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Ferric reducing antioxidant parameter (FRAP) activity, separation of the flavonoids fraction and folin-ciocalteau assay of *Achilleaoligocephala*

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Abstract

Background and objective: *Acheliaoligocephala* DC. is recommended by traditional healers of the Kurdistan Region of Iraq for treating wound healing and gastrointestinal complaints. In medicinal applications, *A. oligocephala* leaves are crushed and topically placed over the wound. The aim of the study is to investigate the phytochemistry and biochemical properties of the plant. We describe for the first time the flavonoid fraction was separated by preparative thin layer chromatography from the leaves methanolic and 70% methanolic extracts of *A. oligocephala* and the total poly phenolic content was determined according to the Folin-Ciocalteau assay using gallic acid as the reference standard.

Methods: The present study describes the scientific exploration of the antioxidant activity and preparative thin layer chromatography (TLC) separation of the flavonoids fraction and Folin-Ciocalteau Assay of *Achelliaoligocephala* DC. that has been collected in the Kurdistan Region of Iraq, in which it has been utilized as herbal medicinal remedy.

Results: Interestingly, the methanol extracts of leaves showed significant total phenolic contents (70.05%). The flavonoid contents are expressed as mg quercetin equivalent/g of extracts. However, the methanol extracts of leaves exhibited higher flavonoid contents (61.77%). Additionally, the FRAP (Ferric Reducing Antioxidant Parameter) of the obtained extracts were investigated. The results showed values of 6.565 and 14.443 (mol/L Fe^{2+}/g extract), of methanolic and hydroalcoholic leaf extracts respectively.

Conclusion: The results obtained in the present study indicated *Achelliaoligocephala* can acts as a potential source of flavonoids and antioxidant.

Keywords: *Achelliaoligocephala;* Flavonoids; Antioxidant (FRAP) assay.

Introduction

In many countries, including Iraqi Kurdistan, traditional medicines still play an important role in healthcare practices. Most of the approximately 1,500 plants utilized in Iraq are highly esteemed for their medicinal and fragrant qualities.[1,2]

The genus *Achillea* (yarrows) is a significant genus within the Asteraceae family, consisting of over 100 species that grow naturally in different regions of the world. These species are native to Eastern and Western Asia, Australia, North America, various areas of Europe, New Zealand, and the Middle East regions. The main habitats of this genus are mostly located in various regions of Serbia, Iraq, Iran, Turkey, and Eastern Europe.[3]

The extracts of the non-volatile fractions and volatile oils of the *Achillea* species exhibit antifungal, antibacterial, nutritive, tonic, anti-flatulence, stimulant, antimicrobial, herbicidal activities, anti-cancer and antioxidant activity. Non-volatile fractions as secondary active metabolites involve complex mixtures of natural bioactive compounds with adaptable and different classes of organic compounds representing effective biomedical and medicinal properties.[4]

Achilleaoligocephala is mainly distributed from Iraq-Iran to Turkey, where it is largely

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used in the traditional medicines in several countries. This plant was collected on Halgurd Mountain-Erbil;[5] Kurds used *Achelliaoligocephala* for treating wound healing and gastrointestinal complaints.[6] In medicinal applications, *A. oligocephala* leaves are crushed and topically placed over the wound or the aerial parts are macerated and distilled (with clay pots and goat-hair rope) for infusion or for topic administration as reported by the Baytars (Baytar or Baytall are “traditional doctors”, known as experts in herbal medicine’s uses).

