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Phytochemical and Bioactivity Variations in *Calobota saharae*: Flavonoid Extraction, Antioxidant, and Anti-inflammatory Assessment

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Abstract

This comprehensive study investigates the physiological growth stage-dependent variations in the chemical composition and bioactivity of extracts from *Calobota saharae* plants. The research explores the changes in carbohydrate, protein, and lipid content across different growth stages and correlates them with the qualitative phytochemical screening results. Flavonoid extracts were obtained and characterized using Fourier-Transform Infrared (FTIR) spectroscopy, highlighting spectral similarities and differences. Additionally, the antioxidant activity was assessed through DPPH free radical scavenging and erythrocyte hemolysis tests, revealing stage-specific efficacy. Furthermore, the anti-inflammatory potential of the extracts was evaluated, showing significant variations across growth stages. Correlation analysis was conducted to elucidate relationships between variables. Overall, this study provides insights into the dynamic chemical profile and bioactivity of *C. saharae* extracts throughout its growth stages, offering valuable implications for further research and potential applications in pharmaceutical and nutraceutical industries.

Keywords: Anti-inflammatory potential, Antioxidant activity, *Calobota saharae*, Flavonoid extracts, Physiological growth stages, Phytochemical screening.

1. Introduction

Plants play a pivotal role in traditional medicine and modern drug discovery due to their diverse range of bioactive compounds (Mustafa *et al*., 2017). Among these, the *Calobota saharae*, a species native to arid regions (Chouikh *et al*., 2018), has attracted considerable interest for its potential pharmacological applications. Understanding the chemical composition and bioactivity of *C. saharae* extracts across different physiological growth stages is essential for effectively utilizing its therapeutic potential effectively. This study aims to examine how variations in growth stages impact the chemical profile and bioactivity of *C. saharae* extracts.

The physiological growth stages of plants affect their metabolic activities, leading to fluctuations in nutrient content and secondary metabolite production (Li *et al*., 2020). Carbohydrates, proteins, and lipids serve as fundamental components of plant tissues (Ofoedum *et al*., 2024), while secondary metabolites such as flavonoids, alkaloids, saponins, and tannins contribute to their pharmacological properties (Kabera *et al*., 2014). By analyzing the variations in these constituents across growth stages, we gain insight into the dynamic nature of plant biochemistry and its implications for medicinal use.

Flavonoids, a class of polyphenolic compounds abundant in *C. saharae*, are well-known for their antioxidant, anti-inflammatory, and other health-promoting properties (Sun and Shahrajabian, 2023). Fourier-Transform Infrared (FTIR) spectroscopy provides a valuable method for characterizing the chemical structure of flavonoid extracts (Noh *et al*., 2017) and tracking spectral changes linked to different growth stages. Additionally, evaluating antioxidant activity through the DPPH free radical scavenging assay and erythrocyte hemolysis test offers key insights into the potential health benefits of *C. saharae* extracts.

Understanding the anti-inflammatory potential of C. saharae extracts is particularly important given the prevalence of inflammatory disorders. By assessing their effectiveness in inhibiting inflammation, we can evaluate their therapeutic relevance and pinpoint the optimal growth stages for extract preparation. Moreover, correlation analysis enables the exploration of relationships between chemical composition, bioactivity, and growth stages, providing insights into underlying mechanisms and guiding future research directions.

This study integrates plant physiology, chemistry, and pharmacology, offering a comprehensive understanding of the chemical composition and bioactivity of *C. saharae* extracts across different growth stages. These findings are vital for maximizing the therapeutic potential of this species and advancing drug discovery efforts in natural product-based medicine.

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2. Materials and Methods

2.1. Plant material

The *C. saharae* plant was harvested at four distinct growth stages: in March, during the initial vegetative and flowering stages; in June, during the fruiting stage; and in September, at the second vegetative stage. These harvests were carried out in the Ghamra region of El Oued State, located at coordinates 33°32′0″N 6°47′0″E. After harvesting, the aerial parts of the plant were subjected to a controlled drying process under ambient temperatures, shielded from light and moisture. Once completely dried, the plant material was milled into a fine powder. This powder was then stored in glass containers, protected from light and heat, until further use.

2.2. Estimated nutritional value

In this experiment, each sample comprised 1 gram of plant material, which was combined with 5 mL of trichloroacetic acid (TCA) and subjected to mixing on a magnetic shaking device for 5 minutes. Subsequently, the mixture underwent centrifugal separation at 3000 revolutions per minute (rpm) for 10 minutes, yielding a supernatant for carbohydrate quantification. The resultant deposit 1 underwent an additional treatment with 2 mL of ether/chloroform (V/V) followed by a second centrifugal separation at 3000 rpm for 10 minutes. The second supernatant was collected to assess lipid content, while the second deposit was treated with 2.5 mL of a 0.1 M solution of sodium hydroxide (NaOH) to determine protein content (Chouikh *et al*., 2020).

