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Phytochemical Characterization and Quantification of Phenolic, Flavonoid, and Tannin Contents in Ethanolic Extract of *Medicago sativa*

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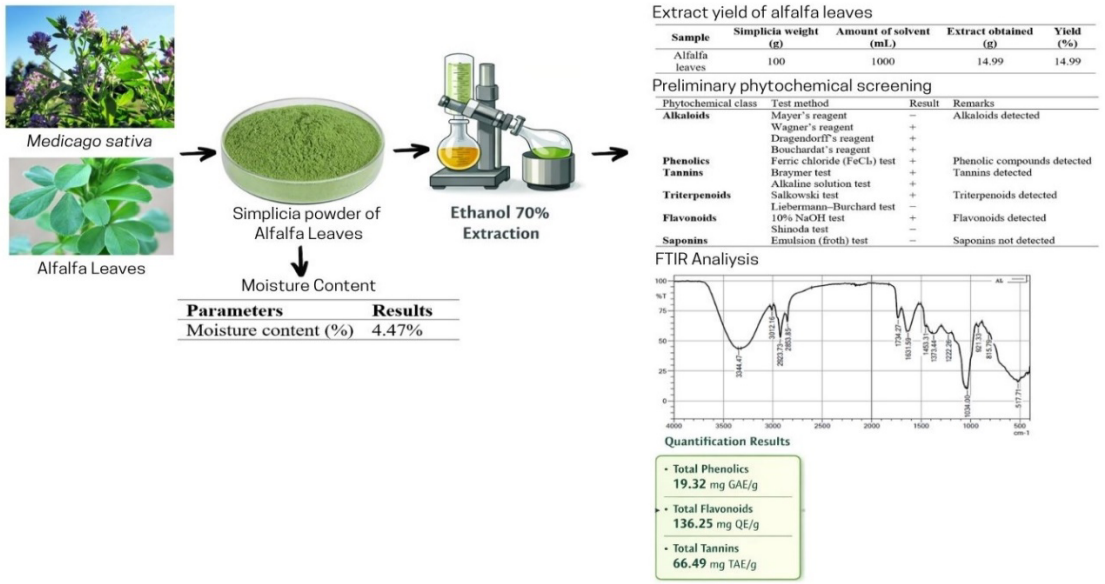
GRAPHICAL ABSTRACT



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ABSTRACT

Medicago sativa (alfalfa) leaves are known to contain diverse bioactive phytochemicals, particularly phenolic and flavonoid compounds that are commonly associated with antioxidant activity. This study aimed to characterize the phytochemical profile of the ethanol extract of alfalfa leaves and quantify its major phenolic constituents to provide chemical evidence supporting its antioxidant potential. Dried alfalfa leaves were extracted using maceration with ethanol 70%, followed by qualitative phytochemical screening, Fourier Transform Infrared (FTIR) analysis, and spectrophotometric determination of total phenolic, flavonoid, and tannin contents. Phytochemical screening confirmed the presence of alkaloids, phenolics, tannins, flavonoids, and triterpenoids, while saponins were not detected. Quantitative analysis showed that the extract contained a high total flavonoid content (136.25 ± 3.165 mg quercetin equivalents/g extract), along with appreciable levels of total phenolics (19.32 ±

0.814 mg gallic acid equivalents/g extract) and tannins (66.49 ± 0.927 mg tannic acid equivalents/g extract). These findings demonstrate that alfalfa leaf extract is a rich source of polyphenolic compounds, which are widely associated with antioxidant properties, thereby providing a scientific basis for further evaluation of antioxidant activity.

1. INTRODUCTION

Medicinal plants are widely recognized as important sources of secondary metabolites with diverse chemical structures and biological activities. Among these metabolites, phenolic compounds, flavonoids, and tannins are of particular interest due to their strong antioxidant properties and their roles as key indicators of extract quality and bioactivity. Accurate phytochemical characterization and quantitative determination of these compounds are therefore essential in chemical analysis studies of plant-derived materials [1, 2].

Medicago sativa (alfalfa) is a leguminous plant traditionally utilized for nutritional and medicinal purposes. Previous studies have reported the presence of various bioactive constituents in *Medicago sativa*, including phenolics, flavonoids, saponins, and tannins. However, the reported phytochemical profiles vary considerably depending on extraction solvents, extraction conditions, and analytical methods employed. This variability underscores the need for standardized chemical analytical methods to obtain reliable, reproducible data on the phytochemical composition of *Medicago sativa* extracts [3].

Ethanol is commonly used as an extraction solvent in phytochemical studies due to its suitable polarity, safety, and effectiveness in extracting a wide range of polar and semi-polar compounds. In addition to conventional phytochemical screening and spectrophotometric assays, Fourier Transform Infrared (FTIR) spectroscopy provides valuable complementary information by enabling rapid identification of functional groups associated with secondary metabolites. The integration of qualitative screening, functional group analysis, and quantitative determination offers a comprehensive analytical strategy for characterizing plant extracts [4].

Therefore, this study aims to characterize the phytochemical constituents of the ethanol extract of *Medicago sativa* leaves through qualitative phytochemical screening and FTIR analysis, and to quantitatively determine total phenolic, flavonoid, and tannin contents using UV–Vis spectrophotometric methods. Notably, this work provides integrated qualitative–spectroscopic–quantitative data for Medicago sativa leaf extract obtained using 70% ethanol, which remain limited in the existing literature and are essential for chemical standardization and future bioactivity-oriented studies.

2. EXPERIMENTAL METHODS

2.1. Material and Apparatus

Fresh alfalfa (*Medicago sativa*) leaves were obtained from the Taman Alquran of Universitas Islam Malang, Malang, East Java, Indonesia. Analytical-grade ethanol was used as the extraction solvent. Gallic acid, quercetin, tannic acid, Folin–Ciocalteu reagent, Folin–Denis reagent, aluminum chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), sodium acetate (CH_3COONa), ferric chloride ($FeCl_3$), potassium bromide (KBr), Wagner's reagent, Mayer's reagent, Dragendorff's reagent, Bouchardat's reagent, Braymer's reagent, Liebermann–Burchard reagent, and Salkowski reagent were used for phytochemical screening and quantitative analysis. All reagents and chemicals used were of analytical grade. Distilled water was used throughout the experiments.

