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# Optimisation of the extraction of secondary metabolites from cypress leaves «Cupressus sempervirens L. »

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**Abstract :**
Our work aims to optimize the extraction conditions of poly phenols from cypress leaves (Cupressus sempervirens L.) by maceration in methanol and distilled water v/v 70% / 30% respectively, after stirring for 24 hours, we proceeded to filtration, a dry evaporation was carried out in order to obtain the dry extracts to be studied.

The results helped to determine the optimal conditions for maximizing the total poly phenol content of extracts. Optimal extraction was achieved with 100 µg EAG/mg DM and 160 µg EAC/mg DM flavonoid. We obtained with a powder concentration of 21.17% and an extraction time of 24 hours. The anti-radical activity demonstrated a strong inhibition of DPPH thanks to the richness of the extract in secondary metabolites.

The HPLC separation chromatographic method used in our work identified 6 secondary metabolites: gallic acid, tannic acid, vanillin, quercetin, Rutin and salicylic acid.and we obtained the major flavonoid that is rutin with a maximum concentration of 8.23 0.01 mg/g MS.

**Keywords:** Cupressus sempervirens L; secondary metabolites; antioxydant activity; HPLC

## Introduction :

The cypress is a tree resistant to harsh natural conditions, adapted to extreme drought or at least to dry and hot summers, in Algeria it is used as a windbreak and in traditional medicine . Phytochemical and pharmacological studies of the genus Cupressus are few compared to their importance in several areas. ***Aboulaïch. (2008).*** In recent years, medicinal and aromatic plants have been known as an inexhaustible source of bioactive compounds. The plant-based chemo-diversity, although evolved as part of plant defence and adaptation mechanisms, is a rich source for the development of new compounds and products of medical and economic

importance, such as drugs, aromas, perfumes, insecticides and dyes. ***Evans. (2002)***
In Algeria, the genus Cupressus is found, except for a few small formations, as an isolated tree or ornamental tree or alignment. Endemic or naturalized species of this genus are: Tassili cypress (Cupressus dupreziana A. Camus), Atlas cypress (Cupressus atlantica Gaussen), Evergreen cypress (Cupressus sempervirens L.)
Medicinal plants are both finished products for consumption and raw materials for the extraction of active substances. Currently, a significant part of scientific research is focused on the study of naturally occurring antioxidant molecules. Among these, polyphenols, compounds widely used in the plant kingdom, are attracting increasing interest because of their beneficial effects on health ***(Boucifet al., 2023).*** They are also popular as additives in various industries such as food, medicine, pharmacy and cosmetics ***(Suhaj, 2006).*** Their structure gives them a remarkable antioxidant activity, comparable to that of vitamins C and E ***(Boucifet al., 2023).*** The method of extraction of bioactive components from plant materials plays a crucial role in their isolation and purification. The steps required to obtain a plant extract include size reduction, extraction, filtration, concentration and drying ***(Azmir et al., 2013); Živkovićet al., 2018)***. Among the various polyphenol extraction methods, maceration is preferred for the determination of polyphenolic compounds due to its simplicity, minimal experimental installation, low cost and environmentally friendly character ***(Ćujićet al. 2016).*** Polyphenols are widely present in the plant kingdom ***(Torreggiani et al., 2005).***

The cypress tree, whose parts are rich in phenolic compounds and essential oils ***(Ben Nouri et al., 2015),*** is a very common medicinal and ornamental plant in Algeria ***(Bouyahyaoui, 2017),*** its parts are widely used in folk remedies to relieve headaches, cold symptoms, cough and bronchitis ***(Ben Nouri et al., 2015).***

The Cypress «Cupressus sempervirens L. » is a very common tree in the Mediterranean and Algeria, which is why we decided to study it and do a phytochemical study for its medicinal properties, cosmetics, pharmaceuticals and agronomics, particularly in plant protection. Our objectives through this study :

Make a dosage of total phenols and flavonoids.

Study the antioxidant activity in relation to our plant.