2.2.1. Carbohydrate estimation

In this method, a solution containing 5% phenol and concentrated sulfurous acid was used, with absorbance measurements recorded at a wavelength of 490 nm. Glucose was utilized as the reference standard, and the carbohydrate content was reported in milligrams per gram of plant material (Chouikh *et al*., 2024b).

2.2.2. Protein estimation

The protein content of plant material was determined utilizing the Folin-Ciocalteu assay (Gurugubelli et al., 2023). This method involved the preparation of a reaction mixture containing Folin-Ciocalteau reagent (in V/V ratio), 0.1 M NaOH, 0.5% $CuSO_4$, and 0.1% $KNaC_4H_4.O_64H_2O$. Bovine Serum Albumin (BSA) was used as the reference standard for quantification. Absorbance measurements were conducted at 750 nm wavelength using a UV spectrophotometer. The obtained absorbance values were then correlated with known concentrations of BSA to determine the protein content in milligrams per gram of the plant material.

2.2.3. Lipid estimation

The analytical procedure employed Sulfophosphovanillinic reagent in conjunction with concentrated sulfurous acid (Chouikh *et al*., 2024a), with subsequent incubation of reaction tubes in a water bath set at 100 °C. Absorbance measurements were conducted at a wavelength of λ= 530 nm, with Soy serving as the reference standard. Lipid content results were quantified in milligrams per gram of plant material.

2.3. Qualitative phytochemical screening

A qualitative phytochemical analysis was carried out on *C. saharae* plant extracts at different growth stages to identify the presence of various secondary metabolites. Standardized procedures were followed, including detection of flavonoids and tannins using ferric chloride ($FeCl_3$) drops, anthocyanins with hydrochloric acid (HCl) and ammonia (NH_3), alkaloids with Wagner's reagent, and saponins through a shaking test for foam formation. Sterols and triterpenes were analyzed using a combination of acetic anhydride, chloroform, and sulfuric acid (Ben Ali *et a*l., 2024; Noudogbessi *et al*., 2013; Sharma *et al*., 2010).

2.4. The Flavonoid Extraction Procedure (Ethyl acetate phase)

Flavonoids were extracted from 20 grams of dried and ground plant material using ethyl acetate fractionation. The plant material was soaked in 100 mL of methanol for 24 hours in the dark at room temperature. After filtration, the solvent was evaporated using a rotary evaporator. The resulting extract was subjected to a second extraction stage (liquid-liquid phase separation) where it was mixed with equal volumes of hot distilled water (50°C) and ethyl acetate (V/V = 120 mL). The solution was shaken well and allowed to settle for approximately 2 hours. The mixture consisting of two aqueous and organic phases was separated. The aqueous phase was discarded, while the organic phase was dried in a rotary evaporator at 50°C. Thus, the flavonoid extract, the ethyl acetate phase, was obtained (Kanoun, 2011).

2.5. Fourier-Transform Infrared (FTIR) Characterization

The extracts were analyzed using Fourier-Transform Infrared (FTIR) spectroscopy with a Shimadzu-00463 spectrophotometer to characterize their molecular composition and structural features. The FTIR analysis was conducted with a high resolution of 4 cm^{-1} to ensure precise spectral details. To enhance the signal-to-noise ratio and obtain reliable spectral data, 64 coadded scans were accumulated. The examined spectral range spanned from 4000 to 650 cm^{-1}, encompassing the mid-infrared region where characteristic vibrational bands of various functional groups and molecular bonds are observed.

2.6. Estimation of antioxidant activity

2.6.1. Free radical DPPH scavenging activity

The assessment of the extracts' $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) scavenging activity followed the protocol outlined by (Chouikh and Alia, 2021). Briefly, varying concentrations of each extract (1 mL) were mixed with an equal volume of DPPH solution (10^{-4} mol) dissolved in methanol. Following a 15-minute incubation at room temperature, the absorbance was recorded at 517 nm. The inhibition activity was determined using the following formula (Faridi *et al*., 2023):

$$Inhibition(\%) = \left[\left(Absorbance_{Control} - Absorbance_{Sample}\right)/Absorbance_{Sample}\right] \times 100$$

The IC_{50} value, representing the concentration at which 50% of the free radicals were scavenged by the extract, was determined using linear regression analysis on the concentration-inhibition percentage curve. A lower IC_{50} value indicates a higher antioxidant capacity (Chouia *et al*., 2018).

2.6.2. Erythrocyte hemolysis test

This assay is utilized to evaluate the protective effects of plant extracts on erythrocyte blood cells against membrane damage induced by oxidative stress and free radicals. The assessment involves measuring the proportion of erythrocytes that have undergone lysis (Dolci and Panteghini, 2014).

To perform the assay, 40 µL of human erythrocytes are mixed with 2 mL of the plant extract and incubated for 5 minutes at 37 °C. Following this, 40 µL of hydrogen peroxide (30 mM), 40 µL of ferric chloride (80 mM), and 40 µL of ascorbic acid solution (50 mM) are added sequentially. After incubating for 1 hour at 37 °C, the mixture is centrifuged at 700 rpm for 10 minutes. The absorbance of the supernatant is then recorded at 540 nm (Ben Ali and Chouikh, 2024).

