

Investigation of the Antioxidant and Anti-Inflammatory Capacities of Different Extracts from *Cistus ladanifer* L. Leaves in the Ait Ammart Region (Northern Morocco)

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ABSTRACT

Plants are a significant source for identifying new compounds (particularly secondary metabolites) with medicinal value for drug development, pharmaceuticals, and food additives. Many of these healing plants are frequently used in conventional medicine to treat and prevent various diseases. The purpose of the current research is to assess the total phenolic content (TPC) and total flavonoid content (TFC) for phytochemical analysis, DPPH and FRAP (Ferric Reducing Antioxidant Power) assays for antioxidant activity (AOA), and the activity of reducing inflammation exhibited by various extracts, which were determined by measurement of absorbance with a UV spectrophotometer. The outcome demonstrated that the *Cistus ladanifer* L leaf aqueous extract (EAqCL) has the highest yield (15.581%) and the highest phenolic content (98.28 ± 0.32 mg GAE/g_{ext}) compared to the other extracts. Whereas, ethanol extract (EECL) exhibited the highest flavonoid content (11.39 ± 1.45 mg QE/g_{ext}), DPPH-radical scavenging activity (RSA = $94.534 \pm 1.24\%$) at 800 µg/ml and FRAP (1.017 ± 0.12) at 400 µg/mL. The extract also showed better anti-inflammatory activity ($95.61 \pm 0.92\%$) at 1000 µg/ml. According to the results obtained, this sticky shrub growing in the Ait Ammart region has great potential for exploiting natural sources of bioactive substances and producing pharmacologically active metabolites.

Keywords: *Cistus ladanifer* L, solvent extraction, polyphenols, antioxidant, anti-inflammatory.

INTRODUCTION

Free radicals are unstable oxygen molecules that are missing an electron from their outer shell, and they can damage cells in the human body and heighten the likelihood of health issues like cancer, heart-related conditions, diabetes, and Alzheimer's disease (Sharifi-Rad et al. 2020).

The formation of free radicals in the body is typically a result of internal or external stimuli such as inflammation, mental stress, aging, and excessive exercise (Pham-Huy et al. 2008). External sources include pollution, radiation, some medicines, cigarette smoke, and pesticides (Lobo et al. 2010). Antioxidants play a very important

role in shielding our cells from harm brought on by oxidative stress and free radicals. They are known to be extracted from plant materials, including fruits, spices, vegetables, and herbs (Lourenço et al. 2019). These natural substances are quite intriguing due to their biological properties.

Cistus ladanifer L. (CL), referred to locally as "Touzalt", is a common perennial shrub found in many parts of the Mediterranean region, pertaining to the family Cactaceae, and is a crucial aromatic plant utilized in the perfumery sector, especially as a fixative and for ornamental purposes (Oliveira et al. 2023). The soft parts of CL are used in traditional Portuguese cuisine to season dishes with rabbit (Tardío et al. 2006).

Additionally, the aerial part of this shrub's decoction is frequently employed in northern Moroccan folk and traditional medicine to treat a range of diseases as an anti-diabetic, anti-diarrheal, and antispasmodic (El Kabbaoui et al. 2017). Scientific investigations confirmed several biological activities of CL extracts, such as antioxidant (Amensour et al. 2010), antimicrobial (Barrajón-Catalán et al. 2010), cytotoxic (Bouothmany et al. 2022), insecticidal (Sosa et al. 2004), anti-inflammatory, and analgesic (El Hamsas El Youbi et al. 2016). Up to now, no studies on the biological activities of extracts from *Cistus ladanifer* leaves have been identified in the literature within the Ait Ammart region. The main objective of this study is to explore the phenolic components, in-vitro antioxidant, and anti-inflammatory properties of CL in the region using various solvents. This investigation aims to compare these findings with studies conducted in different regions. Additionally, it serves as a reference for future research on CL leaf extracts, offering valuable insights for their potential applications in various fields.

