
<td>0.308</td>
<td>0.360</td>
<td>0.330</td>
<td>0.027</td>
<td>0.082</td>
</tr>
<tr>
<td>0.5</td>
<td>0.366</td>
<td>0.396</td>
<td>0.408</td>
<td>0.390</td>
<td>0.022</td>
<td>0.055</td>
</tr>
<tr>
<td>1</td>
<td>0.531</td>
<td>0.534</td>
<td>0.581</td>
<td>0.549</td>
<td>0.028</td>
<td>0.052</td>
</tr>
</tbody>
</table>

Within the established conditions, the limit of detection is 93 μg / ml, the limit of quantification is equal to 310 μg / ml and the sensitivity is 0.29.

Repeatability was assessed by analyzing the same sample under the same conditions: same laboratory, same operator, same batches of reagents, same equipment and in a short time interval, the results are presented in Figure 10.

![Figure 11: Fidelity Test](image)

Table II: Fidelity Test

<table>
<thead>
<tr>
<th>Concentration of ATR (mg/ml)</th>
<th>Day n°1</th>
<th>Day n°2</th>
<th>Day n°3</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>0.304</td>
<td>0.280</td>
<td>0.260</td>
<td>0.281</td>
<td>0.022</td>
<td>0.078</td>
</tr>
<tr>
<td>0.06</td>
<td>0.309</td>
<td>0.274</td>
<td>0.264</td>
<td>0.282</td>
<td>0.024</td>
<td>0.084</td>
</tr>
<tr>
<td>0.12</td>
<td>0.323</td>
<td>0.280</td>
<td>0.283</td>
<td>0.295</td>
<td>0.024</td>
<td>0.082</td>
</tr>
<tr>
<td>0.25</td>
<td>0.359</td>
<td>0.308</td>
<td>0.321</td>
<td>0.329</td>
<td>0.026</td>
<td>0.080</td>
</tr>
<tr>
<td>0.5</td>
<td>0.428</td>
<td>0.396</td>
<td>0.366</td>
<td>0.397</td>
<td>0.031</td>
<td>0.078</td>
</tr>
<tr>
<td>1</td>
<td>0.587</td>
<td>0.534</td>
<td>0.531</td>
<td>0.550</td>
<td>0.031</td>
<td>0.057</td>
</tr>
</tbody>
</table>

Table II provides a summary of the results having to do with precision methods (inter-day values) for the determination of Atractyloside and Carboxyatractyloside. In accordance with the results found in repeatability, values recorded from day to day kept the same trend in linearity with a coefficient of determination \( r = 0.99 \).
IV. Discussion
The dried roots are used for medicinal purposes or as an insect repellent (Georgiou et al., 1988). They are used as therapeutic fumigation against colds, vertigo and headache and infusion against hemorrhage during delivery. Furthermore, these dried roots are also used as anti-syphilitic and against boils, purgative, emetic and as dewormer, but unfortunately, the root of the thistle has already been incriminated for hepato and fatal nephrotoxicity either in humans or in domestic animals feeding on plants containing atractyloside (Stuart et al 1981, Martin et al, 1986) and with great resemblance between the histological results of human and animal organs.

As stated in (Capdevielle and Darraq, 1980), in humans, symptoms begin 6-36 h after ingestion of rhizome extracts of Atractylis gummifera. Palpable symptoms are gastrointestinal problems, including nausea, vomiting, epigastric and abdominal pain, and diarrhea (Capdevielle and Darraq 1980, Georgiou et al., 1988).

Along similar lines, some reports also describe general anxiety, headaches, drowsiness, arrhythmia, and seizures (Hamouda et al., 2000). Generally, these symptoms are followed by coma (Capdevielle and Darraq, 1980).

Laboratory results (marked increase in SGOT, SGPT, and bilirubin) may pinpoint severe hepatocellular injury and acute renal failure (Georgiou et al, 1988; Nogue et al, 1992). Histopathological autopsy examination affirms massive gastrointestinal hemorrhage, necrosis of the hepatic parenchyma with collapse of interstitial connective tissue and accumulation of macrophages (Caravaca-Magarinos et al., 1985).

However, several assay techniques have been described earlier but they lacked validity. The first of the chromatographic techniques that was developed is that done by Calmes et al, in 1994. After a liquid extraction with a water-methanol mixture from fresh roots of Atractylis gummifera L., reverse phase ATR and CATR assay with a water-acetonitrile mixture was performed using a dispersed light detector. The detection limit of the technique is estimated at 9 μg ATR (Calmes et al 1994).