The percentage of hemolysis is determined using the following formula:

$$Hemolysis\% = \left(Absorbance_{Control}/Absorbance_{Sample}\right) \times 100$$

2.7. Estimation of anti-inflammatory activity

The albumin denaturation model was employed to assess the in vitro anti-inflammatory properties of Flavonoid extracts derived from *C. saharae*. Specifically, 1 ml of the extract at various concentrations was combined with 1 ml of human albumin (5%). Following a 15-minute incubation period at 27°C, the mixtures were subjected to further incubation in a water bath set to 70°C for 10 minutes. Subsequently, after cooling to room temperature, the absorbance of the solutions was measured at 660 nm. Diclofenac sodium served as the standard reference. The findings were quantified as milligrams of diclofenac sodium equivalent per milligram of extract to facilitate comparison (Ben Ali et al, 2023).

2.8. Statistical analysis

The results were analyzed using Microsoft Office Excel 2007. Mean values were calculated along with the standard error of the mean (SEM), denoted as ±, with a sample size of n = 3. To determine significant differences in the results, a one-way analysis of variance (ANOVA) was conducted at the significance levels of 0.001, 0.01, and 0.05.

3. Results

With the aim of understanding the effect of changing physiological conditions during the stages of plant growth on flavonoids, this study focused on tracking the chemical composition of the *C. saharae* plant in the laboratory throughout its various growth stages. The results are presented as follows:

3.1. Estimated nutritional value

3.1.1. Carbohydrate estimation

The carbohydrate content of the plant declines gradually as it matures physiologically. The highest carbohydrate content, estimated at 24.41±0.08 mg/g of plant material, occurs during the first vegetative stage. Subsequently, during the flowering stage, the carbohydrate content decreases to 21.23±0.05 mg/g of plant material, and further declines to 16.95±0.25 mg/g during the fruiting stage. The lowest carbohydrate content, 13.34±0.04 mg/g, is observed during the second vegetative stage. Statistical analysis reveals highly significant differences at a significance level of α=0.001 (Figure 1).

3.1.2. Protein estimation

During various stages of plant growth, significant variations in protein content were observed. The highest amount of protein was recorded during the flowering stage, with an estimated value of 12.10±0.14 mg/g of plant material. Conversely, the second vegetative stage exhibited the lowest protein content, with a value of 2.37±0.05 mg/g. The protein content during the first vegetative stage and fruiting stage was estimated at 8.57±0.31 mg/g and 10.53±0.09 mg/g, respectively. The results of the analysis of variance indicate significant differences in protein content across the stages of plant growth, with a significance level of α = 0.001.

3.1.3. Lipid estimation

The analysis revealed minor differences in fat content across the various stages of *C. saharae* growth. Specifically, during the first vegetative, flowering, and fruiting stages, the fat content was measured at 0.97±0.07, 0.77±0.01, and 0.88±0.04 mg/g of the plant sample, respectively. The lowest fat content was observed during the second vegetative stage, with a value of 0.12±0.01 mg/g of the plant sample. Statistical analysis demonstrated highly significant differences at a significance level of α = 0.001.

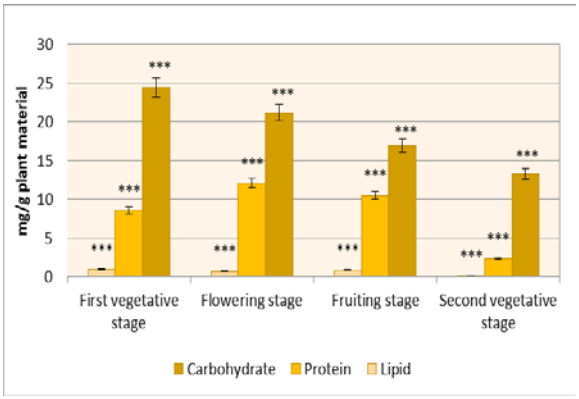

Figure 1: Nutritive values of *C. saharae* plant.

***: Statistically significant differences at the confidence level (α=0.001).

3.2. Qualitative phytochemical screening

Chemical detection of secondary metabolites was carried out based on the dry plant matter of the samples, and the results obtained are represented in Table (1).

Table 1. Phytochemical screening results of secondary metabolites.

Extract stage / Metabolites	*First vegetative*	*floral*	*Fruiting*	*Second vegetative*
flavonoids	+++	+++	+++	+++
Anthocyanin	-	-	-	-
Alkaloids	+++	+	++	++
Saponosides	+++	+++	+++	+++
Sterols and terpenes	-	+	-	+
Tannins	+++ Gallic	+++ Catechol	+++ Catechol	+++ Gallic

+++: The presence of the active ingredient in large quantities. ++: medium amount. +: a small amount. -: Absence of the active ingredient.

