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Research Article

Morphological, anatomical, secondary metabolites investigation and physicochemical analysis of *Cistus creticus*

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ABSTRACT: **Background:** The aim of this work is to study the secondary metabolites and the physicochemical properties of *Cistus creticus* in addition to morphological characteristics for better knowledge. The literature indicates that this plant shows anti-inflammatory and antiviral properties. Locally it is used as sedative drink. **Methods:** The plant was collected from mountains surrounding the city of Batna (East of Algeria). Morphological characteristics of leaves and trichomes type were determined; leaves were then separated from stems, cleaned of impurities and dried for a few days at room temperature in shaded and ventilated place before being grounded to a powder. The powder was then subjected to the following analyses: i) phytochemical analysis using extraction with solvents of increasing polarity and qualitative determination of secondary metabolites and ii) chemical and physicochemical analysis where the humidity, percentage of ash and metals content are calculated. **Results:** The solvent extraction showed a high yield of polar compounds (5.76%) compared to apolar compounds (1.08%). Chemical tests revealed the presence of tannins, heterosides, triterpenes, flavonoids, and saponosides. In addition, it was found that the ash of the plant contains low amounts of toxic metals such as Hg and Pb which makes the plant safe to use. The anatomical study showed the presence of two types of trichomes: glandular and stellate.

KEYWORDS: Folk medicine, Heavy metals, Phytochemistry, Solvent extraction, Trichomes

INTRODUCTION

Medicinal plants constitute the main source of new pharmaceutical and healthcare products. Their use in industrialized societies has been traced to the extraction and development of several drugs from these plants as well as from traditionally used folk medicine.[1,2]

A recent survey conducted by the World Health Organization (WHO) reported that globally around 20,000 medicinal plants are used profusely either in pharmaceutical industry or in folk medicines, but only 1.4% of these used plants do possess well-established active constituents.[3] According to the same organization (WHO), 60–80% of the world population relies on non-conventional or alternative medicines, mainly of botanical origin, as their primary form of healthcare due to its availability and the lack of confidence in commercialized medicine.[4,5]

Plants are rich in natural chemicals; among them are secondary metabolites which are used in modern medicine and as pesticides. Several authors have mentioned that using such chemicals can stop germination and growth of certain micro-organisms.[6–8] These chemicals can be found in all part of the plant or are only concentrated in the aerial parts, usually the leaves.[9] They are accumulated in the vacuoles or cell walls during the life of the plant.[10]

Beside secondary metabolites, plants may contain other elements such as heavy metals, some of which are necessary for plant growth and others are toxic. Toxic heavy metals are confined in plants during growth or after

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collection and transformation; they can enter the human body causing disturbance in the functions of many organs leading to illnesses.[5] As recommended by world health organization, medicinal plants must be checked for the presence of toxic metals before use.[3]

Cistaceae is a family of eight genera and 200 species. Members are herbs and shrubs, particularly found on chalk or sand. The genera include Helianthemum (100 spp.), Cistus (20 spp.) and Halirnium. Species of Cistus yield the oleo-gum resin ladanum, used in perfumery and for embalming.[11]

The genus Cistus (Cistaceae) comprises several medicinal plants of perennial shrubs which grow widely in the Mediterranean region.[12] In Algeria, this genus is widely spread and its species were described by Battandier and Trabut since 19th century.[13] All the Cistus species are frequently used in many traditional medicines for their antimicrobial, antitumor, antiviral and anti-inflammatory properties.[12] Among the species of this genus, we chose *Cistus creticus* which is locally claimed to be useful as tea in the treatment of anxiety, cough, stomach ache and cold.[14]

To our knowledge, no work has been done on *Cistus creticus* in Algeria, therefore to identify chemical composition (active compounds and heavy metals) and establish good knowledge of this plant, an investigation of secondary metabolites, physical chemistry characteristics, anatomical and morphological characteristics was carried out.

MATERIALS AND METHODS

Plant materials

Aerial parts of *Cistus creticus* were collected during flowering period at the end of May 2011 from mountains surrounding the city of Batna (Nord 35° 29′ 86.5″, East 5° 56′ 95.1″) at an altitude of 1700 m. Samples were identified at the plant biology laboratory, department of Biology and Plant Ecology, university of Setif. Leaves were separated from stems, cleaned of impurities and dried for a few days at room temperature in shaded and ventilated place before being ground to a powder.

Anatomical study

Young fresh stems and leaves were chosen and sections were taken manually using sambucus wood and sharp riser blade. Light microscope images were then taken after double coloration of sections according to Locquin and Langeron[15] method.

