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Research Article

Pharmacognostical Investigations on Medicinal Plants

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ABSTRACT

Plants have always been used by humans to find cures for its diseases. After a prosperous period for synthetic molecules, the craze for natural plant substances is now a fact. The expanding market for medicinal plants, because of globalization, requires technical botanical identifications, standardized, to allow a secure commerce. The aim of our work is to establish a pharmacognostical standard methodology, through various examples, leading to useful standards trade of medicinal plants. This study, divided into three parts, include macroscopic and microscopic analysis of Origanum compactum, Opuntia ficus indica, Vitex agnus castus and Stephania tetrandra. Another part of this work concerns the screening phytochemicals such as TLC for Viburnum, Hieracium pilosella and Secale, HPLC chromatographic profiles for Vitex agnus castus and Stephania tetrandra and UV dosage on Hieracium pilosella, which allowed us to highlight the main phytochemical markers for each plant, for example. These results provide on the one hand, qualitative characterization by TLC and also quantitatively by HPLC and UV dosage. The last part of our work concerns the evaluation of physico-chemical characteristics of Hieracium pilosella, Piper nigrum and Piper longum. This work shows the usefulness of such analysis to create a standardized method for botanical and chemical identification, always necessary before using medicinal plants.

Key-words: Anatomical identification, TLC, UV dosage, HPLC, Drug Standardization.

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INTRODUCTION:

Medicinal plants, or more specifically, their active ingredients, were the source, direct or indirect, of most drugs used by man, until the early twentieth century. Subsequently, the chemical industry has developed synthetic molecules identical to the active plant molecules. Among 250 000 higher plants recorded almost 22,000 species are used in traditional medicine, WHO says. From the same source, 80% of the world's population yet uses only vegetable raw materials or their crude extracts for healing. We know that 50-60% of pharmaceuticals are natural or synthesized from natural products (vitamins, minerals, traditional medicines and herbal medicines, homeopathic remedies, probiotics, amino acids and essential fatty acids). An initial list of 34 medicinal plants that could be sold to the public outside the pharmaceutical channel was published in 1979. A new list containing 148 plants was published in 2008 and replaces the previous one. In France, the herbalist diploma was abolished in 1941 and the sale of medicinal plants included in the Pharmacopoeia is reserved to pharmacists. For all other medicinal plants, a scientific record, stating the botanical identification (morphological and anatomical) and phytochemical characterization, is necessary for any pharmaceutical use. We know that the extraction of active ingredient from plant is often less expensive than its synthesis. Therefore, our work aims to develop a standardized methodology for plant identification, botanical and phytochemical, for medicinal purposes. To complete this methodological study we will publish later, an "anatothèque", i.e. a kind of library containing all the standard data of specific botanical identification of key medicinal plants.

MATERIALS AND METHODS:

***Plant material*:**

The plants, identified by us, were dried at room temperature, in darkness and were cut into small pieces and were grounded into fine powder using a household blender.

Preparing slides:

Observations are based on microscopic studies of sectioned and stained material of tissues Transverse sections are prepared with a sliding microtome (MSE) and stained in “alun carmine-green” combination or Mirande reagent[1] during 2 to 3 minutes then washed with water. Following staining, the transverse sections are mounted on glass slides using glycerin gel. Powder observations were made using Chloral hydrate solution R (Ph. Eu.). Observations were made with a LEICA Microsystems DMLB microscope, and pictures were taken with Digital Camera Power Shot S40 CANON photo-micrographic system. For the description we have used some help books[3].

Total ash (Ph. Eur. 2.4.16)

Heat a cup of red silica or platinum for 30 min, cool in a desiccator, and weigh. Uniformly distribute in the crucible 1.00 g of the drug substance or vegetable spray, to be examined. Dry at a temperature of 100 ° C to 105 ° C for 1 h and calcined to constant weight in a muffle furnace at 600 ° C ± 25 ° C, cooling the crucible in a desiccator after each ignition. No fire should occur at any time of the operation. If, after a prolonged calcinations ashes still contain black particles, the ash in hot water, filtered by a filter paper without ashes and calcined again with the filter residue. Collect the filtrate and the ashes carefully evaporated to dryness and ignite to constant mass.