Phenolic compounds found in medicinal and nutritional plants are containing variety of substances such as flavonoids, phenolic acids, coumarins, tannins, lignans, stilbenes, quinones, and other active chemical constituents.[7]

Flavonoids are a prominent and now trendy category of phytochemicals. In order to comprehend the importance of flavonoids in both human health and plant physiology, it is imperative to carry out study on the flavonoid composition found in plant-based meals.[8]

Flavonoids have variety of pharmacological activities including anticancer, antimicrobial, anti-inflammatory, and antioxidant activity, as well as reducing capillary fragility and demonstrating anti-diarrheal capabilities, these effects have been documented.[9]

There are multiple analytical techniques available for studying flavonoids, including Thin Layer Chromatography (TLC) and Capillary Electrophoresis (CE). The analytical potential has been significantly expanded with the implementation of hyphenated HPLC techniques. Gas chromatography (GC) is typically not feasible because of the poor volatility of numerous flavonoid compounds and the requirement to prepare the derivatives. Liga, S. et al.[10] have documented the isolation of chalcones, flavanols, flavones, and flavanones using gas chromatography (GC). The selection of the procedure is contingent upon the polarity of the compounds and the accessible sample amount.[10]

The objective of the study is to examine the non-volatile bioactive components, specifically Flavonoids, found in the leaves of *Achelliaoligocephala*. The total polyphenolic content was measured using the Folin-Ciocalteau test. Additionally, the Ferric Reducing Antioxidant Parameter (FRAP) of the extracted sample was examined for the first time.

Methods

The compounds were obtained from Alfa Aesar, Fluorochem, or Sigma Aldrich and used directly, without further purification. The solvents underwent purification via distillation and were subsequently dried using standard procedures. The experiment involved conducting Thin Layer Chromatography (TLC) using 0.2 mm precoated plastic sheets (Merck LiChroprep RP-C18, 25-40 nm) or Merck silica gel (230-400 mesh). The results were observed using UV examination (254 nm) and staining with 5% H_2SO_4 in methanol, followed by heating. The process of column chromatography was conducted using Merck silica gel 60 with a particle size range of 70-230 mesh, or Merck LiChroprep RP-C18 with a particle size range of 25-40 nm.

Achelliaoligocephala leaves were collected in April 2023 on Halgurd Mountain which belongs to Rawanduz– Erbil, Kurdistan Region – Iraq. The plant has been identified by the botanist “Prof. Dr. Abdulhussain Al-Khayyat” at Salahaddin University-Erbil with a voucher specimen number 7236.[10]

Drying and grinding:

The fresh leaves were subjected to a cleaning process and then dried naturally in a shaded area at room temperature (20-25°C) until they reached a stable weight.

Once the plant component was dried, it was pulverized into a fine powder using a laboratory grinding mill. The resulting powdered materials were then stored in

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brown glass vials at room temperature until they were ready for analysis.

Defatting:

100g of *Achelliaoligocephala* leaves were suspended and defatted with (3x500mL) of petroleum ether, with occasional shaking in an ultrasonic bath (room temperature) for 20 min, then macerated for 3h under continuous stirring at room temperature, the mixture was decanted and filtered.

Extraction with Methanol:

The residue from the defatted leaves was extracted with MeOH (3x500 mL) in an ultrasonic bath for 20 minutes and then left at room temperature for 3 hours under continuous stirring. Subsequently, the mixture was decanted and filtered. The extracts were pooled, and the solvent was removed under vacuum to yield the methanol extract (15.24 g).

Extraction with aqueous Methanol:

After methanol extraction, the filtered biomass leaves were extracted with a mixture of MeOH-H2O (70:30, 3x500 mL), following the procedure previously described for the methanol extract. The dry residue (5.33 g) was transferred to a vial, protected from light and heat sources.

Solid phase extraction (SPE) technique:

Chlorophylls were removed from the methanolic extracts of leaves by dissolving individual batches (each weighing 1 g) of the two residues, mix them separately with 50 mL of a solution containing methanol (80%). Then, running the resulting solution down a solid-phase extraction (SPE) column that contains 100 g of RP-18 phase. The elution process involved using 50 mL of a mixture of methanol and water in a ratio of 80:20, followed by 100 mL of acetone, before proceeding with the separation steps.