Perform high-performance liquid chromatography to see the major secondary metabolite in the leaves of Cypress «Cupressus sempervirens L. ».

## Material and methods:

### Plant material:

The plant material used in this work consists of cypress leaves (Cupressus sempervirens L.). Our study area is located in the BLIDA university experimental station. It is at the foot of the Atlas Blidéen 4.4 km north-east of the city of Blida, bounded to the west by the commune of Soumâa, to the east by the commune of Ouled Yaiche, to the north by the commune of Guerouaou and to the south by the mont de Chrea.

The experimental station is located in the southern part of the Mitidja plain, which is the largest sub-coastal plain in Algeria. It extends over a width of 05 to 20 km and a length of 100 km, covering an area of 140,000 ha ***(Mutin 1977).*** It is bounded to the north by the Sahel ridge, which isolates it from the Mediterranean Sea, to the west by the Chenoua mountains, to the east by a set of mountains and hills of Kabylie and to the south by the Blidéen Atlas. It is located between the parallels 36°29 and 36°30 and the longitudinal 3°35 and 3°45. The collected plant material was dried at room temperature and sheltered from light and moisture. After drying, the leaves and fruits are ground using an electric grinder until a powder is obtained. The botanical identification was carried out by Dr. DEGAICHIA Hoceme. **Permanent researcher at the Agropastoralism research center (Djelfa, Algeria).**

### Maceration extraction:

Preparation of 10g of powder of cypress leaves (Cupressus sempervirens L) mixed with 70ml of methanol and 30ml of distilled water. Maceration is a process that consists of letting a solid body remain in a liquid or in a wet environment, to extract certain active ingredients from this body ***(larousse,1989).*** Secondary metabolites are low molecular weight aliphatic or aromatic molecules with a hydrophobic character. When they are extracted, they are therefore found mainly in the apolar or medium polar fractions. The extraction of secondary metabolites is carried out taking into account the nature of the solvent, time and temperature of extraction. Ethyl ether ***(Culberson et al., 1982),*** dichloromethane, chloroform (***Carlos et al., 2008; Polovinka***

***et al., 2012)*** may be used. Powder concentration and maceration time were subject to optimization. After filtration of the mixture, a dry evaporation was carried out in order to obtain the dry extract to be studied.

## Determination of total phenols (TP)

TP was measured according to the Folin-Ciocalteumethod,modified by ***Fattouchet al.(2007).*** Methanolic extract (50µl,1mg/ml )was mixed with 1.58ml of distilled water and 400µl of the Folin-Ciocalteau reagent. After a 5-min rest period at room temperature, 300µl was added to the saturated solution of $Na_2CO_3$ followed by incubation at 20°C in the dark for 30min.The absorbance was measured at 725nm. The absorbance of the measurement standard (Gallicacid) was described by the equation y=0.0013x($R^2$=0.9997).For each sample three replications were performed.

## Determination of total flavonoids (TF) :

Flavonoids were quantified by the colorimetric method described by ***Popovaet al.(2004).*** A volume of 500µl of sodium hydroxide (NaOH, 1M) was added to the solution obtained after incubation (dark, room temperature, 40min) of 1ml of aqueous methanol extract (1mg/ml) mixed with 1ml of aluminum trichlo-ride (AlCl3, 2%).

The absorbance of the extract was measured at 420nm using a UV spectrometer/VIS (Mecasys Optizen Pop). TF content of the extract was expressed in the milligram(mg) equivalent of quercetin per gram(g) of the weight of the dry matter(QE/gDM).Quantitative analyses of TF were determined using the equation of the linear regression of the calibration curve :y=0.003x+0.025;$R^2$ =0.998.For each sample, three replications were performed.