MATERIALS AND METHODS

Plant material

The CL leaves were harvested in July 2021 from shrubs in the Ait Ammart region in Al Hoceima Province, north of Morocco (N 34°52'48.43207; W 4°10'45.42594). The plant material dries naturally for twelve days at ambient temperature (298 K), then powdered (using the mini electric grinder NM-8300) and stored before extraction. Then, 300 mL of the following solvents were added to a flask in ascending order of polarity: ethyl acetate, ethanol, and distilled water (dH₂O). After each extraction, the resulting solution was concentrated in rotavaps under a vacuum to produce the crude extract. These extracts were labeled as ethyl acetate extract (EACL), ethanolic extract (EECL), and aqueous extract (AqCL) of CL, respectively. They were then stored at four °C until needed.

Chemicals and solvents

L-ascorbic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), folin-ciocalteu reagent (FCR), potassium ferricyanide (III) K₃Fe(CN)₆,

trichloroacetic acid (TCA), iron trichloride (FeCl₃), and sodium carbonate (Na₂CO₃) were bought from Sigma-Aldrich (St Louis, USA). While aluminium (III) chloride (AlCl₃) and sodium acetate (C₂H₃NaO₂) procured from Merck Life Science (Merck KGaA, Germany). BSA solution (bovine serum albumin), phosphate buffer saline (PBS), all other chemicals and solvents utilized in our research were of analytical grade.

Yield of extraction

The extraction yield of each extract was represented as a percentage of the weight of the extract obtained concerning the dry matter of the initial sample used, as shown in the equation (1).

$$\text{Extraction yield \%} = \frac{\text{mass of extract (g)}}{\text{mass of dry matter (g)}} \times 100 \quad (1)$$

Quantification of TPC and TFC in extracts

TPC assay

The TPC of each CL leaf extract was analyzed using the FCR following the method outlined by (Li et al. 2008) with slight alterations. In brief, 200 µl of the solution extract (100 µg/ml) is a mixture of 1 ml of Folin-Ciocalteu (10%). After 5 minutes of dark incubation, 800 µL of sodium carbonate Na₂CO₃ (7.5%) was added. The mixture remained at ambient temperature for 2 hours. The absorbance of sample intensity was determined at 765 nm with a UV-visible (UV-1800 Series, Shimadzu, Japan). The standard was the gallic acid (GA) solutions (0–400 µg/mL). TPC values were quantified in milligrams of GA equivalents per gram of extract (mg GAE/g_{ext}).

TFC assay

The aluminum trichloride (AlCl₃) method is commonly used to determine the flavonoid content, as reported by (Djeridane et al. 2006). The test consisted of adding 1 mL of sample solution prepared in ethanol (100 µg/ml) with 1 ml of 2% aluminum (III) chloride ethanolic solution. The whole mixture was vortexed, and following a half-hour incubation period, the absorbance (Abs) was read at 430 nm. The concentration of TFC was quantified and expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g_{ext}).

Antioxidant activity (AOA)

DPPH assay

The assay was conducted following the modified method of Butsat et al. (2010). A 0.2 mL aliquot of each extract (0 to 800 µg/ml) was mixed with 1.8 mL of 0.1 mM DPPH. After thoroughly mixing the samples, they were kept in a dark environment for 30 min. The Abs of the mixture was read at 517 nm using a UV-Visible instrument. The ensuing formula was employed to calculate the percentage of AOA (RSA %):

$$\text{RSA \%} = \frac{\text{Abs blank} - \text{Abs sample}}{\text{Abs blank}} \times 100 \quad (2)$$

where: Abs blank – Abs of the DPPH solution, Abs sample – Abs of the tested extract solution (sample or standard).

FRAP assay

The FRAP assay differs from the DPPH assay in that it does not involve free radicals but monitors the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The test was conducted using the method outlined by (Złotek et al. 2016) with a little modification. Five dilutions ranging from 0 to 400 µg/ml were prepared for both the extract and the standard (ascorbic acid). Each dilution combined one milliliter of the sample at different concentrations (diluted in ethanol) with 2.5 ml of PBS (0.2M; pH 6.6) and 1.5 ml of 1% potassium hexacyanoferrate solution. The resulting mixture was then incubated in a water bath at 50°C for 20 minutes. Following incubation, 2.5 mL of a 10% trichloroacetic acid (TCA) solution was added to cease the reaction. Subsequently, the tubes were centrifuged for 10 minutes at 3000 rpm. After centrifugation, 2.5 ml of the upper layer was combined with 2.5 ml of distilled water (dH_2O) and 0.5 ml of FeCl_3 (0.1%). The absorbance of the resulting mixture was measured at 700 nm.