Loss on drying of plants (Ph. Eur. 2.8.17)

In a flat-bottomed capsule about 50 mm in diameter and approximately 30 mm in height, weighing 1 g of the plant quickly to examine, finely pulverized. Dry in an oven at 100-105 ° C for 3 h. Allow to cool

in a desiccator over diphosphorus pentoxide R or anhydrous silica gel R and weigh. Calculate the result as a percentage by mass.

Determination of derivatives ortho-dihydroxycinnamic Totals: (Ph. Eur. 2.2.25)
Solution (a). In a flask, place laughingstock (355). Add 90 ml of alcohol R and 50 percent V / V. Heat to reflux in a water bath for 30 min. Cool and filter, collecting the filtrate in a volumetric flask of 100 ml. Rinse the flask and the filter with 10 ml of alcohol R and 50 percent V / V. Add them to the filtrate to 100.0 ml with the same solvent.
Solution (b). In a 10 ml volumetric flask, introduce successively with stirring after each addition, 1.0 ml of solution (a), 2 ml of 0.5 N hydrochloric acid, 2 ml of a solution containing 10 percent m / V of sodium nitrite R and 10 per cent m / V sodium molybdate R, 2 ml of dilute sodium hydroxide R and dilute to 10.0 ml with water. Immediately measure the absorbance at 525 nm of the solution examined (b) compared to the compensation liquid obtained by introducing a volumetric flask of 10 ml, 1.0 ml of solution (a), 2 ml of 0.5 N hydrochloric acid, 2 ml of dilute sodium hydroxide R and dilute to 10.0 ml with water. Calculate the percentage content of derivatives ortho-di-hydroxy-cinnamic total, expressed as chlorogenic acid using the expression: $\frac{A \times 1000}{188 \times m}$. Take 188 as the value of the specific absorbance of chlorogenic acid at 525 nm. A represent absorbance at 525 nm and m represent mass of the sample in grams.

Determination of essential oils in vegetables (Ph. Eur. 2.8.12)
The determination of essential oils in vegetable drugs made by distillation with water steam distillation in a special apparatus, under the conditions described in 2.8.12 of the European Pharmacopeia. The distillate is collected in the graduated tube, using xylene to fix the essential oil, the aqueous phase is automatically returned to the distillation flask.
Place the prescribed volume of liquid in the distillation flask, add porous porcelain and adapt the set of condensation. Introducing water through the funnel R filler N until you get to good level. Heat the liquid in the flask until it boils and adjusts the distillation rate to 2-3 mL / min. Place in the flask the quantity of drugs prescribed and continues the distillation as described above at the time and the speed indicated. Stop heating and after 10 min to read the volume of fluid collected in the graduated tube and subtract the volume of xylene determined above. The difference represents the amount of essential oil in the mass of the drug taken. Calculate the results in milliliters per kilogram of drugs.

Thin layer chromatography (Ph. Eur. V.6.20.2)
The test solution consist of 1.0 g of powdered drug (355) in 10 mL of methanol R. Heat in water bath at 60 to 100 °C for 10 min. Allow to cool and filter. TLC was developed on Silica gel plate F254 (5-40 microns) with mobile phases. 10-20 µL was deposited on the TLC as band and developed up to 8-10 cm and dried in air. For detection the TLC was revealed with spray reagents.