From the results obtained, it is clear that the *C. saharea* plant is rich in bioactive compounds, the most notable of which are flavonoids, alkaloids, saponins, and tannins. The abundance of these compounds varies depending on the physiological stage of the plant, while the absence of anthocyanin compounds was evident across all growth stages. Sterols and terpenes were absent during both the first and second vegetative stages but were present in the flowering and second vegetative stages.

3.3. Yield of flavonoid extract (ethyl acetate phase)

Flavonoid extracts were obtained from various physiological growth stages of *C. saharae* using the organic solvent ethyl acetate. The results, shown in Figure 2, indicate a significant impact of growth stage on extraction yield. The highest yield, 1.4%, was observed in the sample collected during the flowering stage, while the lowest yield, 0.74%, was noted in the extract from the first vegetative stage. Extracts from the fruiting and second vegetative stages exhibited similar yields, estimated at 1.18% and 1.3%, respectively.

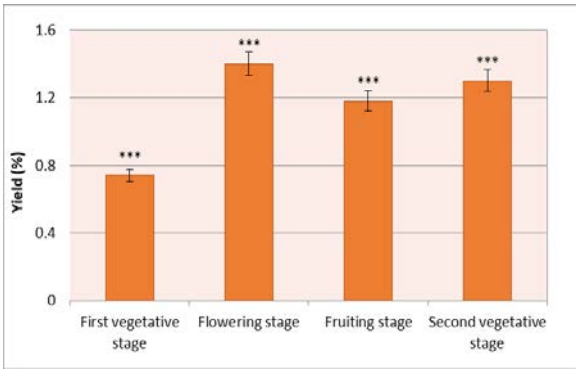

Figure 2: Yield results of flavonoid extracts for different growth stages of *C. saharae.*

***: Statistically significant differences at the confidence level (α=0.001).

3.4. Fourier-Transform Infrared (FTIR) Characterization

Flavonoid extracts from various physiological growth stages of C. saharae were analyzed spectroscopically. To facilitate spectral interpretation and monitor spectral changes, the spectrum was divided into two regions. The first region encompasses wavenumbers above 1600 cm^{-1}, typically featuring a limited number of peaks. Within this range, a broad absorption band was observed spanning 3700-3100 cm^{-1}, indicative of O-H bond presence. Additionally, two prominent absorption bands were noted between 2800 and 3000 cm^{-1}, corresponding to the C-H group, while spectral broadening at 1700-1750 cm^{-1} signifies the C=O functional group.

Conversely, the second spectral region, below 1600 cm^{-1}, is characterized by numerous peaks of varying intensities, many of which are challenging to identify. The bending observed in the 1200-1600 cm^{-1} range signifies the presence of aromatic rings in the studied extracts. The region from 1300-600 cm^{-1} is highly intricate and is known as the "fingerprint region," with the absorption observed in the spectral range of 700-900 cm^{-1} corresponding to the C-H functional group.

Analysis of the spectral curves revealed striking similarities among extracts from all physiological growth stages of the *C. saharae* plant, with only minor differences observed in transmittance intensity and the fingerprint region (Figure 3).