In vitro anti-inflammatory potential

The anti-denaturation activity of CL was studied by inhibiting the denaturation of albumin, which was studied according to (Labhar et al. 2023), followed by slight modification. A mixture comprising 500 µl of a 0.2% w/v BSA solution and 500 µl of CL extract or a reference standard (Diclofenac Sodium (DS)) at various concentrations (100–1000 µg/mL). The samples were incubated at a specific temperature (37°C)

for 15 minutes and then raised to 72°C for an additional 5 minutes. Once the samples had cooled, 2.5 mL of PBS (pH 6.6) was added to all the solutions. The Abs was measured at 660 nm, and the percentage of denaturation-inhibited protein was evaluated like this:

$$\% \text{ inhibition} = \frac{\text{Abs [Control]} - \text{Abs [Test]}}{\text{Abs [Control]}} \times 100 (3)$$

where: Abs [Control] – Abs value of control, Abs [Test] – Abs value of the test sample.

Statistical analysis

Three replicates of each experiment, and the findings were presented as the mean ± standard deviation ($n = 3$). OriginPro 2018 (OriginLab Corp., USA) was used to analyze the obtained data.

RESULTS AND DISCUSSION

Yield of extraction

Figure 1 shows the percent yield of extraction using different solvents. The aqueous extract yield is noticeably higher at 15.81% compared to the ethanolic and ethyl acetate extracts, which stand at 7.59 and 2.57%. In general, the efficiency of extraction increased with increasing polarities of the solvent used; this result is almost consistent with the extraction yield of *Mentha spicata* L. (Zekri et al. 2020). Polar solvents can diffuse inside the plant powder and recover as many metabolites as possible.

Total phenolic and flavonoid contents

Phenolic compounds (PCs) are secondary metabolites important for the body's upkeep (R. Sathya et al., 2013) and contain the antioxidants for eliminating excessive ROS (reactive oxygen species) caused by most stressors. It is also known that a class of phenolic compounds called flavonoids possess antioxidative properties (Tohidi et al. 2017). The TPC of several fractions was determined by extrapolation from the calibration curve ($Y = 0.0033x + 0.0066$; $R^2 = 0.9915$). The obtained results of TPC are given in Table 1 with different fractions, which varied from 51.45 ± 0.09 to $98.28 \text{ mg GAE/g}_{\text{ext}}$. The EAqCL had the highest amount of phenolic compounds ($98.28 \pm 0.32 \text{ mg GAE/g}_{\text{ext}}$), and the other two extracts varied and decreased in the following sequence:

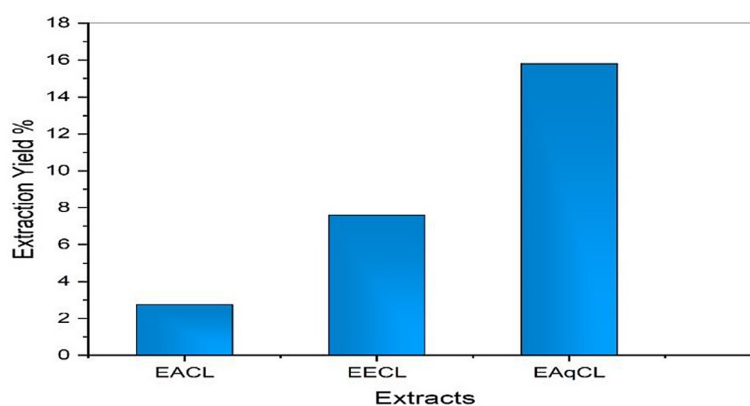


Figure 1. Yields after soxhlet extraction of CL leaves

EAQCL > EAQCL > EECL. On the other hand, total flavonoid content values were acquired from the calibration curve $Y = 0.035x + 0.031$ with $R^2 = 0.098$. As depicted in Table 1, the TFC values of the plant extracts range from 4.2 ± 0.97 to 11.39 ± 1.45 mg EQ/g_{ext}, and they reduce in the subsequent sequence: EECL > EAQCL > EAQCL. The results showed that the extractant's polarity and solubility are crucial part in increasing the recovery of phenolic and flavonoid compounds (Labhar et al. 2023). The findings reported by Bouothmany et al. (2022) from Taza, indicating that ethanolic extract has 73.9 ± 8.7 mg EAG/g_{ext} of polyphenols and 35.634 ± 1.734 mg EQ/g_{ext} of flavonoids, differ from those obtained in the present study. This disparity could be attributed to environmental variables or the plant genotype (Pacheco-Hernández et al. 2021).