## DPPH radical scavenging:

In the presence of free radical scavengers, purple DPPH (1.1-diphenyl-2-picrylhydrazyl) is reduced to yellow 2.2-diphenyl-1-picryl-hydrazine ***(Maataouiet al.,2006).*** The activity of DPPH radical scavenging was measured according to the protocol described by ***(Fattouchet al.(2007).*** To each 1ml sample dilution was added 1ml of an methanolic solution of DPPH concentration equal to 100µM. The resulting

solution was placed in the dark at room temperature for 30min. The absorbance was measured at517nm.The results were expressed using the following formulation:

I% = [1 − (AE − ACn)] × 100; where I%: Percentage ofthe anti-radical activity; AE: absorbance of the sample; ACn: Absorbance of Negative Control. For each sample, three replications were done.

## Identification and quantification of the major phenolic compounds (MPC) by HPLC:

Qualitative and quantitative analyses of MPC present in the different studied extracts were performed using a Varian Prostar HPLC equipped with a C18 reverse-phase column (Varian, 150 mm × 4.6 mm, particle size of 5 m), a ternary pump (Prostar 230) and a Diode Array Detector (Prostar 330). Two eluents were used (A: 100% ethanol; B: aqueous acetic acid solution 0.05%) for the following gradients: 35% A and 65% B, 0 min; 50% A and 50% B, 30 min; 90% A and 10% B, 40 min. The flow rate was 1 ml/min with an injection volume of 20 l at 25 ◦C. MPC were determined by comparing retention times and spectral data obtained with those of authentic measurement standards (Phenolic acids: Gallic acid, Catechin acid, Caffeic acid, epicatechin acid, Vanillic acid, p-coumaric acid and Cinnamic acid; flavonoids: rutin, quercetin and kaempferol). The identification was carried out at 360 nm. All analyses were run in triplicate.

## Results and discussion :

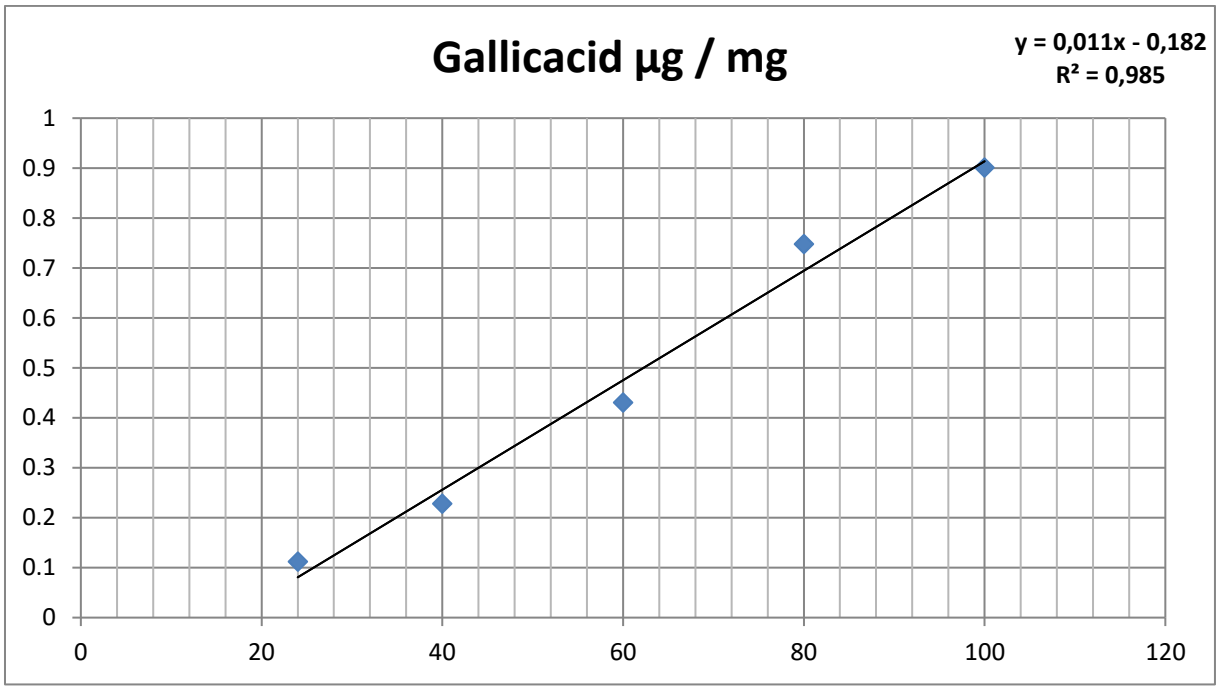
## Yield:

The extraction technique is a crucial step in extracting phytochemicals from plant material ***(Quy Diem Do et al, 2014).*** Each solvent has different characteristics that are the consequences of its molecular structure such as its miscibility to water, its dipole moment or polar character, its density and volatility ***(Abe et al., 2010).***

## Contents of total polyphenols (TP):

The calibration curve was tested at ranges of concentrations **(20 - 100 µg/mg)** of Gallicacid as standard solutions of total phenolic content equation obtained from the linear calibration graph of the studied concentrations.

The yield of 10g of leaf powder from Cupressus sempervirens is: **2.117g or 21.17%.**




**Gallic acid calibration curve**

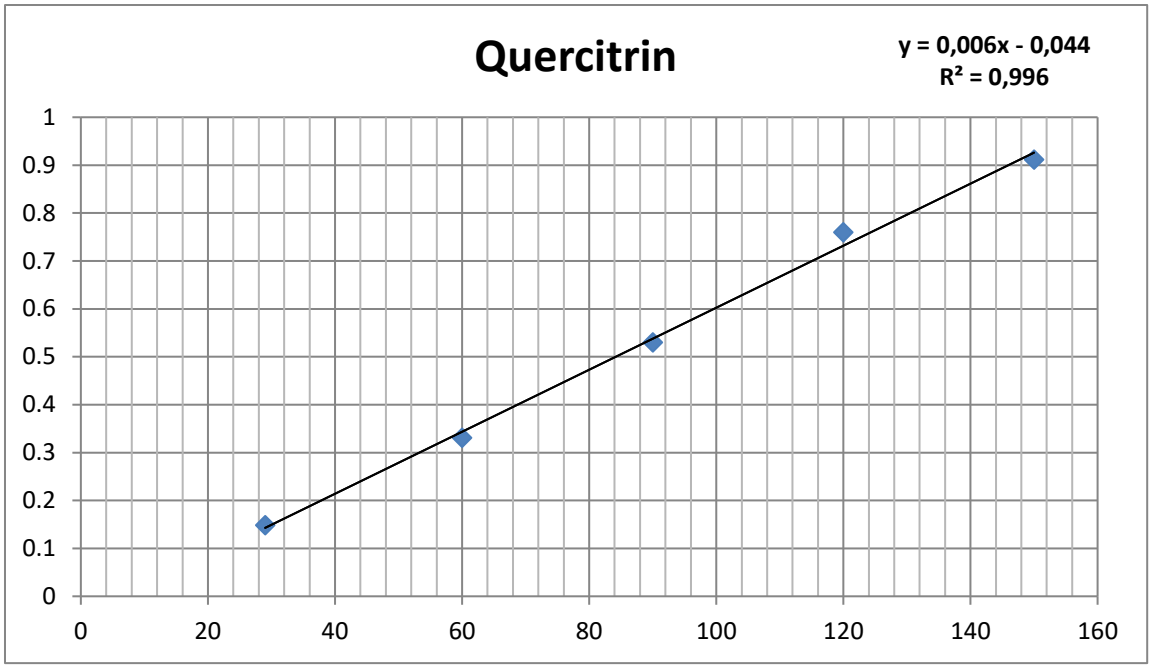
The total phenol assay by the Folin-Ciocalteu test is a non-selective assay

in relation to phenols, because it is involved that all reducing molecules, such as

reducing sugars or vitamin C.