Liquid chromatography (Ph. Eur. 2.2.27)***:***
HPLC analysis of Vitex:
All solvents and reagents from various suppliers were of the highest purity needed for each application and were purchased from Merck and Sigma–Aldrich. The analytical HPLC system employed consisted of a Dionex P680 HPLC high performance liquid chromatography coupled with a UV–vis multiwavelength detector (Dionex UVD 170U detector). The analytical data were evaluated

using a Dionex data processing system. The separation was achieved on a Lichrosorb 100 Å RP 18 (octadecylsilyl silica gel for chromatography R) (5 m), 0.150 m x 4.6 mm ID at ambient temperature. The mobile phase consisted of methanol R (solvent A) and 5.88 g/l solution of phosphoric acid R (solvent B). The gradient used was: 50 to 65 % A 0–13 min, 65 to 100 % A 13–18 min, 100 to 50% A 18-23 min; post-time 10 min before next injection. The flow rate was 1.0 ml min_1 and the injection volume was 20 ml. The identification of each compound was based on a combination of retention time and spectral matching. The Sample preparation method used for dried samples was as follows:
For the test solution, dissolve 50.5 mg of *Vitex agnus castus* standardized dry extract CRS in 20 ml of methanol R, sonicate for 20 min and dilute to 25.0 ml with the same solvent. Filter through a membrane filter (0.2 Mm). For the reference solution (a) – Dissolve 2.5 mg of casticin in methanol and dilute to 10.0 ml with the same solvent. For the Reference solution (b) – Dilute 1.0 ml of Reference solution (a) to 100.0 ml with methanol R. Both test solutions and reference solutions (a) and (b) were injected three times each. Calculate the percentage content of casticin in *Vitex agnus castus* fruit standardized dry extract CRS, from the expression: T = C2 x (P/100) x (100 – d)/100) x (F1/F2) x (100/C1) . F1 mean area of casticin peak in the test solutions , F2= mean area of casticin peak in reference solutions (b), C1 mean concentration of test solutions (mg/ml) , C2 mean concentration of reference solutions (b) (mg/ml) , P mean purity of casticin determined above by LC , D mean water content by coulometry according to CoA= 0.1 %.

HPLC analysis of Stephania:

Tetrandrine and fangchinoline (not available): For the test solution weigh 0.5055 g of powdered roots of *Stephania*. Insert the sample and 25 ml of a methanol solution of hydrochloric acid 2% and weigh. Refluxed in a water bath at 60 ° C for 30 min., then cool and weigh again. Refill with methanolic hydrochloric acid to 2% for the loss of solvent and filter. Place 5 ml of the filtrate into a 10 ml volumetric flask and fill with the mobile phase and mix thoroughly. For the reference solution dissolve tetrandrine (CRS and CRS fangchinoline - not available) in methanol to obtain, respectively, a solution of 0.1 mg/ml (0.05 mg/ml). Column: dimensions: L = 0.25 m and ∅ = 4.6 mm, Stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m). Mobile phase: a solution of 40 ml of acetonitrile R, 30 ml of methanol R, 30 ml of water R and 1 ml of glacial acetic acid R containing 0.41 g/100 ml of sodium dodecylsulphonate. Flow rate: 2 ml/min. Detection: spectrophotometer at 280 nm. Injection volume: 20 μ l. Calculate the percentage content m / m in dry tetrandrine, using the expression: (A_1 x m_2x p x100)/ (A_2 x m_1 x (100 - h)). A_1 represent area of peak corresponding to the tetrandrine in the test solution, A_2 represent area of peak corresponding to the tetrandrine in the control solution, m_1 represent mass of the sample in grams, m_2 represent mass of tetrandrine in the control solution, in grams, P represent purity of tetrandrine SCR after drying in percent, h represent percentage loss on drying.

RESULTS:

ANATOMICAL ANALYSIS:

Microscopic features of *Origanum* :