Antioxidant activity (AOA)

To assess the AOA of various extracts, two different in-vitro methods have been developed; DPPH and FRAP.

DPPH-RSA assay

An acknowledged method for assessing the AOA of plant extracts is DPPH free radical scavenging; due to the relatively short time required for examination, this method has been extensively used to predict AOA. In this current investigation, the results show that all the extracts could scavenge DPPH radicals in a dose-dependent manner. Figure 2 shows the RSA values of the extract samples and standard, ranging from $57.05 \pm 0.28\%$ to $96.864 \pm 1.05\%$ at 800 µg/ml. At this concentration, A higher capacity

Table 1. Determination of TPC and TFC of various solvent extracts of CL

Extracts	TPC (mg GAE/g _{ext})	TFC (mg QE/g _{ext})
EAQCL	67.07 ± 2.02	4.2 ± 0.97
EECL	51.45 ± 0.09	11.39 ± 1.45
EAQCL	98.28 ± 0.32	10.35 ± 0.53

for scavenging DPPH radicals was found in the EECL fraction ($94.534 \pm 1.24\%$), followed by the EAQCL fraction ($90.989 \pm 0.98\%$). These values are close to those of the reference ascorbic acid ($96.864 \pm 1.05\%$). At 250 µg/ml, EECL showed a higher RSA value of 92% compared to the results reported by Amensour et al. (2010) in Chefchaouen which showed an RSA of 50.10%. According to Zeng et al. (2023) the AOA of phenolic compounds in extracts depends on their chemical structure, in particular the number and location of the hydroxyl groups linked to the aromatic cycle and the nature of the aromatic substituents. Phenolic -OH groups possess the ability to provide oxygen atoms, creating pairs with unpaired electrons in radical structures. This action helps decrease the overall count of unpaired electrons and facilitates the scavenging of DPPH•.

FRAP assay

At the level of 400 µg/mL, the absorbance of all extracts was in the range of 0.324 ± 0.014 to 1.309 ± 0.03 . Moreover, heightened absorption indicates increased reducing power. Among the extracts illustrated in Figure 3, the ethanolic extract exhibited a higher AOA than the other extracts, and the order of reducing potential on ferrous ions was