Preliminary phytochemical analysis

In order to identify the phytochemical active principles, several tests have been performed on various extracts of the crude dry powder using colorful specific reactions. Protocols described by Cannell[16] and Evans[11] are used to reveal the presence of the following groups: tannins: gallic tannins, catechin tannins (Ferric chloride test, Stiasny test), sterols and triterpens (Liebermann-Buchard test), flavonoids (ammonia test, Pew test, Shinoda test), saponosides (foam test), alkaloids (Mayer test, Wagner test, Dragendorf test), glycosides (Keller-Killani test) and mucilages.

Chemical and physicochemical analysis

Humidity and ash rate

To measure the humidity rate of the plant, the powder was dried to constant mass in the oven at a temperature of 105 °C. The rate was then estimated as follow:

$$H(\%) = \frac{W_i - W_f}{W_i} * 100$$

Where W_i is the initial weight of the plant material and W_f is the final weight of the plant material.

The inorganic matter content was estimated as a measure of the ash rate after the calcination of the plant material. This was carried out by introducing a known mass of dried powder (in a pre-weighted quartz crucible) in a muffle furnace at 550 °C for 6 hours until the total incineration. The ash is then left to cool down and the crucible is reweighted.

The percentage of inorganic matter is calculated using the following equation:

$$A(\%) = \frac{W_f}{W_i} * 100$$

Where W_i and W_f are, respectively, the initial weight and the final weight of the plant material.

Microelement content determination

Essential and non-essential heavy metals such as copper (Cu), cobalt (Co), iron (Fe), cadmium (Cd), zinc (Zn), lead (Pb) and mercury (Hg) were quantified in *C. creticus* by using flame atomic absorption spectrometry (Shimadzu AA 6800) after mineralization of the ash in HNO_3 (14 N). The digest was washed and diluted with distilled water to 100 ml and the total concentration of the microelements were then determined. For each metal a calibration curve was constructed. All calibration curves showed good linear regression ($R^2 > 0.98$) within test ranges. Reported values are the average of three measurements.

Solvent extraction

Powdered dried plant parts were subjected to successive solvent extraction using petroleum ether, dichloromethane and methanol. In each solvent, the plant was macerated for 24 hours. Each time, the marc (exhausted plant material) was air dried and later extracted with the next solvent. All the extracts were concentrated by distilling the solvent in a rotary flash evaporator (Rotavapor Büchi). The yield was then calculated using the following equation:

$$\text{Yield}(\%) = \frac{W_{extr}}{W_{plant}} * 100$$

Where W_{extr} is the weight of the extract and W_{plant} is the weight of the plant material.

In addition, the aspect and the color of the extracts were noted.

RESULTS AND DISCUSSION

Morphological description of *Cistus creticus*

Figure 1 represents *Cisctus creticus* in both flowering and non-flowering stages. *C. creticus* is an evergreen Shrub growing to 1 m tall by 1 m wide. It is in flower in Mid-end of May. The flowers are hermaphrodite (have both male and female organs) and are pollinated by bees. The plant is self-fertile and it is noted for attracting insects. This species is suitable for rocky and clay-loam, sandy-clay-loam and sandy-loam soils.[11,17] It prefers dry or moist soil and can tolerate drought.[17] The plant under study grows in slightly alkaline sandy-loam soil, with a long season of dry weather.

As described by Sweet[18] this plant is branched, the branches are thickly covered with unequal entangled hairs. The hairs are short and irregularly stellate trichomes. Flowers terminal, solitary or in pairs of purplish red. Peduncles solitary or sometimes binate densely covered with wooly hairs, generally one or two flowered. Calyx of 5 sepals, vellosely hairy. Petals 5, imbricate at the base, with a pale yellow mark. Stamens numerous: filaments pale yellow, unequal in length. Stigma capitates flattened.

Figure 1. *Cistus creticus* plants at flowering and non flowering times.

Anatomical study

The anatomical study which was carried out on young stems showed the presence of pith, transporting tissues, cortex and epidermis (Figure 2). Cross section of leaf under light microscope, shows mesophyll tissue between the upper and lower epidermis. It is differentiated into two distinct layers: palaside parenchyma and spongy parenchyma and it is traversed by veins (Figure 3).Vascular bundles form a network that extends throughout the leaf. They are surrounded by layers of parenchyma cells. Both leaves and stems bear on their surface two types of trichomes: glandular and non-glandular. Glandular trichomes are elongated and consist of a group of cells, from which those being close to the base are flattened, whereas the ones approaching the tip become fine cylindrical. The apical region ends in a small globular head. Non-glandular trichomes are of two types, i.e. stellate

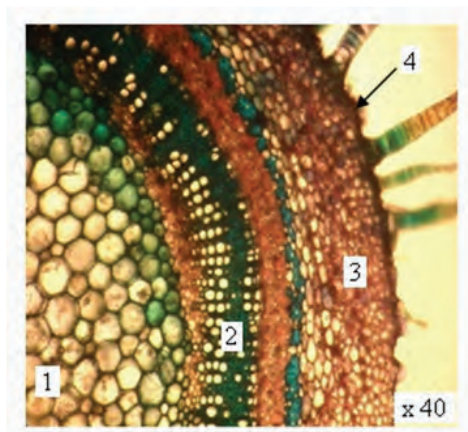

Figure 2. Cross sectional view of *C. creticus* stem 1: Pith, 2: Transporting tissues, 3: Cortex, 4: Epidermis.