The other chromatographic techniques described all conform to a coupling with mass spectrometry: a general method for the determination of the main vegetable toxicants in the blood by LC-MS-MS was developed by Gaillard and Pepin in 1999. The separation is carried out on a C18 column using an ammonium formate buffer at pH 3 and an elution gradient with acetonitrile. The detection of ions is generally carried out in negative mode (Gaillard et al in 1999).

Steenkamp et al. suggested another method. They used a mobile phase that is made up of a mixture of ammonium formate buffer, methanol and acetonitrile, with a linearity range ranging from 1 ng / ml to 160 μg / ml. (Steenkamp et al 2006)

A GC-MS method has been developed for the detection of atractyloside in gastric lavage fluids and Callilepsis laureola root extracts, with a detection limit of 0.0451 mmol / L [Laurens et al 2001].

A competition ELISA immunoenzymatic technique has been developed for diagnostic purposes. The results generated have been confirmed by different chromatographic techniques [Bye et al 1990].

The dosage of sugars in solution can be carried out by the so-called anthrone method. It is a colorimetric method based on the intramolecular dehydration of oses in acid medium (H2SO4). The furfural derivatives obtained condense with the anthrone to give colored products (violet). The color differences obtained give a certain specificity to the technique. The results are read on a spectrophotometer where the principle of our method has been validated according to ICH.

However, the whole of the parts of the plant contain the active ingredient Atractyloside (Atr) with variable content. The root contains the highest toxic content followed by the stem, bracts, flower, seed and finally the leaf contains the least (Obatomi and Bach, 1998). Several factors determine the toxicity of Glu thistle (the dose, the age of the subject being intoxicated, the amount and nature of the food substance ingested). Indeed, according to Lefranc, a dose of 100g of fresh root milk infusion or taken in kind, would be fatal for an adult. While according to Charnot, an individual of 60 kg would be killed by 480g of root approximately.
ATR and CATR were also isolated from other plants of different genera, including Callilepis laureola, Xanthium strumarium, Iphiona aucheri and Wedeila glauca (Obatomi and Bach, 1998). ATR analogues have also been found in aqueous extracts of green beans and roasted Coffea arabica. In particular, three glycosides have been identified: 2-O-(2-O-isovaleryl-beta-D-glucopyranosyl) -attractyligenin, 2-O-beta-D-glucopyranosyl-atractytylenin and 2-O- (3-O- beta d-glucopyranosyl-2-O-isovaleriy beta-D-glucopyranosyl) -tracytylenin (Obermann and Spiteller, 1976, Richer and Spiteller, 1978).

Several factors, including climate, extraction method, soil composition, timing of harvest and genetic factors, influence the diterpenoid glucoside content in the rhizome of Atractylis gummifera (Errai S and al 2018). For example, differences in ATR content in the rhizome of Atractylis gummifera grown in Sardinia or Sicily (Fassina et al., 1962). A greater amount of ATR was found in the rhizomes harvested in autumn or winter, than in spring or summer, in agreement with a higher content in reserve substances and compounds active in the underground part of a plant during the quiescence period. And finally, a quantitative evaluation study showed that Atractylis gummifera of Sicilian origin had a higher ATR content than that of Sardinian origin, even though both plants were acclimatized in the same territory different from the native habitat origin (Toth, 1964).

ATR contains multiple polar groups and is soluble in water but has relatively low solubility in organic solvents. CATR differs from ATR because of the presence of a second carboxylic group at the C-4 'position. CATR of diterpene is present in fresh plants, not in dried plants, because it is decarboxylated from ATR during aging or desiccation. CATR is also more toxic than ATR (Luciani and Carpenedo, 1978).

V. Conclusion

The new method of plasma determination of atractyloside (ATR) and carboxyatractyloside (CATR) in the roots of thistle glue (Atractylis gummifera) showed a coefficient of determination of 0.99. The limit of detection, limit of quantification, replicability and repetability are respectively 93 µg/ml, 310 µg/ml, 6.97 % and 7.71 %., while the sensitivity is 0.29, which reduces the risk of interference in the dosage of the active ingredient of the thistle glue plant. Many methods for the determination of atractyloside and carboxyatractyloside are published, often based on the use of liquid chromatography, gas or techniques cumbersome and complex.

This new method is validated according to ICH norms and has the advantage of being simple, practical and applicable to all laboratories regardless of their levels.

References