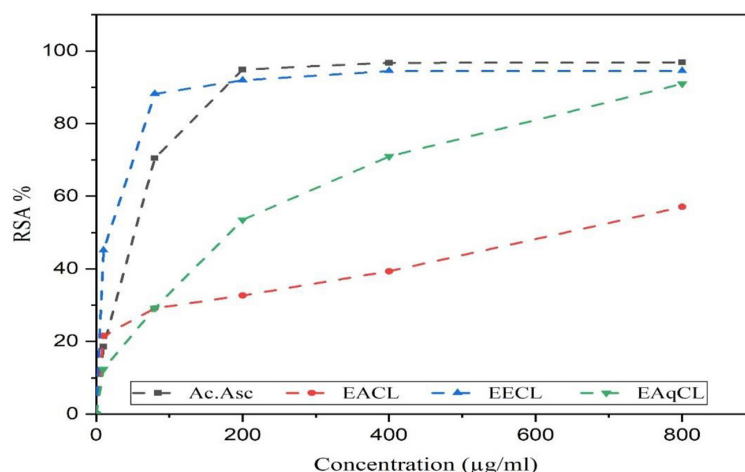


Figure 2. Percentage DPPH-RSA of different extracts of CL

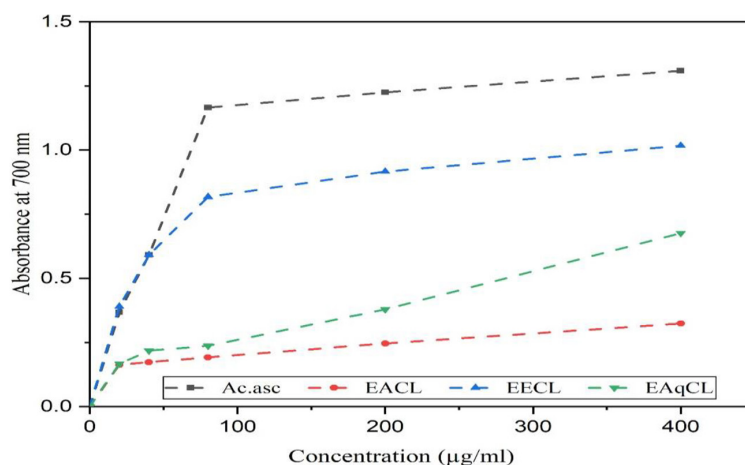


Figure 3. Profiles of the reducing power (FRAP test) of CL extracts

Table 2. Effect of CL extracts on percentage inhibition of protein (BSA) denaturation

Concentration (µg/ml)	%Inhibition			
	EACL	EECL	EAqCL	DS
100	21.74 ± 0.53	71.08 ± 0.54	70.56 ± 0.86	72.22 ± 0.05
200	27.06 ± 0.11	75.45 ± 0.65	74.41 ± 0.85	77.67 ± 0.47
400	31.72 ± 0.86	84.03 ± 0.27	85.02 ± 0.96	85.33 ± 0.45
800	46.22 ± 0.40	88.82 ± 0.41	89.71 ± 0.23	93.06 ± 0.8
1000	51.03 ± 0.21	95.61 ± 0.92	95.01 ± 0.08	96.45 ± 0.51

as follows: EECL > EAqCL > EACL, closely mirroring the findings observed in the DPPH assay. In addition, the TPC and flavonoid seem to have strong and positive links to the AOA (Alara, Abdurahman, et Ukaegbu 2021). Our study showed that the ethanolic extract (EECL) possessed a higher reducing power of 0.93 ± 0.02 compared to those reported by Amensour et al. (2010) of 0.15 at a concentration of 250 µg/mL. The denaturation

of proteins is the main cause of inflammation. In this current research, the anti-inflammatory activity of diverse extracts from the leaves of CL was performed using the bovine albumin denaturation method, and the percentage inhibition of denaturation of BSA of CL extract and DS (standard) were compiled in Table 2. The findings indicate that the EECL and EAqCL fractions demonstrate the highest percentage inhibition, followed by the EACL

fraction, with recorded values of $95.61 \pm 0.92\%$, $95.01 \pm 0.08\%$, and $51.03 \pm 0.21\%$, respectively (tested at $1000 \mu\text{g/ml}$). However, it's noteworthy that both the EECL and EAqCL fractions demonstrate anti-inflammatory activity almost equal to the standard DS. Our results (95.01% for EAqCL) came in the same trend as those reported by Tavares et al. (2020), who indicated that CL hydrolat had a powerful 94% ability to reduce inflammation in vitro through the inhibition of protein (albumin) denaturation, and this signified that the CL plant effectively inhibits inflammation. It is known that the phenolic compounds of plants serve as antioxidants and reduce the oxidative stress caused by free radicals associated with chronic inflammatory diseases (Saleem, Saleem, et Akhtar 2020; Spisni et al. 2020).

CONCLUSIONS

This work describes the antioxidant potential, phytochemical characterization, and anti-inflammatory efficacy of CL extracts with different polarities using in vitro methods. The results showed that polar extracts, such as EECL and EAqCL from CL, have strong anti-inflammatory and antioxidant potencies. Furthermore, the data indicated the enormous potential of this medicinal shrub as a supplier of phenolic compounds with advantageous properties and an encouraging reservoir of health products for the pharmaceutical and nutraceutical sectors. As the bioactive compounds found in the plant substance consist of multi-component mixtures, further scientific studies are needed to isolate the compounds accountable for the antioxidant and anti-inflammatory effects and to demonstrate their mode of action.

Acknowledgments

The authors express gratitude for the assistance extended by the technical and support staff in the Research Unit of Applied Chemistry at Al Hoceima Faculty of Sciences and Techniques.

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