Origanum compactum Benth. leaf midrib, concave at upper side and slightly prominent at bottom shows the following elements. In the upper face there is a piliferous upper epidermis consisting of cellulosic thin-walled isodiametric cells, covered with a thin cuticle (Photo 1). Below epidermis is the

parenchyma, collenchymatous in its outermost layer, consisting of cellulosic, slightly ovoid, thick-walled cells, separated by intercellular spaces (Photo 2). The sieve is driven by a bundle arranged in semi-arch formed by the superimposition of xylem, with medium sized vessels, arranged radially with xylem pole near the upper face and of phloem with small rounded riddled tubes surrounded by phloem parenchyma with large cells (Photo 2). The lower epidermis is consisting of rounded cells layer, cellulosic thin-walled, covered with a cuticle and three types of trichomes. The first type is uniseriate, multicellular curved, tapered covering trichome which measures 180 to 300 µm long (Photos 3-4). The other two types are glandular trichomes (Photos 5-7); the first one is a unicellular headed (19-20 µm long) and bicellular stalk (30-33 µm long), glandular trichome (Photo 5). The other one is an octocellular gland, labiatae type (Photos 6-7) which measures 60-70 µm long and 85-90 µm wide. The lamina leaf, with an average width of 220-230 µm, shows an upper epidermis, with diacytic stoma (25-33 µm long, 15-20 µm wide) (Photo 8). This epidermis is consisting of layer of ovoid cellulosic thin-walled cells, covered with cuticle and covering-glandular trichomes identical to those of midrib lower epidermis (Photo 3).Glands mainly distributed on the lower face are lodged in the crypts of 80-90 µm deep, spaced more or less steadily from 600 to 800 µm. The covering trichomes, present on both sides, are more abundant for their part on the upper epidermis. The mesophyll is asymmetrical, heterogeneous and consists of palisade and spongy parenchyma (Photo 9). The lower epidermis similar to the upper epidermis present wall cells taking an original "moniliformous" shape. It is the first time that a such complete botanical analyze was done as a tool of identification of leaf oregano. Aromatic plants usually sequester essential oils in special structures like cells or glands that are special kinds of plant trichomes that have an enlarged cavity that becomes filled with the essential oils. The glandular apparatus anatomy, among other leaf features like wall parenchyma, vascular bundle, collenchyma, mesophyll, covering trichomes type as well as the essential oil content were proposed to be elements to help the recognition of oregano.

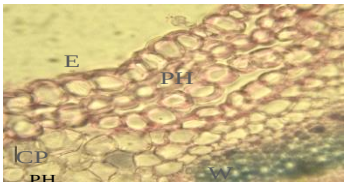
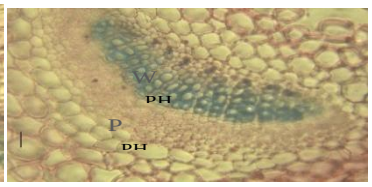


Photo 1(x 40)



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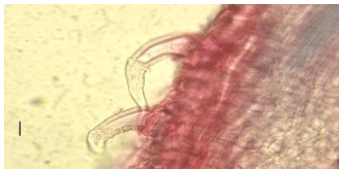

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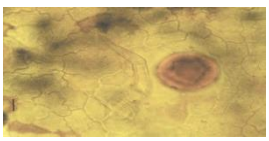

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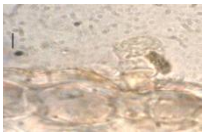


Photo 5 (x 100)

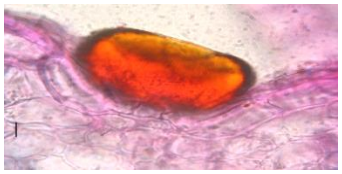


Photo 6 (x 100)

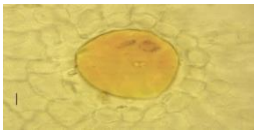


Photo 7 (x 40)

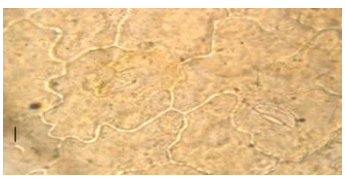


Photo 8 (x 40)

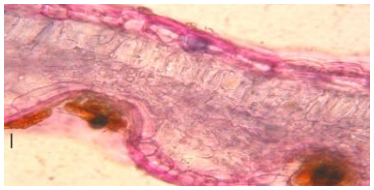


Photo 9 (x 40)

Photos 1-9. Leaf sections of O. compactum Benth. (Scale bars: 14 µm)