Analytical TLC:

The authors have suggested that methanol -water mixtures, with or without TFA, can be employed as effective mobile phases for TLC screening of polar molecules. An optimal initial approach for exploring the polar flavonoid fraction would have involved using a mobile phase composed of a mixture of methanol and water in a ratio of 70:30. Flavonoids are commonly detected using UV radiation at either 254 nm (as all flavonoids result in fluorescence quenching) or at 366 nm (where flavonoids of different structural types exhibit dark yellow, green, or blue fluorescence). The flavonoid fraction, separated via preparative TLC method, was identified under UV light at 254 and 366 nm respectively as light blue fluorescent spots. Subsequently, the desired spots were further identified by being passed through ammonium vapor, resulting in yellowing of the blue fluorescent spots. The spots of interest were then scratched with a stainless-steel spatula, and the scratched Flavonoids containing the stationary phase were filtered with the mobile phase on a micro filter.

Determination of Total Polyphenol Content (Folin-Ciocalteau Assay) (TPC)

The Folin-Ciocalteu reagent consists of a yellow solution containing sodium tungstate ($Na_2WO_4 \cdot H_2O$), sodium molybdate ($NaMoO_4 \cdot H_2O$), lithium sulfate ($LiSO_4$), phosphoric acid (H_3PO_4), and hydrochloric acid (HCl). The spectrophotometric assay was conducted following the method outlined in the referenced literature.[11]

Preparation of Reagents:

Folin-Ciocalteau's reagent: 1 mL of the reagent combined with deionized H_2O up to the mark to make a total volume of 10 mL. The Folin-Ciocalteau reagent kept in a dark bottle away from light. Na_2CO_3 20% (w/v): 20 g of Na_2CO_3 dissolved in 100 mL of H_2O. EtOH 10% (v/v): 10 mL of ethanol concentrate diluted to a final volume of 100 mL with deionized H_2O.[11]

Assay Procedure:

The reaction mixture consisted of 1 mL of the reference standard or sample solution dissolved in 10% ethanol, 6 mL of deionized water, and 500 µL of Folin-Ciocalteau reagent. The mixture was agitated and allowed to undergo a chemical reaction for a duration of 3 minutes. Subsequently, 1.5 mL of

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a solution containing 20% w/v of Na_2CO_3 was introduced. Finally, necessary modifications to the volume were made by adding deionized water until it reached a total of 10 mL.

Securely close the flask and place it in a light-free environment for a duration of two hours at the ambient temperature.

Adjust the concentration of the plant extracts as needed to ensure that their antioxidant levels at the end are within the range of the standard's calibration curve. Obtain absorbance readings at a wavelength of 760 nm using a blank solution containing 10% ethanol (1mL). The calibration curve is represented by the equation:

$$y = 0.0785x + 0.0253 \quad \ldots\ldots\ldots\ldots\ldots \quad Eq\ 1$$

Each sample analyzed twice (Figure 1).

The total polyphenols content in each extract was expressed as gallic acid equivalent (w/w%) and the following formula is used for the calculation:

$$C = c.\ V/m \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad Eq\ 2$$

The formula for calculating the total polyphenolic content (C) in milligrams of gallic acid equivalent (GAE) per gram of dry extract is as follows:

$$C = c * V / m \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad Eq3$$

Here, c represents the concentration of gallic acid obtained from the calibration curve in milligrams per milliliter, V represents the volume of the extract in milliliters, and m represents the mass of the extract in grams.[12]