The apparatus used in this study included a UV–Vis spectrophotometer, Thermo Scientific GENESYS, a Fourier Transform Infrared (FTIR) spectrophotometer Shimadzu FTIR 8400S, a rotary evaporator, a moisture analyzer, an analytical balance, an oven, a blender, 80-mesh sieve, vortex mixer, glassware (beakers, volumetric flasks, test tubes, pipettes, and Erlenmeyer flasks).

2.2. Preparation of raw materials

Alfalfa leaves were collected from Taman Alquran of Universitas Islam Malang, Malang, East Java, Indonesia. The samples were harvested from healthy plants at the vegetative growth stage, characterized by fully expanded green leaves and the absence of visible disease or pest infestation. The plant material underwent wet sorting, washing, chopping, drying, and dry sorting. The leaves were then dried in an oven at 60 °C until a constant dry state was achieved. Subsequently, the sample's dry weight was measured, and the moisture content was calculated to ensure it did not exceed 10%. After drying, the leaves were ground into a fine powder using a blender [5, 6]. The resulting powder was sieved through an 80-mesh sieve to increase the surface area, thereby facilitating solvent penetration during the extraction process [7].

2.3. Determination of Moisture Content

One gram of simplisia powder was weighed and placed into a moisture analyzer. The sample was analyzed for 10 minutes to determine its moisture content. The maximum allowable moisture content for simplisia was set at 10%, in accordance with established quality standards [8].

2.4. Extraction

Alfalfa leaves were extracted using the maceration method with a solvent-to-material ratio of 1:10, employing ethanol 70% as the extraction solvent. Ethanol 70% was selected due to its ability to dissolve a wide range of bioactive compounds, including alkaloids, flavonoids, tannins, saponins, steroids, and terpenoids. The polarity of ethanol is compatible with that of secondary metabolites, facilitating the efficient solubilization of phytochemicals. This is consistent with the “like dissolves like” principle, whereby compounds are more readily dissolved in solvents with similar physicochemical properties. In addition, ethanol is readily available and more cost-effective than other organic solvents [9]. Initially, 100 g of simplisia powder was weighed and placed into a 1 L glass reagent bottle. The first maceration stage employed a solvent ratio of 1:7, followed by a second stage with a ratio of 1:3. Both maceration stages were homogenized in a 1000 mL Erlenmeyer flask. The combined filtrate was then concentrated using a rotary evaporator at 40 rpm and 60–70 °C for 1 hour, until a viscous, paste-like extract was obtained [5].

2.5. Determination of Extraction Yield

The extract yield of alfalfa leaves was calculated according to the AOAC method (1999) using the equation: extraction yield (%) = (W1/W2) × 100, where W1 represents the mass of the crude extract and W2 represents the mass of the initial sample [10].

2.6. Phytochemical Screening

Qualitative phytochemical screening was conducted to identify the presence of secondary metabolites in the extract, including alkaloids, phenolics, tannins, flavonoids, triterpenoids, and saponins. Alkaloid detection was carried out using Wagner’s, Mayer’s, Dragendorff’s, and Bouchardat’s reagents. Phenolic compounds were identified using ferric chloride ($FeCl_3$) reagent, while tannins were detected using Braymer’s reagent. Triterpenoids were analyzed using Salkowski and Liebermann–Burchard tests. Flavonoids were identified using aluminum chloride and 10% NaOH reactions, whereas saponins were detected using the froth formation method. The appearance of characteristic color changes, precipitates, or stable foam indicated positive results [12].

2.7. FTIR Analysis

Functional group analysis was performed using Fourier Transform Infrared Spectroscopy (FTIR). The dried extract was thoroughly mixed with potassium bromide (KBr) and compressed into pellets. FTIR spectra were recorded over a wavenumber range of 4000–400 cm^{-1}. Spectral interpretation was carried out by identifying characteristic absorption peaks corresponding to functional groups such as –OH, –C=O, and –C–O, as these groups are structural features commonly associated with major classes of plant secondary metabolites, including phenolics, flavonoids, and

glycosides. Therefore, the presence of these absorption bands provides a rational basis for inferring the occurrence of secondary metabolites in the plant extract [11].

2.8. Determination of Total Phenolic Content [13]

2.8.1. Determination of Maximum Wavelength

The maximum absorption wavelength was determined using the Folin–Ciocalteu method. A volume of 0.1 mL of a 100 ppm gallic acid solution was mixed with 1 mL of Folin–Ciocalteu reagent and incubated for 5 minutes. Subsequently, 1 mL of 7.5% Na_2CO_3 solution was added, and the mixture was incubated at room temperature for 90 minutes. The absorbance was measured using a UV–Vis spectrophotometer over 600–850 nm to determine the maximum absorbance wavelength.

2.8.2. Preparation of Gallic Acid Standard Solutions

10 mg of gallic acid was dissolved in distilled water and diluted to 10 mL to obtain a stock solution. Aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 mL were pipetted and diluted with distilled water to a final volume of 10 mL, yielding standard solutions at 20, 40, 60, 80, and 100 ppm, respectively. 0.1 mL of each standard solution was mixed with 1 mL of Folin–Ciocalteu reagent and incubated for 5 minutes, followed by the addition of 1 mL of 7.5% Na_2CO_3 solution. The mixtures were incubated for 90 minutes at room temperature in the dark. Absorbance was measured at the maximum wavelength of 786 nm using a UV–Vis spectrophotometer. A calibration curve was constructed by plotting absorbance against gallic acid concentration.

2.8.3. Determination of Total Phenolic Content of Alfalfa Leaf Extract

Ten milligrams of alfalfa leaf extract were dissolved in 10 mL of distilled water until homogeneous. A volume of 0.1 mL of the solution was mixed with 1 mL of Folin–Ciocalteu reagent and incubated for 5 minutes. Subsequently, 1 mL of 7.5% Na_2CO_3 solution was added, and the mixture was incubated for 90 minutes at room temperature in the dark. Absorbance was measured at 786 nm using a UV–Vis spectrophotometer. Total phenolic content was expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g), calculated based on the linear regression equation of the gallic acid standard curve.