1. *Upper epidermis: Epidermis (E), Collenchyma (PH), cortical parenchyma (CP) - Wood (W)*

2. *Vascular bundle: Wood (W), Phloem (P)* ***3-4***. *Covering trichomes*

5. *Glandular trichome* ***6-7***. *Octocellular, Labiatae type, gland*

8. *Diacytic stoma*

9. *Lamina*

Microscopic features of *Ficus*:

The stem of *Opuntia ficus indica* L. has a triangular section, with rounded corners and bearing tufts of spines. Stained with "Carmino-green alum R" reagent, the cross section of the rod has, from outside to inside (Photos 10-11): an epidermis thick cuticle composed of ovoid cells, thickened, curved to its outside; a collenchymatous hypodermis; a cortical parenchyma consists of polyhedral cells, thin-walled cellulose, some mucilage; a ring of vascular bundles, secondary structure, separated from each other by cellulosic pluriseriate rays material; a marrow consisting of polyhedral cells with thin cellulose walls, many of which are mucilaginous.

Macroscopic and microscopic characters of *Vitex*:

The fruit of *Vitex agnus castus* L., also called chaste tree, is oval to nearly globular with a diameter up to 5 mm. The calyx is persistent, gray-green, finely pubescent, ending in 4-5 short teeth. It surrounds the fruit on two-thirds to three quarters of its surface. The fruit, brown-black, consists of a pericarp becoming progressively until the sclerotic endocarp. The scar of the style is often visible. Some fruits are provided with the stems, about 1 mm long. The section of fruit shows four cells, each containing an elongated seed.