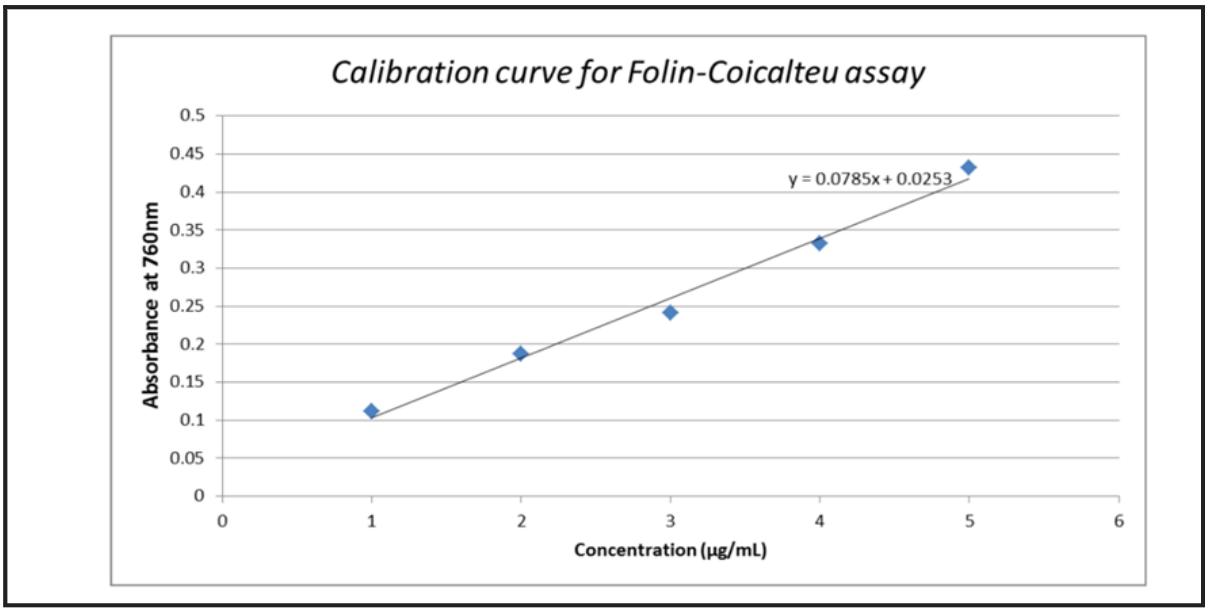

Figure 1 Calibration curve of Folin-Coicalteu for total polyphenol determination

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Determination of Total Flavonoid Content (TFC)

The total flavonoid content was estimated spectrophotometrically.[12,13] A diluted extract (50 mg in 0.1 mL H_2O) was further diluted with distilled water to a final volume of 5 mL. Subsequently, 0.3 mL of 5% $NaNO_2$ was added, followed by 3 mL of 10% $AlCl_3$ in distilled water, 5 minutes later. Following a duration of 6 minutes, a volume of 2 mL of a 1 M NaOH solution was introduced, and the absorbance was then determined at a wavelength of 415 nm. Quercetin used as the reference substance for creating a calibration curve. (Figure 2)

(y = 0.0368x + 0.0017) …..... Eq 4

Where, y= absorbance at 415nm, x= conc. of the TFC corresponds to qurecitine (TFC in mg).

*TFC % average= (TFC in mg) /(original extract mass (50 mg)) *100 ..... Eq 5*

The total flavonoid content (TFC) was quantified in:

mg quercetin equivalent

g of dry sample

The data were presented as the mean ± standard deviation (SD) for duplicate measurements.

Ferric Reducing Antioxidant Parameter (FRAP) Assay:

All the reagents and chemicals utilized in the experiment were of highest purity and analytical grade. They were obtained from reputable suppliers such as Sigma, Merck, Aldrich, Fluka, or Reanal (Budapest, Hungary). The leaves were fragmented into small bits and pulverized using a cool mortar and pestle, together with quartz sand and 9 mL of cool 0.1 M phosphate buffer (pH 7.6) with 0.1 mM EDTA. The mixture underwent filtration using filter paper and was subsequently subjected to centrifugation at a speed of 15,000 revolutions per minute for a duration of 10 minutes. The liquid portion, known as the supernatant, was utilized for the measurements.