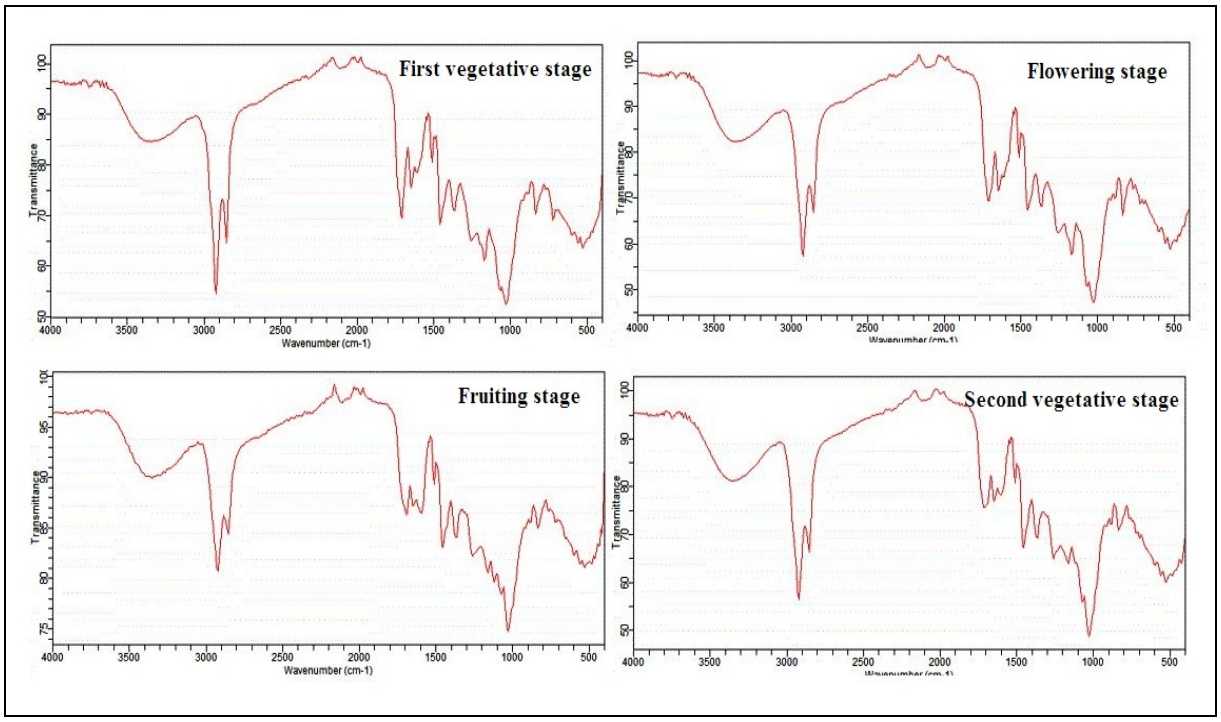

Figure 3: FTIR spectrum of extracts of different growth stages of *C. saharae*.

3.5. Estimation of antioxidant activity

3.5.1. Free radical DPPH scavenging activity

In the DPPH assay, noticeable discrepancies were observed in the IC_{50} values of *C. saharae* plant extracts. Statistical analysis using ANOVA revealed significant differences at a significance level of $\alpha = 0.001$. Notably, the extract obtained during the fruiting stage exhibited the highest efficacy in free radical scavenging, recording an IC_{50} value of 0.138±0.026 mg/mL. The IC_{50} values for the extracts obtained during the first and second vegetative stages, as well as the flowering stage, were recorded at 0.177 ± 0.031 mg/mL, 0.187 ± 0.036 mg/mL, and 0.213 ± 0.047 mg/mL, respectively (Figure 4).

Furthermore, the efficacy of these extracts was considerably lower compared to ascorbic acid, which demonstrated a significantly lower IC_{50} value of 0.005±0.001 mg/mL.

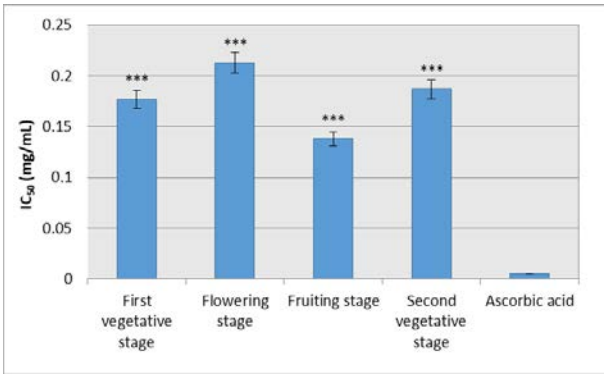

Figure 4. Value of IC_{50} of DPPH free radical scavenging test of extracts of different growth stages of *C. saharae* and Ascorbic acid.

***: Statistically significant differences at the confidence level (α=0.001) when compared to ascorbic acid.

3.5.2. Erythrocyte hemolysis test

The results indicate that the protective capacity of different plant extracts on red blood cells is relatively similar ($\alpha = 0.05$) at a concentration of 1 mg/mL. Table 2 shows that the first vegetative stage extract exhibited the lowest hemolysis rate, measured at 41.72±2.2%, followed by the second vegetative stage extract with a hemolysis rate of 48.96±1.9%, and then the fruiting stage extract with a hemolysis rate of 51.44±1.7%. Conversely, the flowering stage extract showed the highest hemolysis rate at 62.2±2.1%. These results indicate that the first vegetative stage extract demonstrates superior efficacy in protecting red blood cells compared to the other extracts studied.

However, it is worth noting that despite this efficacy, it remains relatively weak compared to ascorbic acid, which exhibited the lowest hemolysis rate of 17.09±1.6% under the same conditions. Therefore, while plant extracts show promise in protecting red blood cells, their effectiveness appears modest when compared to the potent effect of ascorbic acid.

Table 2. Percentage of Hemolysis (C: 1 mg/ml) of of extracts of of different growth stages of *C. saharae* and Ascorbic acid.

Extract stage	*First vegetative*	*floral*	*Fruiting*	*Second vegetative*	*Ascorbic acid*
Hemolysis (with C: 1 mg/ml)	41.72±2.2%***	62.2±2.1%***	51.44±1.7%***	48.96±1.9%***	17.09±1.6%***

***: Statistically significant differences at the confidence level (α=0.001) when compared to ascorbic acid.

3.6. Estimation of anti-inflammatory activity

Statistical analysis revealed significant differences in the anti-inflammatory efficacy of plant extracts across different growth stages, with a significance level of α = 0.001. The highest inhibitory capacity was observed during the flowering stage, estimated at 5.63±0.81 mg Diclofenac E/mg extract. Following this, the first vegetative stage exhibited a value of 4.15±0.87 mg Diclofenac E/mg extract. In comparison, both the fruiting and second vegetative stages showed similar inhibitory capacities, estimated at 3.45±0.32 and 3.96±0.58 mg Diclofenac E/mg extract, respectively. When comparing the effectiveness of these extracts to the reference compound Diclofenac, it can be concluded that their anti-inflammatory activity is significantly high (Figure 5).