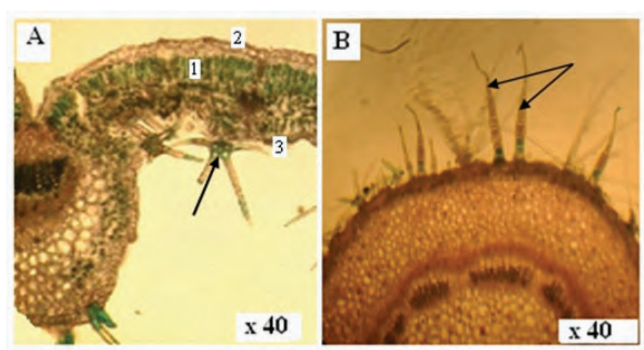

Figure 3. Cross sections of (A) leaf (Stellate trichomes), (B) stem (Glandular trichomes). 1: Mesophyll, 2: Upper epidermis, 3: Lower epidermis

multicellular trichomes composed of unicellular rays and simple long trichomes with acute tips.[19]

Gulz et al.[20] also reported the presence of glandular trichomes (sites of secondary metabolites biosynthesis, secretion and storage) on this plant. However, Demetzos et al.[21] suggested that the presence of several glandular trichomes on the leaf surface of *C. creticus* could explain the production of essential oil. Secondary plant metabolites are accumulated in the vacuoles or cell walls during the life of the plant.[10]

Preliminary qualitative phytochemical screening

Plants produce several secondary metabolites. Some of these are responsible for the characteristic odours, pungencies and colours of plants, others give a particular plant its culinary, medicinal or poisonous virtues, or aid to the defense of the plant. However, the role of high number of these compounds to the plant (and to the human race) is still obscure.[11]

The phytochemical screening of *C. creticus* (Table 1) revealed a strong presence of tannins, catechin tannins, gallic tannins, and catechols. However, tests showed moderate presence of linked-anthraquinones (*C*-hétérosides, *O*-hétérosides), sterols and triterpenes, flavonoids and mucilage. The other secondary metabolites like alkaloids and saponosides were present in trace amounts in the plant. Free anthraquinones were absent. Most of these compounds are known for their medicinal activities. This information will be helpful as a primary platform for further phytochemical and pharmacological studies.

Chemical and physicochemical characteristics

Humidity and ash rate

Fresh plants contain 60 to 80% water and to ensure their proper conservation for long time, the water content must be less than or equal to 10%.[22] The moisture content of *C. creticus* was calculated at 8.33% (<10%) which gives to our plant material a better long-term conservation.

Ash may be defined as a complex mixture of charcoal and minerals (i.e inorganic compounds) and generally it contains significant amounts of most plant nutrient elements.[23] Our result shows that after incineration of the plant material, the ash content was 23.85% while the organic matter was 76.15% and the ash was of light color. The light colored ash, as reported by Knicker,[24] consists mostly of crystalline or amorphous inorganic compounds.

Metal contents

Concentration of essential and non-essential heavy metals in plants beyond permissible limits is a matter of great concern to public safety all over the world. Some of elements are necessary to human health, such as Ca, Mg and Zn whereas others have been shown to be toxic, such as Pb, Cd and Al.[25,26]

Comparison of the chemical elements values of *C. creticus* with those given by Kabata-Pendias and Pendias.[27] (Table 2) showed that cobalt (Co), iron (Fe) and lead (Pb) contents are within the normal range. However, copper (Cu), cadmium (Cd) and zinc (Zn) contents seem to be lower than normal. Results show also the absence of

Table 1: Phytochemical screening of *Cistus creticus*

Compounds		Result	Color in test tube
Tannins		+++	Blue-black
Catechin tannins		+++	Red ring
Gallic tannins		+++	Blue-black
Alkaloids	Mayer	+	Precipitates
	Dragendorff	–	/
	Wagner	+	Precipitates
Sterols and triterpenes		++	Violet ring
Free anthraquinones		–	Green
Flavonoids		++	Yellow brown
Flavones		++	Pink orange
catechols		+++	Red-brown
Saponosides		+	Visible foam
Anthraquinones	*C*-hétérosides	++	Red
	O-hétérosides	++	Red
Mucilage		++	Flocculent precipitate

+++ : Strongly positive reaction, ++ : Moderate positive reaction + : Weakly positive reaction, – : Absent.