2.9. Determination of Total Flavonoid Content [5]

2.9.1. Determination of Maximum Wavelength

A volume of 0.5 mL quercetin solution (100 ppm) was reacted with 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M CH_3COONa, and 2.5 mL of distilled water. The mixture was homogenized and incubated for 40 minutes under light-protected conditions. The maximum wavelength was determined using a UV–Vis spectrophotometer over 415–440 nm to obtain the highest absorbance.

2.9.2. Preparation of Quercetin Standard Solutions

Quercetin standard solutions were prepared by dissolving 10 mg of quercetin in 96% analytical-grade ethanol to a final volume of 10 mL, yielding a stock solution of 1000 ppm. The stock solution was diluted in series to obtain standard solutions at 20, 40, 60, 80, and 100 ppm. Each standard solution was reacted with $AlCl_3$ and CH_3COONa, incubated in the dark for 40 minutes, and the absorbance was measured at 431 nm.

2.9.3. Determination of Total Flavonoid Content of the Sample

10 mg of the extract was dissolved in 96% analytical-grade ethanol and diluted to a final volume of 10 mL to obtain a stock solution at 1000 ppm. The sample solution was reacted with 10% $AlCl_3$ and 1 M CH_3COONa, homogenized, and incubated for 40 minutes under light-protected conditions. Absorbance was measured at 431 nm using a UV–Vis spectrophotometer, and total flavonoid content was calculated based on the quercetin calibration curve.

2.10. Determination of Total Tannin Content [14]

2.10.1. Determination of Maximum Wavelength

The maximum absorption wavelength was determined using gallic acid as a reference standard. A volume of 1 mL of a 100 ppm gallic acid solution was reacted with 0.5 mL of Folin–Denis reagent and incubated for 3 minutes, followed by the addition of 1 mL of 7.5% Na_2CO_3 solution. The mixture was incubated at room temperature in the dark for 40 minutes to ensure stable color complex formation. Absorbance was measured over 500–800 nm to determine the wavelength with the highest absorbance.

2.10.2. Preparation of Tannic Acid Standard Solutions

Tannic acid standard solutions were prepared by accurately weighing 10 mg of tannic acid and dissolving it in distilled water to obtain a stock solution of 1000 ppm. The stock solution was diluted in series to obtain concentrations of 100, 80, 60, 40, and 20 ppm. Each standard solution was reacted with Folin–Denis reagent, homogenized with a vortex mixer, incubated for 3 minutes, then treated with Na_2CO_3 solution and incubated for an additional 40 minutes before absorbance was measured using a UV–Vis spectrophotometer. This method is based on the oxidation–reduction reaction of phenolic compounds with the Folin reagent, producing a blue-colored complex that can be measured by spectrophotometry.

2.10.3. Determination of Total Tannin Content of Alfalfa Leaves

Ten milligrams of alfalfa leaf extract were dissolved in 10 mL of distilled water, followed by ethanol addition and dilution until a homogeneous solution was obtained. Sample solutions were prepared at 1000 ppm and 500 ppm, and each was analyzed in triplicate. The samples were reacted with Folin–Denis reagent and Na_2CO_3 solution, incubated as described, and their absorbance was measured at 647 nm using a UV–Vis spectrophotometer. Total tannin content was calculated based on the tannic acid calibration curve and expressed as milligrams of tannic acid equivalents per gram of extract (mg TAE/g extract).

3. RESULTS AND DISCUSSIONS

3.1. Characterization Determination of Moisture Content of Alfalfa (*Medicago sativa*) Leaf Simplicia

The quality of simplicia is a critical factor in ensuring the safety and efficacy of the final product. One of the key parameters used to assess simplicia quality is moisture content. Moisture content can significantly influence the stability, purity, and effectiveness of simplicia. The results of the moisture content determination are presented in Table 1.

TABLE I. Characteristics of alfalfa (*Medicago sativa*) leaf simplicia

Parameters	Results	Reference
Moisture content (%)	4.47%	≤10% [15]

A moisture content below the maximum permissible limit substantially contributes to the quality and shelf life of simplicia. Elevated moisture levels, as reported by Fahmi, Herdiana, and Rubiyanti [16], fundamentally increase the susceptibility of simplicia to microbial growth, including bacteria and fungi. The metabolic activity of these microorganisms can lead to the degradation of bioactive compounds, the formation of toxic metabolites, and alterations in organoleptic properties, thereby reducing therapeutic efficacy and potentially posing risks to consumers. Furthermore, excessive moisture may trigger hydrolytic reactions in labile compounds, such as glycosides and esters, accelerating chemical degradation and shortening the storage period.

Therefore, the moisture content of 4.467% obtained in this study indicates that the drying process was performed optimally and complies with the quality requirements of the Indonesian Herbal Pharmacopoeia. Moisture levels within the established standard play a crucial role in inhibiting microbial activity and slowing enzymatic and chemical degradation reactions, thereby

maintaining the stability of bioactive constituents during storage. High-quality simplicia ensures optimal preservation of bioactive compounds in alfalfa leaves, minimizes the risk of enzymatic and microbiological deterioration, and maintains the simplicia's purity throughout storage [16]. Compliance with moisture content standards represents a fundamental aspect of ensuring the initial quality of simplicia prior to further processing. This condition is essential to minimize variability in raw material quality, thereby enabling the production of extracts with more consistent potency and safety profiles. In addition, a low moisture content indicates that the simplicia is in an adequate condition to preserve its physical and chemical integrity during storage.

3.2. Characterization of Alfalfa (*Medicago sativa*) Leaf Extract

3.2.1. Determination of Extract Yield

In this study, the maceration technique was employed to obtain the extract because, compared to other extraction methods, maceration is a simpler process, requires less specialized equipment, and can be widely applied on a large scale [17]. Alfalfa (*Medicago sativa*) leaf extract was prepared using this method. The extraction yield data for alfalfa leaves extracted with 70% ethanol are presented in Table 2. The extract yield was determined by weighing the concentrated extract obtained after solvent removal using a rotary evaporator and dividing it by the initial weight of the powdered plant material.