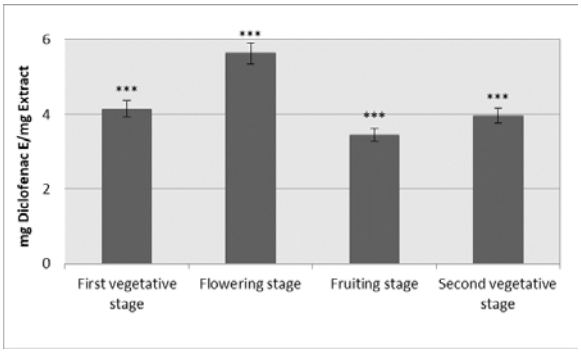

Figure 5. Estimation of anti-inflammatory activity of extracts of different growth stages of *C. saharae*.

***: Statistically significant differences at the confidence level (α=0.001).

3.7. Statistical study

To evaluate the strength and direction of the relationships between the studied variables, a correlation analysis was conducted using the Pearson Linear Correlation Coefficient test. This analysis produced correlation coefficient (R^2) values, which are presented in Table 3.

Table 3. Correlation coefficient (R^2) between the different variables studied.

Variable	Carbohydrate	Protein	Lipid	Yields	DPPH	Hemolysis	Anti-inflammatory
Carbohydrate	1						
Protein	0.627	1					
Lipid	0.685	0.883	1				
Yields	-0.606	0.001	-0.405	1			
DPPH	-0.234	0.122	0.438	-0.259	1		
Hemolysis	-0.812	-0.696	-0.929	0.708	-0.376	1	
Anti-inflammatory	0.428	0.428	0.027	0.394	-0.829	0.071	1

4. Discussion

Primary metabolic compounds such as carbohydrates, proteins, and fats play pivotal roles in the growth and development of organisms, particularly plants (Ofoedum *et al*., 2024). Their concentrations fluctuate across different physiological growth stages, as demonstrated in studies on *C. saharae* plants, revealing significant differences ($p < 0.001$) (Liu *et al*., 2022), as evidenced by the study on *C. saharae* plants, with significant differences observed ($p<0.001$). Conversely, (Ammar *et al*., 2004) observed that the protein content in leguminous shrubs is less susceptible to seasonal variations compared to other species.

Carbohydrates, as products of photosynthesis, are fundamental for synthesizing cellular compounds, providing energy, and aiding plant defense against pathogens (Bolton, 2009). The decline in carbohydrate content with plant maturation is influenced by factors such as maturity stage, utilized plant parts, and physiological condition. The initial vegetative stage typically exhibits the highest carbohydrate content due to increased development of young branches, which are crucial for the formation of structural components like semi-cellulose and pectin (Nour El-Din and Ahmed, 2004). Sugars also collaborate with proteins in glycoprotein formation, contributing to membrane stabilization and various cellular functions (Nguema-Ona *et al*., 2014).

This association elucidates the positive correlation ($R^2 = 0.617$) between carbohydrates and proteins. Conversely, the negative correlation ($R^2 = -0.606$) between carbohydrates and yield percentage is attributed to their structural relationship. Carbohydrates act as energy substrates for flavonoid biosynthesis; however, their content decreases as plants progress to allocate resources for organic compound synthesis (Cheynier *et al*., 2013), meeting the demands essential for growth and sustainability.

Crude protein serves as a key indicator of the nutritional quality of desert plants for ruminants (Nour El-Din and Ahmed, 2004), with legumes particularly valued for their rich protein and nutrient content (Semba *et al*., 2021). The protein levels in *C. saharae* plants fluctuate throughout their biological life cycle, influenced by factors

such as climate, harvest season, and physiological stage. Research indicates that protein content peaks during the early vegetative and flowering stages due to increased photosynthesis and nutrient absorption (Zhang *et al*., 2009). The flowering stage is characterized by heightened metabolic activity, typical of leguminous plants, which maximizes protein production. In the fruiting stages, protein is stored for seed development and enzyme activation, while declines in later vegetative stages may result from reduced biological activity or tissue aging (Ammar *et al*., 2004). Genetic, environmental, and soil factors contribute to the overall variation in protein content.