## Contents of total flavonoids (TF):

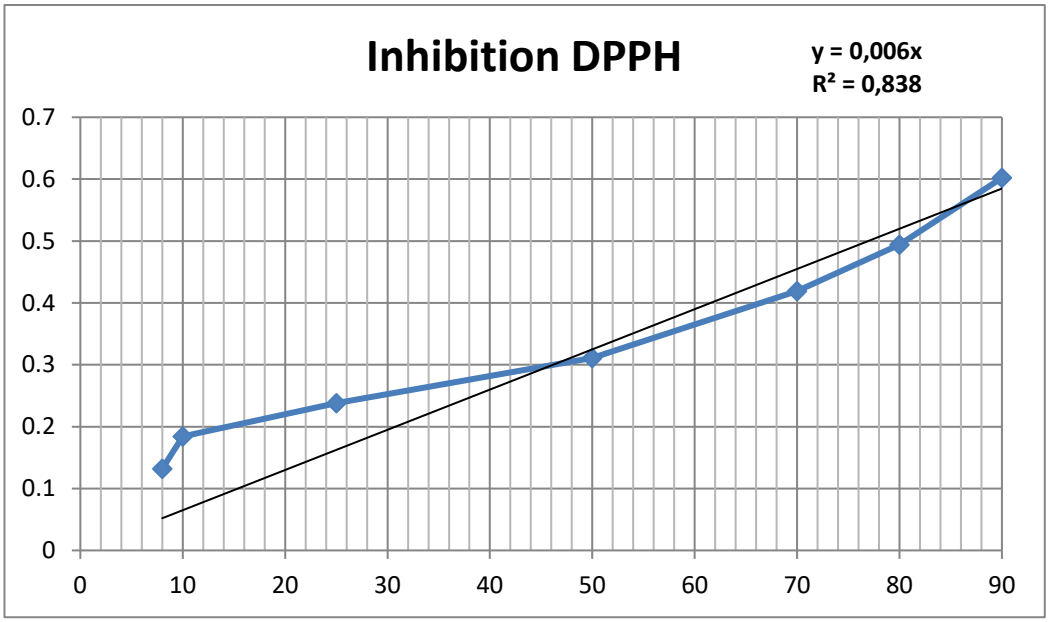
The regression line of the calibration curve was evaluated by preparing standard solutions of quercitrine and we obtained concentrations between **30µg/ml and 150µg/mg.**




**Quercitrin calibration curve**

## Antioxidant activity:

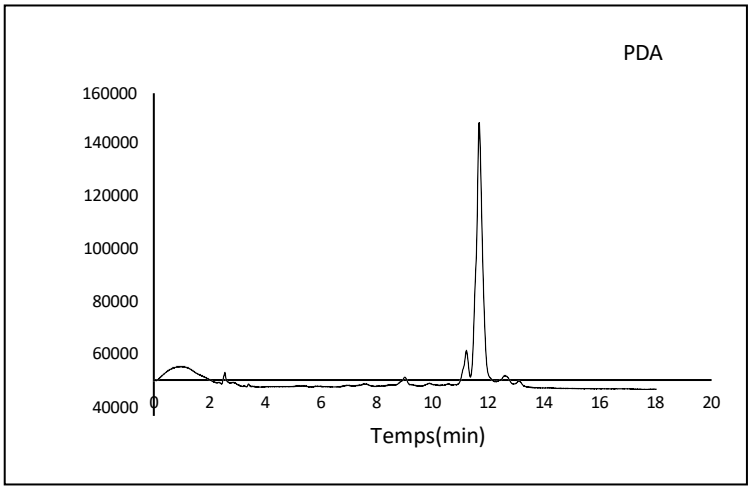
Numerous studies demonstrate the importance of natural antioxidants in the food and medical industry, but also their protective roles against oxygen reactive species, as well as the correlation between bioactive compounds in plant matter and their antioxidant capacity (Kong et al. (2010). The antioxidant activity was verified by following the rate of reduction of the free radical DPPH by the extract of Cupressus sempervirens L prepared at (8, 10, 25, 50, 70, 80 and 90µg/ml) by the maceration method, after measurements of the absorbance at 517 nm of the extracts. The results of measurement of the percentage inhibition of the radical DPPH as a function of the concentration of the extract of Cupressus sempervirens L showed that the percentage inhibition of the free radical of the extract by the maceration method increases with the increase of concentration of the extract, as observed for the reference molecule Quercitrin.