TABLE II. Extract yield of alfalfa (*Medicago sativa*) leaves

Sample	Simplicia weight (g)	Amount of solvent (mL)	Extract obtained (g)	Yield (%)
Alfalfa leaves	100	1000	14.99	14.99

One of the critical parameters in the extraction process is extract yield, which reflects the efficiency of the extraction method. In plant material extraction, yields typically range from 5% to 30%, depending on the raw material's chemical composition. A higher yield generally indicates a higher abundance of extractable bioactive compounds, whereas a lower yield may suggest a lower concentration of target compounds or suboptimal extraction efficiency. The 14.998% extract yield obtained with 70% ethanol indicates that this solvent system is sufficiently effective for extracting secondary metabolites from alfalfa leaves.

Several factors, including extraction time, temperature, method, solvent type, and phytochemical composition of the plant material, influence extraction efficiency. The use of mixed solvents, such as distilled water (Aquadest) and ethanol, during maceration is commonly used to maximize the extraction of bioactive compounds from plant materials. This approach is effective because the use of multiple solvents facilitates the extraction of compounds with different polarity profiles, including polar, semi-polar, and slightly non-polar constituents. Distilled water, as a highly polar solvent, promotes cell wall disruption and solubilization of polar compounds, whereas ethanol, a solvent with intermediate polarity, enhances the extraction of semi-polar constituents. Consequently, this solvent combination enables the extraction of a broader spectrum of phytochemicals. Polar compounds tend to dissolve more readily in water, while semi-polar compounds exhibit higher solubility in ethanol. The use of mixed solvents has been shown to improve extraction efficiency and enhance extract characteristics, such as total phenolic content and antioxidant activity [18-20].

3.2.2. Preliminary phytochemical screening of the Extract

Plants are an invaluable source of secondary metabolites that can be used to develop novel therapeutic agents. However, a large proportion of their pharmacological and biological potential remains unexplored; therefore, researchers worldwide continue to investigate the bioactive constituents of medicinal plants [24, 25]. The results of the preliminary phytochemical screening of single and combined extracts are presented in Table 3.

Phytochemical screening was conducted to assess the presence of secondary metabolites in the extracts qualitatively. All tests were performed according to standard phytochemical screening

protocols. The positive reactions observed for alkaloids, phenolic compounds, tannins, triterpenoids, and flavonoids indicate that the extracts contain diverse classes of bioactive secondary metabolites. Conversely, the absence of saponins suggests that these compounds were either not present or present at concentrations below the detection limit of the applied qualitative assays.

TABLE III. Preliminary phytochemical screening of the extracts

Phytochemical class	Test method	Result	Remarks
Alkaloids	Mayer's reagent	−	Alkaloids detected
	Wagner's reagent	+	
	Dragendorff's reagent	+	
	Bouchardat's reagent	+	
Phenolics	Ferric chloride ($FeCl_3$) test	+	Phenolic compounds detected
Tannins	Braymer test	+	Tannins detected
	Alkaline solution test	+	
Triterpenoids	Salkowski test	+	Triterpenoids detected
	Liebermann–Burchard test	−	
Flavonoids	10% NaOH test	+	Flavonoids detected
	Shinoda test	−	
Saponins	Emulsion (froth) test	−	Saponins not detected

3.2.3. FTIR (Fourier Transform Infrared) Analysis of the Extract

FTIR spectroscopy is a rapid, simple, and non-destructive analytical technique that enables comprehensive identification of chemical characteristics within a sample through spectral interpretation [21]. FTIR analysis of alfalfa leaf extract was performed over a wavenumber range of 4000–400 cm^{-1}. The FTIR results indicated that the extract exhibited absorption bands corresponding to various functional groups. The FTIR spectrum of Alfalfa Leaf extract (AL) is presented in Figure 1, showing the characteristic peaks observed in the spectrum.

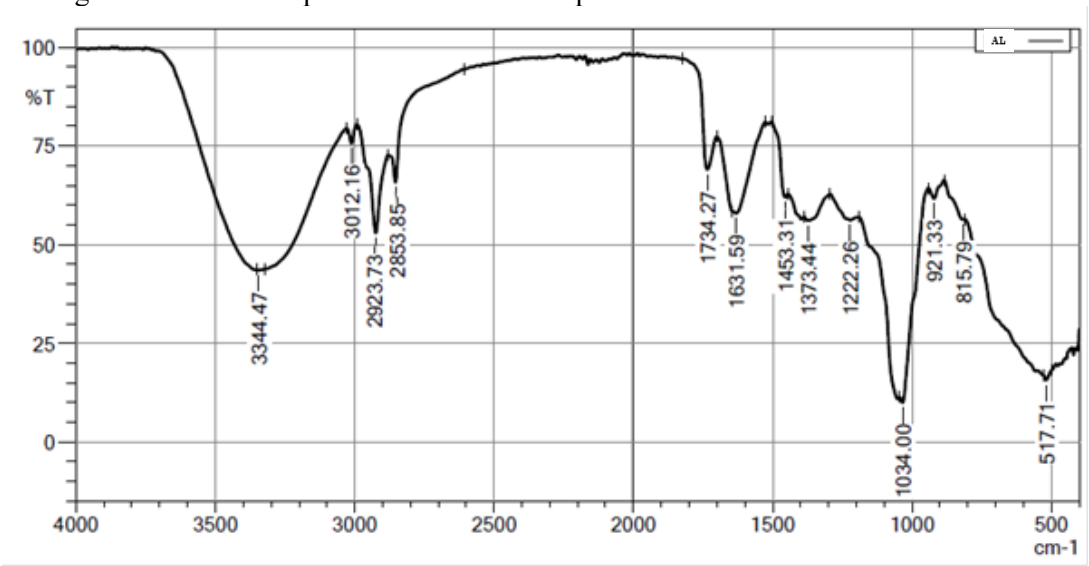

Figure 1. FTIR spectrum of Alfalfa Leaf extract (AL). The observed absorption peaks are summarized in Table 3.

A detailed interpretation of the infrared spectrum of the extract is provided in Table 4. The functional groups identified by FTIR analysis can be used to predict the classes of phytochemicals present in the extract.