Table 2: Metal contents in *Cistus creticus*

Elements	Concentration in *C. creticus* (ppm)	Normal content in plants (ppm)[27]	Toxic threshold in plants (ppm)[27]	Permissible limits[27]
Copper	0.1174	5–30	20–100	5–20
Cobalt	0.2392	0.02–1	15–50	5
Iron	173.52	18–340	>500	/
Cadmium	0.0057	0.05–0.2	5–30	0.05–0.5
Zinc	6.22	27–150	100–400	50–100
Lead	7.2957	5–10	30–300	0.5–10
Mercury	0	/	1–3	0.2

Table 3: Elements daily need for person weighing 70 kg[28]

Element	ADDI (mg/day) (Range)
Copper	2.5 (2–3)
Cobalt	0.04
Iron	15 (10–28)
Cadmium	0.057
Zinc	15
Lead	0.415
Mercury	/

mercury (Hg). Most of these elements may exert toxic effects on plants at elevated concentrations[28] and as consequence can affect the human health. Therefore, the plant under study was deemed to be safe to use as a beverage.

To demonstrate the contribution of *C. creticus* as a beverage on the average daily dietary intake (ADDIs), Table 3 is used to indicate the daily intake recommended for a person weighing 70 kg. When the intake values listed in Table 3 were compared with those given in Table 2, it is noted that the contents of the elements in the plant are below average. This makes *C. creticus* safe to consume in reasonable amounts, yet a good source of essential elements.

Solvent extraction

Solvent extraction is the separation of medicinally active compounds present in the plant tissues using selective solvents.[29] In such extraction, the plant material is extracted using a solvent of an appropriate polarity following the principle of "like dissolves like".[30]

Results in Figure 4 and Table 4 show the yields and characteristics respectively of extracts obtained using increasing polarity solvent extraction applied on *C. creticus* as plant material. From Figure 4, it can be seen that the nonpolar solvent (petroleum ether) gave a yield of 1.08% which consists of mostly lipophilic compounds (e.g., alkanes, fatty acids, pigments, waxes, sterols, some terpenoids, alkaloids, and coumarins). The medium-polarity solvent used in this study

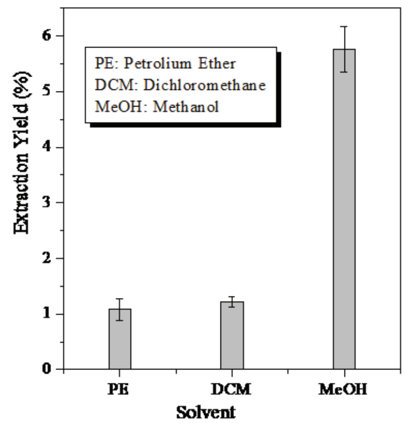

Figure 4. Yields of extraction using solvents with increasing polarity.

Table 4: Results of solvent extraction

Extract / Solvent	Aspect	Color
Petroleumether	Pasty	Dark greenish brown
Dichloromethane	Pasty	Dark green
Methanol	Highly viscous liquid	Caramel

was dichloromethane and it resulted in a yield of 1.22%, which represents compounds of intermediate polarity (e.g., some alkaloids, flavonoids). The value of 5.76% represents the yield obtained when methanol which is the more polar solvent is used. The last extract contains the more polar compounds such as flavonoids, glycosides, tannins, some alkaloids, etc. These results indicate that there are more polar compounds in *C. creticus* than nonpolar ones.

The products so obtained from plants are relatively complex mixtures of metabolites, in liquid, semisolid state or in dry powder form (Table 4), and are intended for oral or external use. This part of work has the advantage of allowing a preliminary separation of the metabolites present in *C. creticus* within distinct extracts and simplifies further isolation.

CONCLUSIONS

The aim of this work was to investigate anatomy, morphology, phytochemistry (secondary metabolites) and physical-chemical characteristics of *Cistus creticus*. Light microscopy shows the presence of glandular trichomes which are generally linked with essential oil production. The phytochemical screening revealed the presence of several secondary metabolite compounds such as mucilage, sterols and triterpenes, flavonoids and saponosides. These are known to have therapeutic effects (gastric dressing, expectorant, antioxidant, etc.) which support the use of this plant as tea drink in the treatment of anxiety, cough and stomach ache. Due to the low presence of metal elements in the plant there will be no toxicity generated from them and therefore, the plant is safe to use. In addition, this plant contains several microelements which are necessary for human body such as copper, cobalt, iron, cadmium, zinc and lead contributing to the daily dietary intake.

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