The automated technique for quantifying FRAP, also known as the assessment of "antioxidant capacity," was adapted to a manual assay based on the approach described by Varga A.[14]

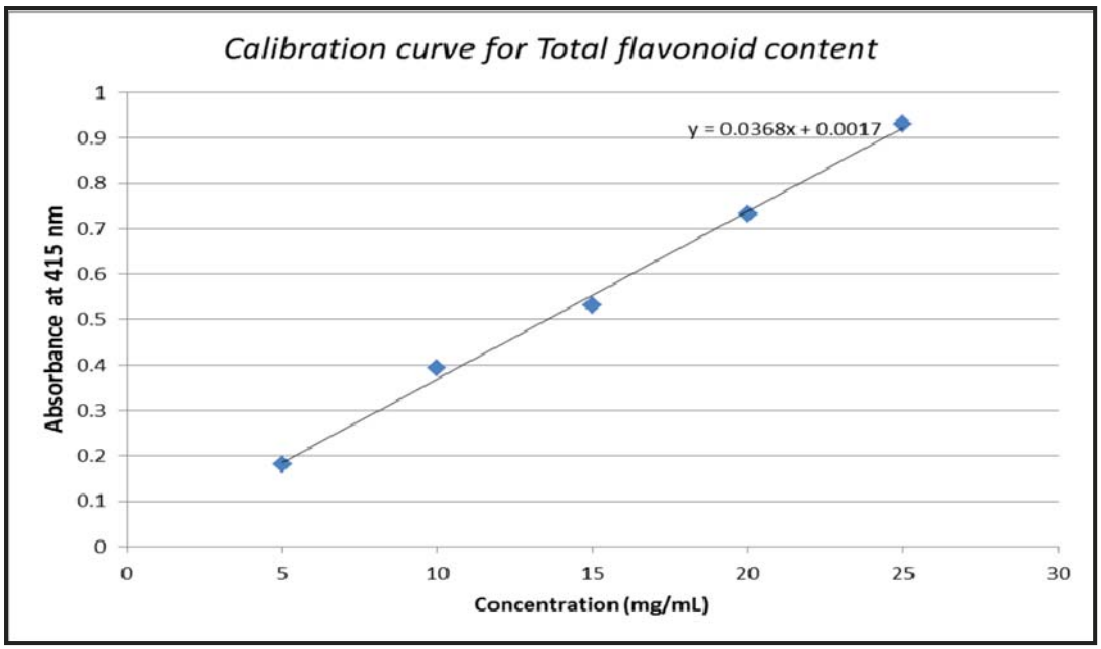

Figure 2 Calibration curve for total flavonoid determination

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Reagents:
Sodium acetate x 3 H_2O
Concentrated acetic acid
2,4,6-tripyridyl-s-triazine (TPTZ)
Hydrochloric acid (HCl)
$FeCl_3$ x 6 H_2O
A calibrated aqueous solution, with a precisely measured concentration of Fe (II), was used for calibration purposes, with a range of 100-1000 µmol/L.

Assays:
The FRAP reagent, along with the volume of the plant extract being 50 mL, and the volume of the plant extract being 1.5 mL, was monitored for a duration of 5 minutes at a wavelength of 593 nm, using a light path of 1 cm and at a temperature of 25°C.

Calculation:
The concentration of the samples was evaluated by comparing them to the activity of Trolox (0.052625 mol/L Fe^{2+}/g Trolox) or L-ascorbic acid, utilizing the calibration curve.

Results

Achelliaoligocephala leaves were collected from Halgurd Mountain in the Kurdistan region of Iraq. The fresh leaves were cleaned and, after drying, the plant material was finely powdered using a laboratory grinding mill. Subsequently, it was defatted with petroleum ether. The residue of the defatted leaves was then extracted with MeOH and MeOH-H_2O (70:30) to obtain extracts weighing 15.24 g and 5.33 g, respectively. Chlorophylls were removed from the methanolic extracts of the leaves by dissolving separate batches (1 g each) of the two residues in 50 mL of MeOH-H2O (80:20) and passing the solution through an SPE column fitted with RP-18 phase (100 g). The recovery yields of the chlorophyll-free extracts were 79.8%. (Figure 3)