TABLE IV. Interpretation of FTIR absorption bands of alfalfa leaf extract

Wavenumber (cm^{-1})	Peak Character	Vibration / Bond Type	Possible Functional Group	Additional Remarks
3344.47	Broad	O–H stretching	Alcohols, phenols, water (–OH)	Characteristic of hydroxyl groups, likely from phenolic or flavonoid compounds
3012.16	Sharp	=C–H stretching (aromatic/aliphatic)	Alkenes or aromatic compounds	Common in aromatic structures
2923.73 & 2853.85	Sharp	Aliphatic C–H stretching	Alkanes (–CH_2–, –CH_3)	Indicates the presence of saturated carbon chains
1734.27	Sharp	C=O stretching	Esters, carboxylic acids, aldehydes, ketones	Typical carbonyl group absorption
1631.59 & 1543.31	Sharp	C=C stretching and N–H bending	Alkene double bonds, amide groups	May also indicate aromatic ring vibrations
1374.53	Sharp	C–H bending	Alkanes (–CH_3)	Indicates methyl groups
1222.26	Sharp	C–O stretching	Esters, ethers, alcohols, phenols	Ether or alcohol functional groups
1034.00	Sharp	C–O–C stretching	Ethers, glycosides	Common in polysaccharides or glycosidic structures
921.33 & 815.79	Sharp	Aromatic C–H bending / out-of-plane vibration	Aromatic or heterocyclic compounds	Indicates aromatic ring structures
517.71	Sharp	C–X stretching (X = halogen) or ring deformation	Halogenated compounds (unlikely) or aromatic rings	Fingerprint region

FTIR analysis of the alfalfa leaf extract revealed several major absorption bands corresponding to O–H stretching, aliphatic C–H stretching, C=O stretching, C–O vibrations, as well as bands in the fingerprint region, indicating the presence of aromatic and glycosidic structures. This spectral pattern is consistent with FTIR profiles reported for various plant extracts obtained using organic solvents such as ethanol or hydroethanolic systems. Furthermore, FTIR spectral studies of eight different plant extracts have reported broad absorptions in the range of 3950–3200 cm^{-1} attributed to O–H functional groups, along with bands at approximately 1636–1550 cm^{-1} and within the 950–1500 cm^{-1} region associated with phenolic and aromatic moieties. These findings are in agreement with the FTIR spectrum of the alfalfa leaf extract observed in the present study [22].

Other studies employing FTIR for phytochemical profiling of various plant materials have demonstrated that absorption bands in the regions of 3300–3200 cm^{-1} (O–H stretching), around 1600–1500 cm^{-1} (aromatic ring vibrations/C=C stretching), and 1200–1000 cm^{-1} (C–O and C–O–C stretching) are characteristic features of phenolic compounds, flavonoids, and glycosides in ethanol-based plant extracts [23].

Overall, FTIR analysis confirms that the alfalfa leaf extract obtained with 70% ethanol is rich in diverse functional groups that represent major classes of phytochemicals, including polyphenols (phenolic compounds and flavonoids), carbohydrates, and possibly proteins or peptides. This functional group profile is highly consistent with previously reported phytochemical data for *Medicago sativa*, which is well recognized for its bioactive constituents with various pharmacological activities, including antioxidant, anti-inflammatory, and hypolipidemic effects. The presence of these functional groups provides a crucial foundation for further studies on the isolation and identification of active compounds, as well as the evaluation of the extract's biological activities.

3.2.4. Total Phenolic Content

The total phenolic content was determined using a UV–Vis spectrophotometer at 786 nm, based on a gallic acid calibration curve. The gallic acid standard curve was used to calculate total phenolic content, expressed as gallic acid equivalents (GAE). Gallic acid was used as the reference standard for total phenolic determination. In this test, gallic acid was used as a benchmark because it is a stable natural phenolic compound, and the results are usually expressed in gallic acid equivalents (GAE). The reaction between gallic acid and the Folin–Ciocalteu reagent is shown in Figure 2. This reaction occurs in an alkaline atmosphere (with the addition of a sodium carbonate solution (Na_2CO_3), where the phenol compound acts as a reducing agent against the heteropoly complex (phosphomolybdate–phosphotungstate) [26].