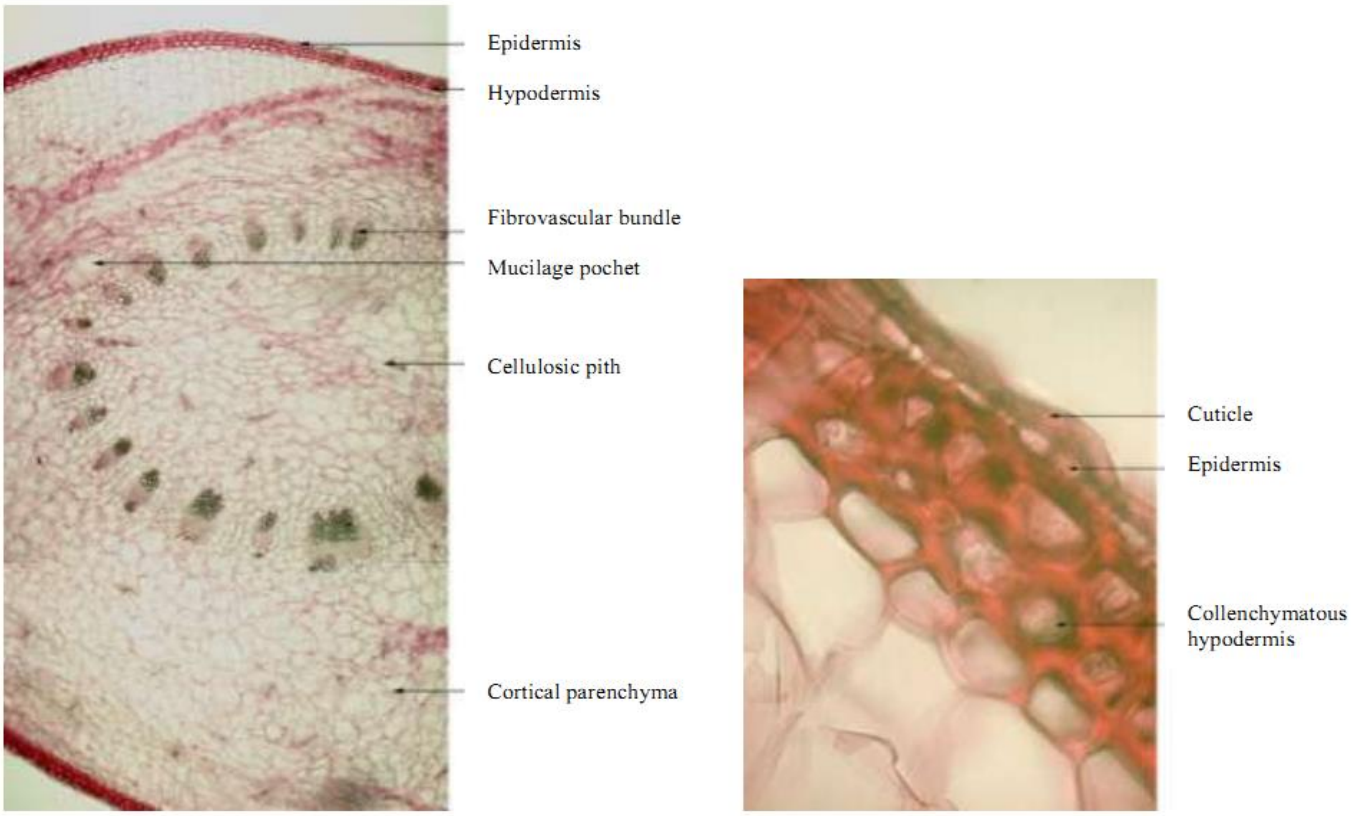


Photo 10 **Photo 11**

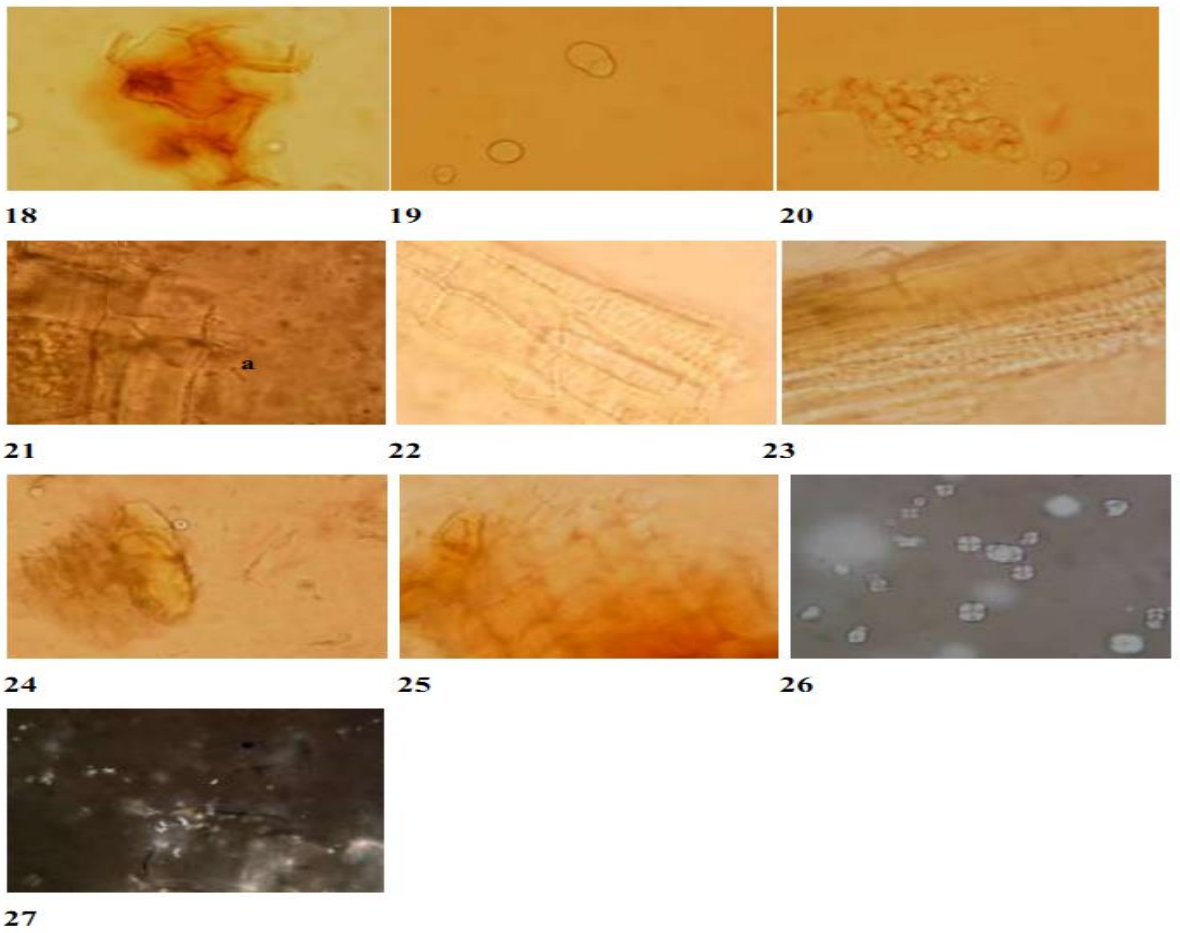
Photos 10-11. *Leaf sections of Opuntia ficus indica L.*