Fats serve as essential raw materials for synthesizing various compounds, including certain vitamins and hormones, and they act as solvents for fat-soluble vitamins (Ravisankar *et al*., 2015). In the *C. saharae* plant, fats are among the least abundant primary metabolites, with slight variations observed across different growth stages, likely reflecting their utilization in physiological functions such as cell membrane formation and energy storage in seeds. Fats can combine with sugars to form glycolipids, which are important for membrane function (Wiegandt, 2011). The correlation between fats and carbohydrates (R^2 = 0.685) suggests their interconnected metabolic roles, while the strong correlation between fats and proteins (R^2 = 0.883) highlights their structural and functional significance, particularly in cell membranes. During the second vegetative phase, fat content decreases significantly, likely due to lipid recycling during senescence an adaptive mechanism in response to environmental stress that aims to conserve resources for survival and subsequent vegetative cycles (Yang and Ohlrogge, 2009).

Chemical analysis of *C. saharae* plant samples revealed the synthesis of various secondary metabolites, including flavonoids, alkaloids, saponins, sterols, triterpenes, and tannins, while anthocyanins were absent. These findings align with previous studies utilizing aqueous extracts of the same plant (Guettaf *et al*., 2016). The presence of these compounds supports the plant's physiological growth, adaptation to environmental stresses, and overall resilience (Nour El-Din and Ahmed, 2004). Factors influencing the chemical diversity within a single plant include the plant part used, growth stage, genetic composition, environmental conditions, soil composition, and time of collection (Chouikh *et al*., 2015).

The fluctuation in the presence of these compounds throughout the plant's life cycle may reflect their vital functions and the plant's adaptive strategies in response to varying needs. Differences in localization and storage within the plant may also contribute to their appearance or absence at different stages (Nour El-Din and Ahmed, 2004). Moreover, variations in extraction methods or reagents used could influence the detection of certain compounds (Chatoui *et al*., 2016).

Flavonoid extracts from C. saharae plant samples were obtained using ethyl acetate, a commonly used solvent for flavonoid extraction (Boumaza and Boukaabache, 2015). The percentage yield of these extracts varied slightly across different physiological growth stages, despite consistent laboratory procedures. This fluctuation can be attributed to several factors, as flavonoid levels tend to increase in aerial parts during the development of new organs such as flowers, leaves, fruits, and seeds (Michalak, 2006). Additionally, the nature of chemical compounds plays a crucial role, with changes in the complexity and length of carbon chains affecting extraction efficiency (Chouikh *et al*., 2018). Furthermore, climatic conditions (Ksouri *et al*., 2008), lead to variations in environmental factors during plant growth stages, impacting flavonoid production. Lastly, the activity and condition of plants at different age levels during the study period can significantly influence flavonoid yields (Chouikh *et al*., 2018). These interconnected factors contribute to the observed fluctuations in flavonoid extraction yields from *C. saharae* plants across various growth stages.

The study revealed that the highest percentage yield was observed during the flowering stage, consistent with previous findings (Chouikh *et al*., 2018; Meriane *et al*., 2014). This increase in yield during flowering may be attributed to factors such as the relationship between flavonoids and pollinators as well as the coloration of flowers due to flavonoids (Chouikh *et al*., 2015; Ketaren Bunga Raya *et al*., 2015).

Conversely, the lowest percentage yield was observed during the first vegetative stage, which differs from some previous studies that reported the lowest yield during the fruiting stage (Chouia *et al*., 2018). This discrepancy may be attributed to the chemical nature of active compounds present in the plant during specific growth stages, the solubility characteristics of the solvent used, variations in harvesting periods and geographical regions, as well as differences in extraction methods and conditions (Abdelmadjide *et al*., 2020; Chouia *et al*., 2018; Feng *et al*., 2017).

Based on Fourier transform infrared (FTIR) spectroscopy, flavonoid extracts were found to contain hydroxyl (OH) groups and lacked aldehyde and methoxy functional groups, indicating that they are non-sugar, polyhydroxy flavonoids. These compounds, possibly belonging to the categories of flavonols, flavanones, or isoflavones (Meriane *et al*., 2014), possess hydroxyl groups and a phenolic ring, making them significant for biological purposes such as free radical inhibition, anti-inflammatory action against bacteria and viruses, and potential anti-cancer properties (Rahman *et al*., 2021)

In the DPPH assay, the results demonstrate a diminished free radical scavenging capacity of the investigated extracts compared to the reference compound, ascorbic acid, as observed in this study, corroborating the findings of (Bouchouka *et al*., 2012). This disparity may be explained by the limited presence of flavonoids in these extracts, which typically contain free radical scavenging entities. The literature suggests that strong antioxidant efficacy stems from an abundance of hydroxyl (OH) groups capable of interacting with free radicals (Chouikh *et al*., 2018).

The results further reveal that the extract obtained during the fruiting stage exhibits the most potent anti-DPPH activity. These findings align with those reported by Bouchouka et al. (2012) in their investigation of the raw methanolic extract from the same plant. This enhanced activity could be attributed to the accumulation of certain active constituents as reserves within the fruits (Chouikh *et al*., 2015), with compounds formed at this stage potentially possessing superior scavenging capabilities. Numerous researchers posit a substantial correlation between the

chemical structure of flavonoids and their free radical scavenging activity. Interestingly, the activity is notably diminished during the flowering stage, which contradicts the findings of Chouikh *et al.* (2018) and Meriana *et al*. (2014). This discrepancy may arise from variations in solvent type and its effectiveness in extracting active compounds, impacting their concentration in the extract or the nature of their chemical structures conducive to DPPH scavenging, such as the presence of hydroxyl groups, double bonds between carbon atoms, and heterogeneous oxo groups (Chouikh *et al*., 2018).