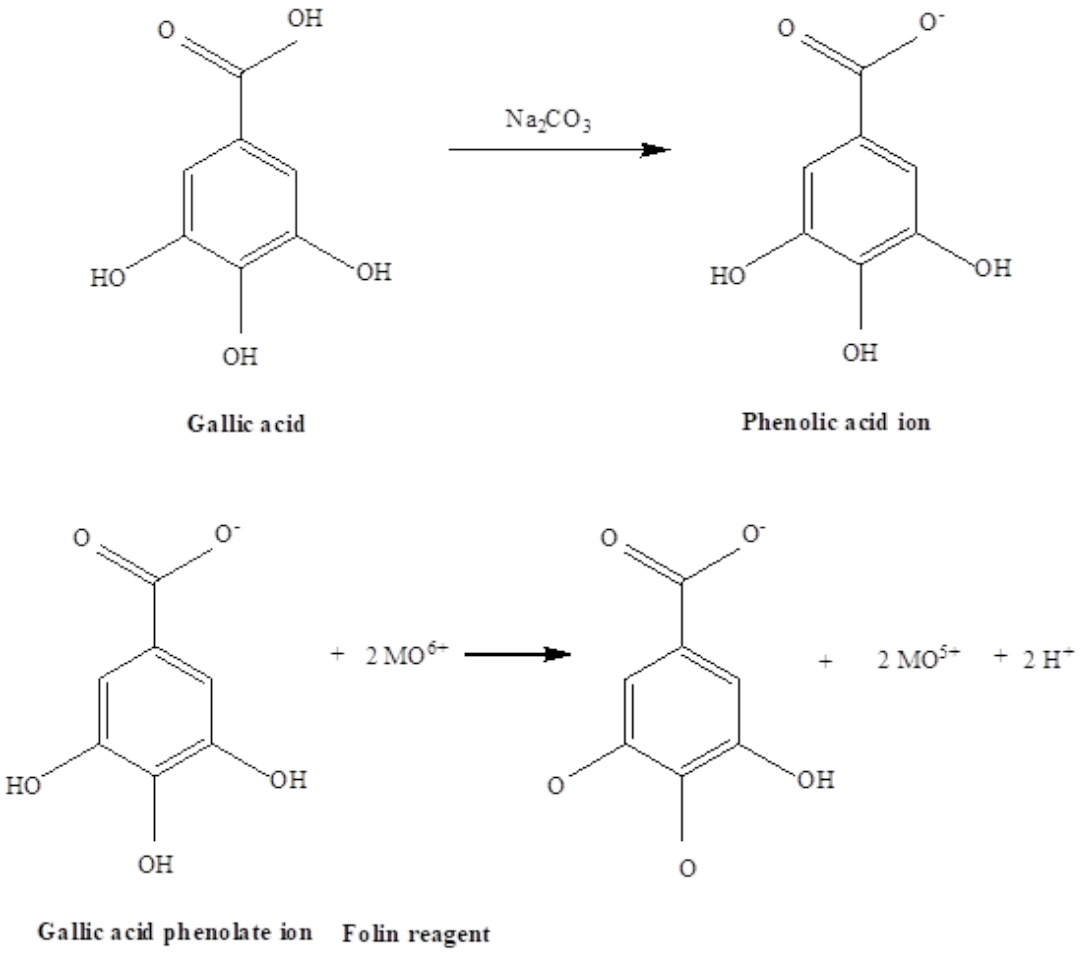

Figure 2. Reaction between Gallic Acid and *Folin-Ciocalteu Reagent*

The calibration curve is presented in Figure 3. The curve was constructed using gallic acid standard solutions at concentrations of 10, 20, 40, 60, 80, and 100 ppm. Linear regression analysis yielded the equation $y = 0.0393x + 0.3542$ with a coefficient of determination (R^2) of 0.9965.

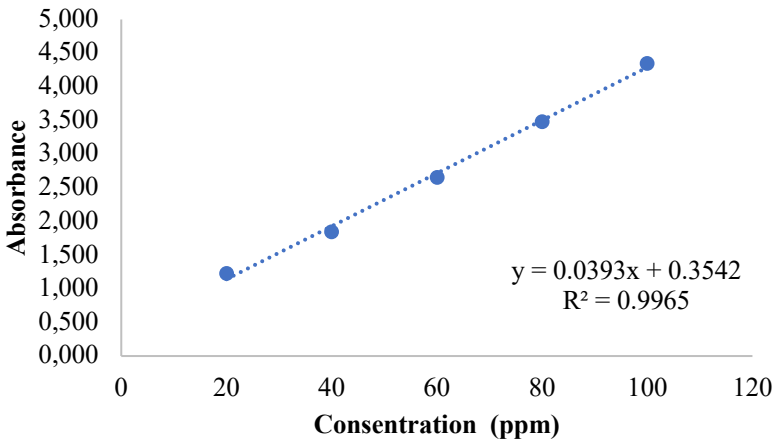

Figure 3. Gallic acid standard calibration curve showing linear regression between concentration 10–100 ppm and absorbance at 786 nm (y = 0.0393x + 0.3542 R^2 = 0.9965).

The determination of total phenolic content was conducted using the Folin–Ciocalteu method. The Folin–Ciocalteu assay is one of the most widely applied methods for quantifying total phenolic compounds in plant materials due to its simplicity and reliability. The Folin–Ciocalteu reagent reacts with phenolic compounds to form a blue-colored complex, the absorbance of which can be measured spectrophotometrically. All measurements were performed in triplicate to ensure analytical reliability. The results of the total phenolic content determination are presented in Table 5.

TABLE V. Total phenolic content of the extract

Sample	Concentration (ppm)	Mean total phenolic content (mg GAE/g)
Alfalfa leaves	1000	19.32 ± 0.814

3.2.5. Total Flavonoid Content

The determination of total flavonoid content (TFC) has been widely used for many years across various scientific fields, including applied chemistry, food science, and, particularly, plant biochemistry and bioactivity studies. Spectrophotometric methods are commonly employed due to their rapid application and the ability to determine target compounds in sample solutions quantitatively. For TFC analysis, spectrophotometry remains the most frequently used analytical approach.

The total flavonoid content was measured based on the presence of quercetin in the extract. Quercetin was used as the reference standard for the calibration curve. Quercetin was selected as the standard because it belongs to the flavonol subclass of flavonoids, which are highly effective free radical scavengers (including hydroxyl, superoxide, and peroxyl radicals) and potent inhibitors of various oxidative reactions. This activity is attributed to its ability to form resonance-stabilized phenoxyl radicals within its aromatic ring system.

In this study, total flavonoid content was determined using a colorimetric method based on the formation of complexes between flavonoids and Al(III) ions. The reaction between Al(III) and flavonoids proceeds optimally under mildly acidic conditions, producing a yellow-colored complex with maximum absorbance in the range of 410–440 nm. The increase in color intensity of this complex was subsequently measured using a UV–Vis spectrophotometer. The formation of the flavonoid–Al(III) complex is primarily influenced by the presence of a carbonyl group at the C4 position and hydroxyl groups at either the C3 or C5 positions of the flavonoid structure. Coordination between the C4=O group and the hydroxyl groups at these positions results in a relatively stable complex (Figure 4). This interaction constitutes the basis of the principal absorbance signal detected in UV–Vis analysis; therefore, flavonoids with C4=O/C3–OH or C4=O/C5–OH configurations generate strong analytical responses.