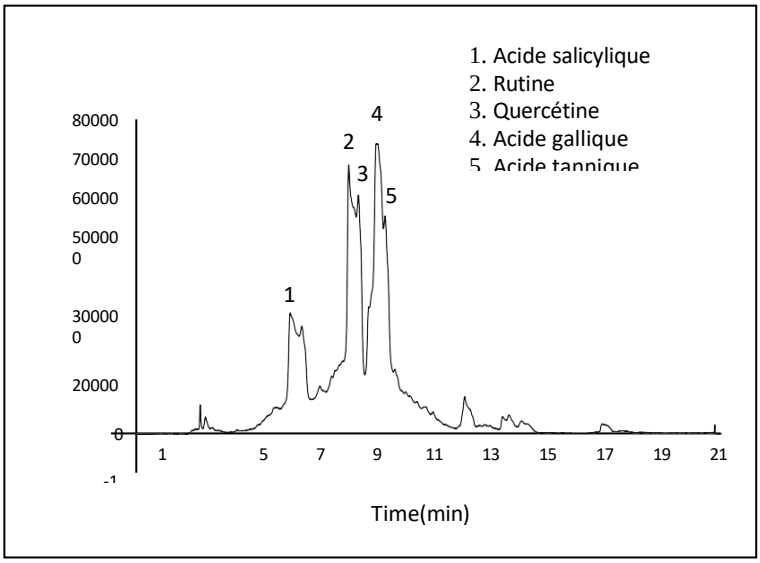



## Quantitative and qualitative analysis of phenolic compounds:

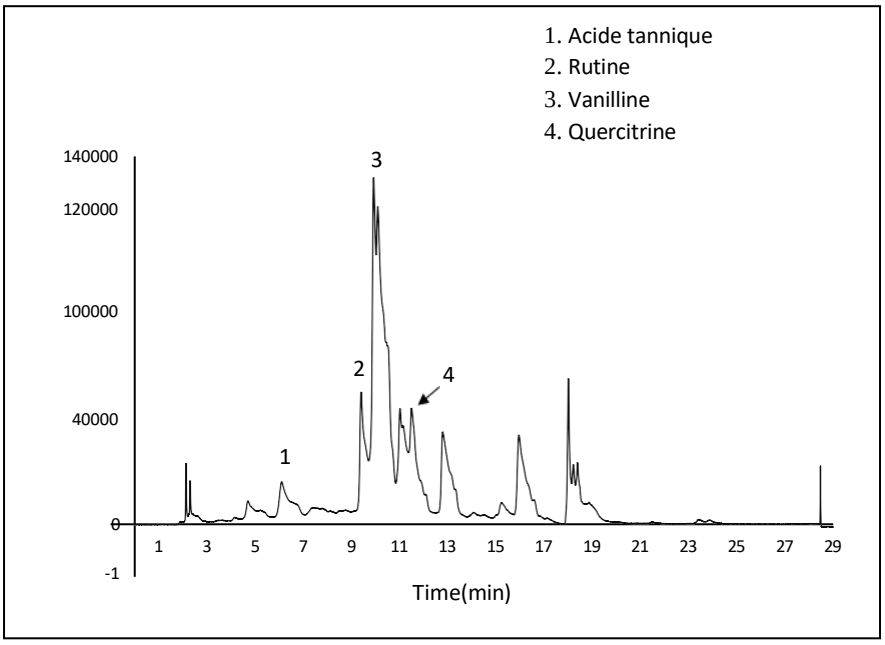
Five phenolic compounds were identified based on their retention time. The chromatogram shows a coelution of rutin and salicylic acid as well as quercetin and tannic acid. This difficulty was solved by their characterization using mass spectrometry (SIM). The two characteristic compounds: quercetin and vanilla are observed for retention times of 8.6 min and 9.5 min respectively.