Reduce Chasteberry Powder (355). Examine under a microscope using the chloral hydrate solution R. The powder has the following characteristic elements: fragments of the outer epidermis of the calyx composed of polygonal cells densely hairy detectors short, uniseriate, uni-, bi-or multicellular, bent or flexuous; epidermal cells of epicarp wall thickened with large punctuations clearly marked, the glandular hairs secreting isolated unicellular stalk and head off uni or multicellular, fragments of the

outer mesocarp composed of layers of parenchyma cells, some of which contain a brown pigment, and others are compartmentalized , fragments of the inner part of sclerenchyma mesocarp composed of cells, some thin-walled punctate, others sclerotic isodiametric cells containing typical, very thick-walled, strongly canaliculate, narrow and light, starry, small dark cells the endocarp; fragments of the seed coat with ranges fairly large thin-walled cells with thickened lignified cross-linked network, and many fragments of endosperm composed of thin-walled parenchyma cells, containing grains of aleurone and numerous oil droplets.

Powder microscopy of Stephania:

The powder of *Stephania tetrandra* S. Moore is whitish gray. Examine under a microscope using the chloral hydrate solution R. The powder has the following elements: fragments of parenchyma cell walls slightly thickened and moniliform; Xylem vessels ornamentation reticulate or dotted along fiber; phelloderm fragments containing sclerotic cells, rare fragments suber; oxalate crystals rod-shaped calcium. Examine under a microscope using a solution of glycerol R and 50 percent V / V, the powder has a large number of starch grains with a diameter of from 10 to 20 μ m, hilum punctiform, rounded or truncated, single or grouped by 2 or 3 elements.



***Photos 18-27**: Fruit powder of Stephania tetrandra*
18. Fragment suber
19. Starch granule
20. amyliferous parenchyma
21. Needle-shaped crystals (a)
22. Parenchyma cells with moniliform walled
23. Xylen vessels and fibers
24. Sclerotic cells
25. Phelloderm with sclerotic cell
26. Starch granule in polarized light
27. Needle-shaped crystals in polarized light

PHARMACOGNOSTICAL ANALYSIS:

Loss on drying

Loss on drying of *Hieracium* is **8.4**% and the one of Vitex is **9.47** %.

Content in percent derived ortho-di-hydroxy-cinnamic total

The percentage of ortho-di-hydroxy-cinnamic total in *Hieracium pilosella* L. is **4.237**

Determination of essential oils in *Piper*:

From Piper nigrum L., we can produce white or black pepper, depending on stage and preparation type. Black pepper, made from berries almost reached maturity, fermented and dried produces 32.67 ml/Kg of essential oil. White pepper consisting of ripe berries without pericarp produces 24.11 ml/Kg of essential oil. Green pepper obtained by conservation of wet immature berries produces 19.24 ml/Kg of essential oil. Long pepper or *Piper longum* L. produces 6.69 ml/Kg of essential oil.