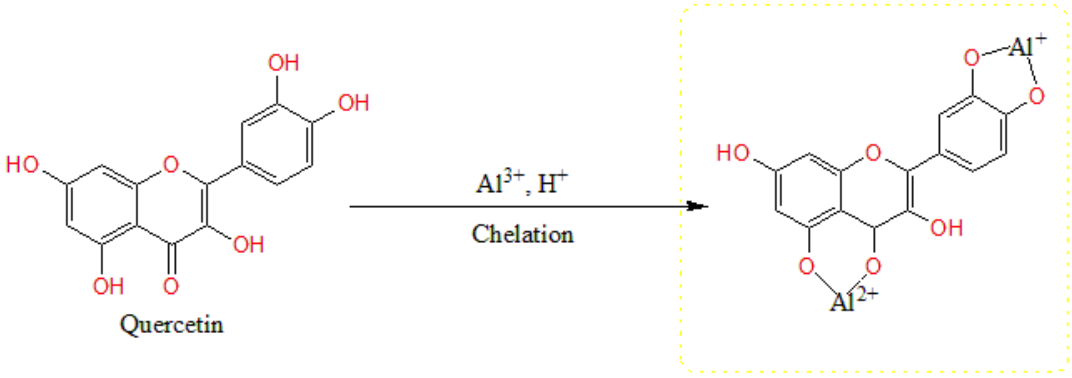

Figure 4. Simple chelation on the molecule of quercetin is possible in three locations on the flavonoid backbone (shown here: 5-hydroxy-chromone and 3′,4′-dihydroxyl groups) [27]

In addition to these interactions, flavonoids containing ortho-dihydroxyl (catechol) groups, particularly on the B ring, are also capable of forming complexes with Al(III). These complexes are more labile to pH variations and are susceptible to degradation when reaction conditions shift toward more alkaline environments (Figure 5). Although their contribution to absorbance is generally weaker than that of C4=O-based complexes, the presence of ortho-dihydroxyl groups enhances the affinity of flavonoids for Al(III) ions, thereby influencing the overall absorbance signal.

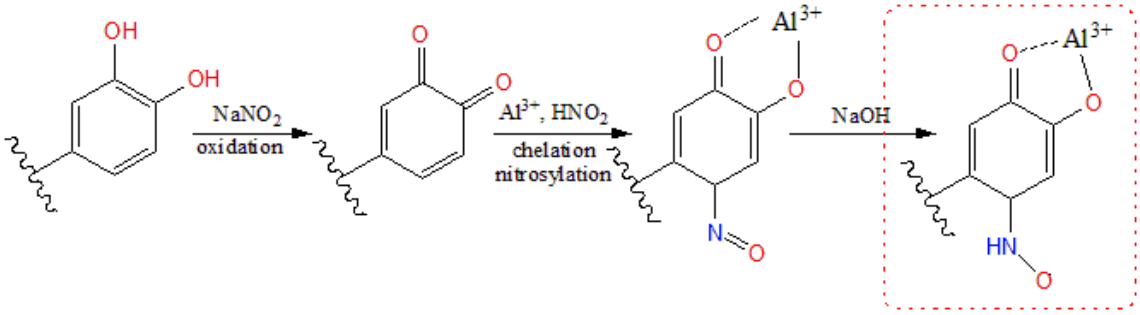

Figure 5. The mechanism of flavonoid-nitroxyl complexation, specific to theo-diphenol moiety [28]

The results of this study showed a maximum absorbance at 431 nm. The standard calibration curve for total flavonoid content yielded a linear regression equation of $y = 0.0292x + 0.1812$ with a coefficient of determination (R^2) of 0.9923, as shown in Figure 6.

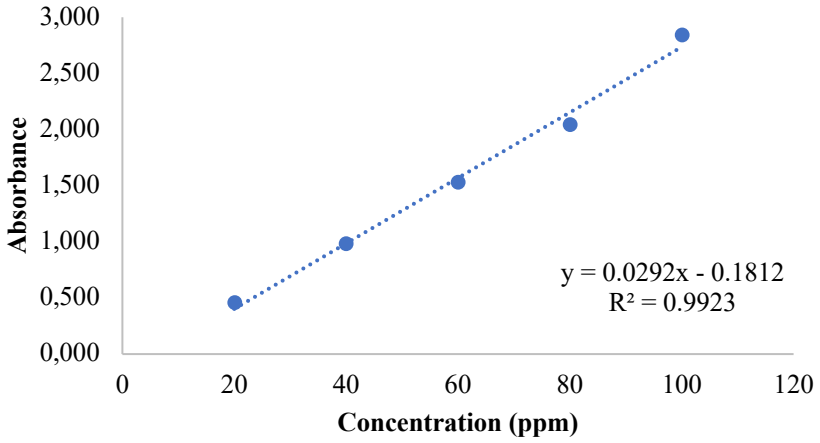

Figure 6. Quercetin standard calibration curve showing linear regression between concentration 10–100 ppm and absorbance at 431 nm (y = 0.0292x + 0.1812 R^2 = 0.9923)

All measurements were performed in triplicate. The results of the total flavonoid content determination are presented in Table 6.

TABLE VI. Total flavonoid content of the extract

Sample	Concentration (ppm)	Mean total flavonoid content (mg QE/g)
Alfalfa leaves	1000	136.25 ± 3.165

The total flavonoid content of *Medicago sativa* (alfalfa) leaf extract determined in this study was 136.25 ± 3.165 mg QE/g extract. This value is considered relatively high compared to several previously reported studies employing the $AlCl_3$ colorimetric method with quercetin as the standard. In a study entitled *Comparison of Various Extraction Techniques of Medicago sativa*, alfalfa leaf extracts were also reported to exhibit high flavonoid content when specific extraction techniques were applied, reaching 139.0 ± 7.1 mg rutin equivalents/g dry matter with supercritical fluid extraction (SFE) using 70% ethanol as the solvent. These findings indicate a phytochemical composition comparable to that observed in the present study [29].

Furthermore, comparisons with other plant species reveal substantial variation in total flavonoid content. For instance, ethanol extracts of *Moringa oleifera* leaves have been reported to contain total flavonoid levels of 155.61 mg QE/g extract, which are slightly higher than those obtained in this study, suggesting that flavonoid profiles are highly dependent on plant species and growth conditions. Conversely, flavonoid analyses of methanolic alfalfa extracts reported in other studies (with total flavonoid contents of approximately 18.55 ± 1.01 mg QE/g extract) showed markedly lower values than those observed here. These differences are most likely attributable to variations in solvent polarity, extraction methods, and the molecular composition of flavonoids present in the analyzed samples [30].

3.5.6. Total Tannin Content

The determination of tannin levels in Alfalfa leaf extract was quantified using a UV-Vis spectrophotometer with the Danish Folin method, with the wavelength set in the visible region. 15% Na_2CO_3 was added to form complex compounds, resulting in a shift in the wavelength towards the visible region. In this test, tannic acid is used as a benchmark because it is a stable natural tannin, and results are usually expressed as tannic acid equivalents (TAE). The principle of the Danish Folin method (Figure 7) is the formation of a blue complex that can be measured at 675 nm. This reagent oxidizes phenolics (alkaline salts) or phenolic hydroxy groups, reducing heteropoly acids (Phosphomolybdate-Phosphotungstate) contained in the Danish Folin reagent into a Molybdenum Tungsten complex [12].