Quercitrine is the major poly phenol at a maximum concentration of 3.04 0.01 mg/g MS in the fraction < 180 µm. The other characterized poly phenols are also concentrated in the same fraction. The contents of poly phenols in the fraction > 500 µm are the lowest. From these results, it is clear that the concentrations of poly phenols in the <180µm fraction are 30% to 50% higher than those of the NT fraction. This proves that the PTC process has a significant effect on poly phenol enrichment in one of the particle size fractions (< 180 µm)






The best concentration of salicyline was recorded for the fraction containing particles smaller than 180 µm: 1.36 0.03 mg/gMS. The other compound concentrated in this fraction is rutin which is the main flavonoid with a maximum concentration of 8.23 0.01 mg/g MS.



This chromatogram indicates the presence of vanillin which is one of the flavonoids markers in the aerial parts of the millet with an intense peak at the retention time of 10,2 min. The other marker compound of this plant that was characterized by ESI/MS at retention time of 22 min is vanillin. Salicylic acid is another compound characteristic of St. John's wort which does not appear under UV because it does not contain a chromophore, so it was detected using its m/z ratio and the tR= 19 min of its external standard.

| **Bioactive Compound (*m/z*)** | **Molecular Family** | **$t_R$( min)** | **Concentrations(mg/gde matière sèche)** | | | | |
|---|---|---|---|---|---|---|---|
| | | | **<180µm** | **180-315µm** | **315-500µm** | **>500µm** | **NT** |
| Tannic acid | Tannin | 6,2 | **0,78**±0,01 | 0,66±0,02 | 0,44 ±0,03 | 0,25 ±0,01 | 0,36 ±0,01 |
| Vanillin | Flavonoid | 9,5 | **1,91**±0,01 | 1,59 ±0,02 | 0,96±0,02 | 0,45 ±0,05 | 0,71 ±0,01 |
| Rutin | Flavonoid | 10,2 | **6,61**±0,02 | 5,45 ±0,02 | 3,89±0,03 | 2,07 ±0,01 | 3,05 ±0,01 |
| Quercétin | Flavonoid | 11,5 | **0,84**±0,01 | 0,83 ±0,01 | 0,51±0,02 | 0,19 ±0,02 | 0,36 ±0,03 |
| Gallic acid | Phenol | 19 | **3,28**±0,05 | 3,03 ±0,02 | 3,02±0,01 | 3,08 ±0,01 | 3,15 ±0,04 |
| **Salicylic acid** | Phenol | 22 | **4,53**±0,02 | 4,17 ±0,05 | 4,02 ±0,04 | 3,74 ±0,01 | 3,92 ±0,02 |

The four compounds identified are: tannic acid at tR=3min, salicylic acid at tR= 7.7 min, rutin at tR= 10 min and quercitrine at tR= 11 min.

Using ESI/MS spectrometry, the presence of tannic acid which is a phenolic acid was proven by its ratio m/z193 and l and R=13min of its external standard.

## Conclusion:

This study extended the value of the plant studied, especially by its chemical composition and its richness in secondary metabolites. The latter were extracted by the maceration method, followed by a dosage of poly phenols and flavonoids necessary to detect these secondary metabolites followed by anti-radical activity and high-performance liquid chromatography (HPLC) to determine the dominant secondary metabolite in the hydro-alcoholic extract of Cypress (Cupressus sempervirens L).

These results highlight the potential of cypress leaf extract as a source of bioactive compounds for applications in plant protection, therapeutics, cosmetics in particular as antibacterial and antifungal agents. In addition, this study demonstrates that the use of

the centered composite plan to optimize poly-phenol extraction conditions is effective, producing accurate results and improved extraction yield.

Several research perspectives can be envisaged:

• Examine other parameters and extraction solvents: Study the influence of various other factors and extraction solvents to compare yields of phenols and other bioactive compounds.

• Perform in vitro and in vivo toxicity tests: to ensure the safety of these extracts for therapeutic applications.

• Explore potential applications in various fields: Investigate the potential use of cypress extracts in areas such as plant protection against biotic and abiotic stress, cosmetics and agri-food.

## Acknowledge:

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