TLC

TLC Pilosella

The chromatogram obtained with test solution has a succession of bands, the main ones in order of R Crescent: a blue-green band similar in position and fluorescence band in the chromatogram obtained with reference solution (a) , a yellow band of low intensity (apigenin glycoside), an orange band similar in position and fluorescence band in the chromatogram obtained with reference solution (b), a blue-green band (chlorogenic acid) of equal importance to that of chlorogenic acid, an orange band of low intensity immediately below the band of the reference solution (c) (luteolin, which is clearly present in the whole plant flowers) and a similar blue band on its position and fluorescence band in the chromatogram obtained with reference solution (c). Conclusion: Presence of chlorogenic acid and umbelliferone. Luteolin is absent in dried plant.

TLC Stephania

The thin layer chromatography of *Stephania tetrandra* S. Moore are made according to the monograph with Chinese use of external controls: T = control solution of tetrandrine, B = control solution of berberine and Aar = control solution of aristolochic acids.

Thin plates with silica gel TLC R (5-40 mm) are cut to proper size, and 20 microliters of solution of *Stephania tetrandra* (ST) to study and reference solutions T and B, are deposited in the form of strips . The mobile phase used is composed of R methylene chloride, acetone and methanol R R (6: 1: 1 V / V / V). The development of chromatography is performed on a path of 10 cm [or 6 cm]. After air drying, the plates are examined either by UV light at 365 nm (A) or UV light at 254 nm (B) or the dry plate is sprayed with a solution of potassium iodobismuthate TS then examined in the light of day. In conclusion, the only reference berberine is chosen because its Rf is correct (0.14).

TLC Secale cornutum

Secale cereale L. parasitized by *claviceps purpurea* Tul. test solution was prepared like explain above. As reference samples, we used alkaloids as sistosterol and a mixture of rusco-neorusco that are not present in *Secale cornutum* solution but these substances of references allow to show the suitability of the system (good resolution, adequate retention factor…). The rusco-noerusco mixture migrates just to the top of the ergotamine. sistosterol migrates to the top of the ergocristine. These analytical markers located in the middle and in the top of the profile, are chosen for the chromatogram description. The chromatographic conditions are optimised and based on a detection of alkaloids using a solution of *sulphuric vanillin* because it offers the best highlighted of the rich composition of the solution test while remaining sufficiently discriminative for the location of the ergotamine and the ergocristine.

TLC Calendula

The best resolution is obtained with the eluent: Anhydrous formic acid / water / ethyl acetate (10/10/80- V / V / V). The more revealing reagent is solution of aminoethanol diphénylborate R with a detection UV at 366 nm for the sample and the three references.

HPLC:

High performance liquid chromatography, coupled with a UV-Vis multiwavelength detector offers a very good alternative for the identification of plant extract.

HPLC Vitex

The percentage content of casticin in *Vitex agnus castus* fruit standardized dry extract CRS is 0.116%.

HPLC Stephania

The percentage content of tetrandrine in *stephania tetrandra* standardized dry extract CRS is 0.266 %.

CONCLUSION

Nowadays there is an increasing return to using natural products (herbs, etc.). This increasing trade brings a major problem, that of security. The botanical identification and chemical essential for safe appears essential. As we saw with botanical studies outlined above, it is possible to identify a plant with some sort of photo identification specific anatomical. The anatomical expertise provides a standardized identification with a certain precision. However, it is recommended to attach to this analysis, identifying a specific chromatographic (TLC, UV and HPLC) when necessary, but in many cases it is sufficient. In the past, confusion in the identification of herbal drugs has caused serious public health problems such as *Aristolochia* used for slimming cures.

Pharmacognostical data are specific and are used to guarantee the quality of the plant at the base of a medicament. They also provide a safe, because the great difficulty in this area comes from the development phase and the vegetative state of the plants that are sometimes unknown elements, especially when you work from wild plants. This work show that with an scientifically methodology, we can confirm the identification and characterization of any medicinal plant, before its safe use.

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