The protective effect of plant extracts against oxidative damage to red blood cells caused by two oxidizing agents, $FeCl_3$ and H_2O_2, was assessed. These radicals initiate lipid and protein oxidation reactions upon attacking red blood cells, leading to membrane damage, eventual cell lysis, and hemoglobin release (Abirami *et al*., 2014; Supriya *et al*., 2013). The observed capacity of the studied plant extracts to safeguard red blood cells can be elucidated through two hypotheses: firstly, flavonoids within the extracts may interact with free radicals, thereby neutralizing them (Nabavi *et al*., 2013). Alternatively, the flavonoids might interact with cellular membrane components, particularly lipids and proteins, shielding them from oxidative damage, as evidenced by the protective effects observed across all extracts (Chaudhuri et al., 2007). Moreover, a concentration-dependent increase in extract concentration correlates with greater red blood cell protection and decreased hemolysis, indicating a potential role of flavonoids in restoring and stabilizing oxidants through electron donation or proton modification (Ebrahimzadeh *et al*., 2014). Additionally, the observed inverse relationship (R^2= -0.929) between lipid degradation rate and flavonoid-lipid interactions, and the moderate relationship (R^2= -0.696) between protein degradation rate and flavonoid-protein interactions, further support the hypothesis of flavonoid-mediated protection against lipid peroxidation, thus reducing the degradation rate as flavonoid interaction with lipid and protein components increases (Khalili *et al*., 2014).

In contrast, the obtained results diverge from the findings of Chouikh *et al*. (2018) in their investigation of raw extracts derived from the same plant species, wherein the crude phenolic extract exhibited notable protective effects against red blood cell hemolysis. This discrepancy may stem from the distinct composition of compounds within the extracts. The crude extract contains phenols and tannins, which likely contributed to its protective efficacy by facilitating electron donation to H_2O_2, converting it into water (Ebrahimzadeh *et al*., 2014). Alternatively, the observed protection could be attributed to the abundance of compounds containing sugar radicals within the extract, which possess a strong membrane-blending capacity, a characteristic absent in the flavonoid extract under study. This was substantiated by the results of FTIR spectroscopy, which indicated the absence of aldehyde functional bonds.

The studied extracts exhibit promising anti-inflammatory activity, as evidenced by their ability to prevent protein denaturation by more than 20%, a hallmark of anti-inflammatory compounds (Williams *et al*., 2008). This activity is likely attributed to the presence of flavonoids containing numerous hydroxyl groups (OH), which facilitate the formation of hydrogen bonds with proteins, thus averting their denaturation.

Remarkably, the flowering stage extract demonstrated the highest inhibition capacity. This could be attributed to the elevated concentrations of non-saccharide flavonoids known for their potent anti-inflammatory properties. Alternatively, the unique nature of flavonoid compounds within this extract might enable interactions with proteins, notably albumin (Dufour *et al*., 2007). These findings align with those of Guettaf *et al*.(2016), underscoring the significant protein degradation inhibition capacity of the aqueous extract of *C. saharae*.

5. Conclusion

This study illuminates the intricate interplay between the physiological growth stages, chemical composition, and bioactivity of *C. saharae* extracts. Our investigation reveals pronounced fluctuations in nutrient content, secondary metabolite composition, and pharmacological efficacy throughout the plant's development. The decline in carbohydrate content with advancing growth stages underscores metabolic shifts, while the peak in protein content during the flowering stage signifies heightened biosynthetic activity. Flavonoid extracts, enriched with secondary metabolites, exhibit stage-specific variations in yield and composition, with the flowering stage yielding the highest flavonoid content. Analysis of FTIR spectra unveils structural insights into flavonoid extracts, crucial for quality assurance and standardization. Moreover, antioxidant assays demonstrate stage-dependent efficacy, with the fruiting stage displaying the highest radical scavenging capacity. The erythrocyte hemolysis test elucidates variable protective effects against oxidative stress across growth stages, hinting at potential health benefits. Additionally, the anti-inflammatory activity of *C. saharae* extracts underscores their therapeutic potential, with the flowering stage extract exhibiting the strongest inhibitory capacity. Correlation analysis elucidates intricate relationships between chemical constituents, bioactivity, and growth stages, offering insights into underlying mechanisms. Overall, our findings emphasize the significance of considering growth stage variability in pharmacological investigations and pave the way for harnessing the medicinal properties of *C. saharae* extracts for diverse therapeutic applications.

Conflict of interests

Authors declare that there is no conflict of interests.

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