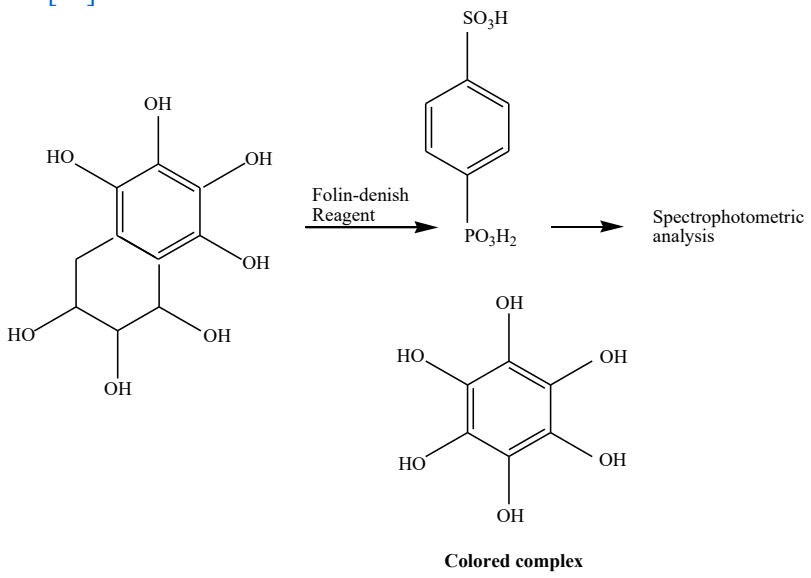

Figure 7. Reaction between Tannic Acid and *Folin-Denish Reagent*

In the determination of tannin content, tannic acid was used as the standard solution. The selection of tannic acid as the reference standard is based on its naturally occurring polyphenolic nature, containing phenolic hydroxyl groups and carboxyl functional groups, and its molecular structure analogous to that of tannins, as well as its widespread distribution in plant materials. The determination of the maximum absorption wavelength revealed a maximum absorbance at 647 nm, at which tannic acid exhibited its highest absorbance. The selection of the maximum wavelength was intended to identify the optimal wavelength at which the tannic acid solution reaches maximum absorbance. Quantification of total tannin content was based on the calibration curve, which yielded a linear regression equation of $y = 0.0151x + 0.3011$ with a correlation coefficient (R^2) of 0.9945 (Figure 8).

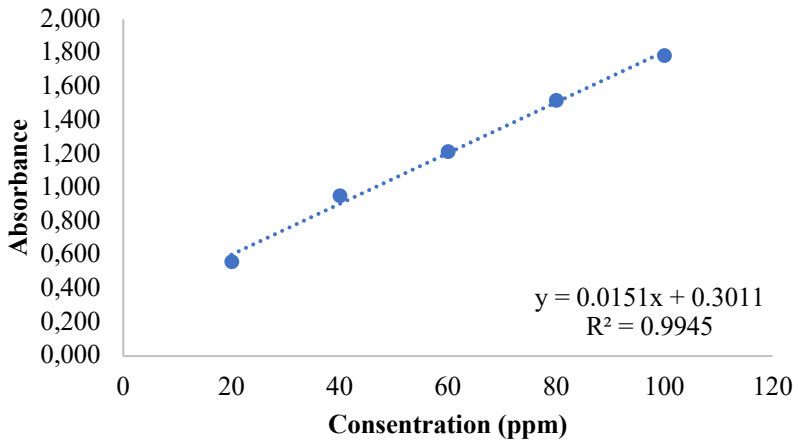

Figure 8. Tannic acid standard calibration curve showing linear regression between concentration 10–100 ppm and absorbance at 675 nm (y = 0.0151x + 0.3011 R² = 0.9945)

All measurements were performed in triplicate to ensure analytical reliability. The results of the total tannin content determination are presented in Table 7.

TABLE VII. Total tannin content of the extract

Sample	Concentration (ppm)	Mean total tannin content (mg TAE/g)
Alfalfa leaves	500	66.49 ± 0.927

The total tannin content of alfalfa leaf extract determined in this study was 66.49 ± 0.927 mg TAE/g extract. This value indicates that *Medicago sativa* leaves are a rich source of tannin-type polyphenolic compounds when extracted using hydroalcoholic solvents. The relatively high tannin content observed is consistent with the phytochemical profile of alfalfa reported in previous studies, which describe the plant as containing substantial amounts of polyphenols, including tannins, flavonoids, and phenolic acids.

Several studies have reported tannin contents in medicinal plant extracts within a comparable range, although variations are commonly observed depending on plant species, extraction solvent, and analytical method. For instance, hydroethanolic extracts of phenolic-rich leaves such as *Psidium guajava*, *Camellia sinensis*, and *Moringa oleifera* have been reported to contain total tannin levels ranging from approximately 30 to 100 mg TAE/g extract when quantified using Folin-based methods. The tannin content obtained in the present study falls within the reported range, suggesting that the extraction conditions applied were effective in solubilizing tannins from alfalfa leaves [31].

4. CONCLUSIONS

The present study demonstrates that the 70% ethanol extract of Medicago sativa leaves

possesses good physicochemical quality and a rich phytochemical profile, as confirmed by FTIR and preliminary screening, which revealed the presence of alkaloids, phenolics, flavonoids, tannins, and triterpenoids. Quantitative analyses, based on calibration curves constructed using gallic acid, quercetin, and tannic acid as reference standards, revealed appreciable levels of total phenolics (19.324 ± 0.814 mg GAE/g), high total flavonoids (136.249 ± 3.165 mg QE/g), and substantial tannin content (66.490 ± 0.927 mg TAE/g). Collectively, these findings indicate that alfalfa leaves contain abundant polyphenolic metabolites with potential biological and pharmacological relevance, providing a strong foundation for subsequent investigations on bioactivity and compound isolation.

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