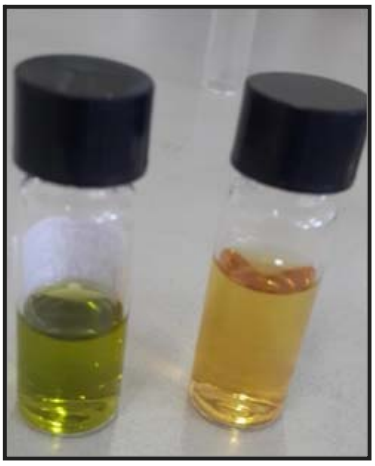

Figure 3 Methanolic extracts of leaves before and after SPE process

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The preliminary phytochemical analysis was performed by using reversed phase TLC chromatography and different solvent systems as eluents. Compounds were detected by fluorescence under UV light and by spraying the plates with vanillin/ H_2SO_4 reagent, followed by heating at 105^{o}C for 1-2 min.

The extracts were further analyzed for the possible presence of flavonoids and other phenolic compounds in chromatographic system: reversed phase C-18 TLC plates eluted with the mixture MeOH-H_2O, 65:35, (Figure 4). The spots were revealed by exposure to NH_3 fumes, followed by spraying with the vanillin reagent and heating at 105 ^{o}C, or by spraying with the $FeCl_3$ reagent (Figure 4 A-B), which is specific for phenolic compounds.

Additionally, the total polyphenol content of the leaf extracts was determined using the Folin-Ciocalteau assay, with gallic acid as the reference standard (Figure 1). The methanol extracts of leaves showed a significant total phenolic content of 70.05% (0.76% S.D) (Table 1).

The flavonoid contents in the studied extracts of *Achelliaoligocephala* were measured by determining the azo-chromophore using the spectrophotometry at the wavelength of 415 nm. The flavonoid contents are expressed as mg quercetin equivalent per gram of dry plant sample. Both leaf extracts exhibited higher flavonoid contents, 61.77% and 53.09%, respectively.

The FRAP assay was conducted to measure the antioxidant power of the methanolic and hydroalcoholic leaf extracts. The results showed values of 6.565 and 14.443 (mol/L Fe^{2+}/g extract), respectively. These findings suggest that the presence of flavonoid fraction in the extracts, to a large extent, contributes to this antioxidant power.

Table 1 Total polyphenolic content of the methanol and methanol 70% extracts of the leaves of *A. oligocephala*

Extract	Polyphenol Content % (S.D)
Methanol extract	70.05 (0.76)
70% methanol extract	63.73 (1.23)

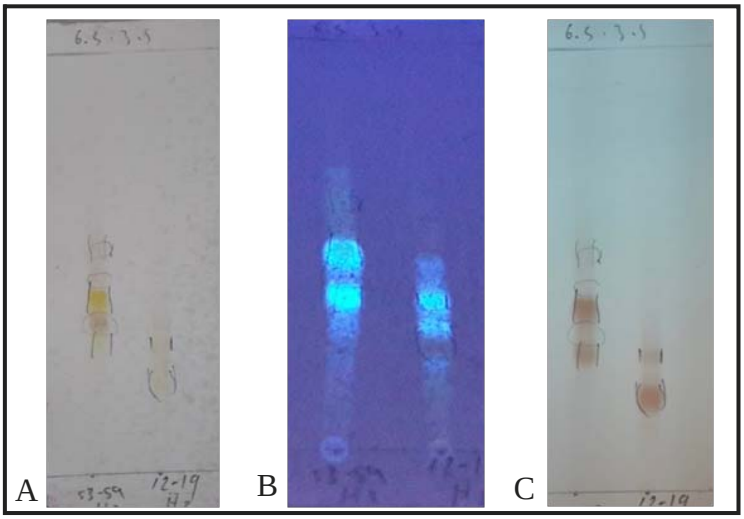

Figure 4 TLC chromatogram of the methanol and methanol 70% extracts of the leaves of *A. oligocephala*. 1: RP phase; MeOH-H_2O (65:35); after reaction with NH_3fumes and spraying with vanillin solution. Observed under UV light (254 nm). 2: RP phase; MeOH -H_2O (65:35); observed under UV light (366 nm). 3: RP phase; MeOH-H_2O (65:35); after spraying with Sulfuric acid 5% solution.

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Discussion

The present study provides the first scientific exploration of the antioxidant activity, preparative thin-layer chromatography separation of the flavonoids fraction, and Folin-Ciocalteau Assay of *Achelliaoligocephala* DC., collected in the Kurdistan Region of Iraq, where it has been utilized as a herbal medicinal remedy.

Solid-phase extraction (SPE) was employed to initially purify *Achelliaoligocephala* extracts by eliminating interfering matrix components, such as chlorophylls and other pigments. These components negatively impacted the clarity of the baseline and posed challenges in accurately identifying and quantifying the desired flavonoids.

Preliminary phytochemical analysis was conducted on methanol and hydro-methanol extracts of *Achelliaoligocephala* leaves using reversed-phase TLC chromatography, employing various solvent systems as eluents. The optimal chromatographic system for separating compounds in methanol extracts was found to be methanol-water (65:35) as the eluent on reversed-phase C18.

Further examination of the extracts was carried out to determine the potential presence of flavonoids and other phenolic compounds. After performing TLC for methanol and methanol-water extracts and exploring various methods as depicted in Figure 4 A-C, the spots were revealed either by exposure to NH_3 fumes or by spraying with the $FeCl_3$ reagent (Figure 4 A-B), which is specific for phenolic compounds. Additionally, spraying with a 5% solution of sulfuric acid was conducted as a final trial. Intense yellow or blue-colored spots were indicative of the presence of flavonoids and phenols, respectively, as observed in Figure 4 A-C. It is evident that both methanol and methanol 70% extracts of *Achelliaoligocephala* contain significant amounts of phenolic and flavonoid compounds.

The antioxidant activity of plant extracts is strongly linked to the concentration of their phenolic components. Therefore, it is essential to consider the influence of the overall phenolic and flavonoid content, as these components demonstrate antioxidant properties in the extracts. M. Amin previously isolated two bioactive compounds from *Achelliaoligocephala*, including (+)-isoorientin, marking the first time this compound was identified in the plant. This supports our findings regarding the flavonoid and phenolic content of the plant, as well as previous research on its bioactivity. The isolated compounds exhibited notable cytotoxic activity, demonstrating stronger inhibition effects than cisplatin against the MCF7 human tumor cell line. They also showed significant total antioxidant activity compared to standard antioxidants.[6]

We observed that both extracts displayed elevated polyphenol and flavonoid levels. These results indicate that the presence of the flavonoid fraction in the extracts significantly contributes to their antioxidant potency.

Conclusion

This is the first study to evaluate the Ferric Reducing Antioxidant Power (FRAP) activity, spectroscopic determination of total polyphenols and flavonoids, and preparative TLC separation of the flavonoid fraction in *Achelliaoligocephala*, a plant species growing in the Kurdistan region of Iraq. The study demonstrated that the leaves of *Achelliaoligocephala* contain significant amounts of phenols and flavonoids. This research highlights the use of thin-layer chromatography in the analysis, detection, and separation of flavonoids in the traditional herbal medicine *Achelliaoligocephala*. The flavonoid fraction was identified, and its percentage was estimated using the Folin-Ciocalteu assay. Additionally, the FRAP assay was conducted to assess the antioxidant capacity of the leaf extracts, concluding that the flavonoid fraction, which is a major

component of the extract, may be responsible for the observed antioxidant activity.

Competing interests

The author declares that he has no competing interests.

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