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Comprehensive Analysis of Phytochemical Composition, Antioxidant Potential, and Antibacterial Activity of *T. polium*

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Abstract: This study aims to thoroughly examine the chemicals and effects of the ethanol extract from *T. polium*'s upper parts. We used the Soxhlet method for extraction, resulting in an extract with a significant yield of 20.6%. Qualitative analysis identified a variety of compounds, such as tannins, saponins, reducing compounds, terpenoids, quinones, and alkaloids. In quantitative analysis using the colorimetric method, we found the extract was rich in total flavonoids (20.78 mg equivalent QE/g DW extract) and total polyphenols (227.43 mg equivalent GAE/g DW extract). To assess antioxidant potential, we used the ferric reducing antioxidant power (FRAP) method, with ascorbic acid and butylated hydroxytoluene (BHT) as standards. The extract showed moderate activity in both the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and FRAP methods at concentrations of 65 µg/mL and 21 mg/mL, respectively. Additionally, we tested the ethanolic extract against various bacteria using the disk diffusion technique on agar medium. The results indicated that the *T. polium* extract had moderate effectiveness against Gram-negative bacteria like *Pseudomonas aeruginosa ATCC 9027* and *Escherichia coli ATCC 8739*, as well as Gram-positive bacteria like *Staphylococcus aureus ATCC 6538* and *Bacillus subtilis ATCC 6633*. We further investigated the composition of the ethanolic extract through LC-MS/MS analysis, establishing a detailed profile of phenolic compounds, with six flavonoids identified as the main polyphenolic constituents. This thorough evaluation provides insights into the potential therapeutic uses of *T. polium*.

Keywords: *T. polium*; total flavonoids; total polyphenols; biological activities; LC-MS/MS

1. Introduction

Phytotherapy involves using plants or herbs as medicine to prevent or treat diseases in both humans and animals. The historical use of plants for healing goes way back, and there

is a growing interest in studying these medicinal plants globally. People commonly use whole plants or extracts with various therapeutic and biological activities. Phytotherapy looks into the use of naturally derived extracts for medicine or promoting health. *T. polium*, an ancient medicinal plant, is commonly found in the Mediterranean regions. Belonging to the Labiatae or Lamiaceae family [1], this genus comprises over 300 species [2], typically aromatic and growing wildly in various parts of the world [3]. Abundantly present in Southwest Asia, Europe, and North Africa [4], it is renowned for its extensive varietal diversity of medicinal and aromatic plants, especially in semi-arid, arid, and Saharan regions [5]. *T. polium*, also known as *tomentose germander*, is a Mediterranean plant [3], appearing whitish, perennial, and herbaceous, with enduring flowers. It can reach a height of 10 cm to 30 cm and emits a pronounced odor. The stems are numerous, woody at the base, revolute, and more or less branched. The leaves are sessile, oblong, or linear", with pale green edges above and white below. The flowers are white or yellowish, globose or ovoid, with a small (3 mm to 4 mm) bell-shaped calyx, almost equally triangular short teeth, and a very hairy texture [4].

Current research on the chemical composition of *T. polium*, based on chromatographic analysis of the extracts [6], reveals that this genus is composed of various chemical substances. It is rich in secondary metabolites such as polyphenols, flavonoids, tannins, and coumarins, well known for their therapeutic properties. This plant has been utilized for centuries to treat inflammatory [7], and cardiovascular diseases [8]. Additionally, it has been employed as a hypoglycemic agent for diabetic patients [1] and for addressing gastrointestinal and anti-ulcer problems [9]. Though extensive research has investigated the effects of *T. polium* against different pathological situations in various organs [10], to the authors knowledge, there is limited research in Algeria evaluating the biological properties of the ethanolic extract of the *T. polium* plant.

The aim of this study is to deepen the understanding of the chemical composition and biological activities inherent in the ethanolic extract of the aerial parts of *T. polium*. The extraction method used, based on the Soxhlet principle, demonstrated a noteworthy yield of 20.6%. A comprehensive qualitative analysis was conducted to identify various compounds, including tannins, saponins, reducing compounds, terpenoids, quinones, and alkaloids. Subsequently, a meticulous evaluation of antioxidant power was carried out using the FRAP and DPPH methods, providing in-depth insights. Simultaneously, antibacterial activity was scrutinized, targeting both Gram-positive and Gram-negative bacteria. These rigorous methodologies established solid foundations, thus paving the way for in-depth discussions on the obtained results and further exploration of the properties of the *T. polium* extract.

In this study, the aerial parts of *T. polium* were meticulously harvested in the Wilaya of Bouira, Algeria during the flowering season. After a 15-day air-drying period, followed by storage at room temperature, the plant material was finely ground to obtain a powder. The ethanolic extract was prepared by extracting 0.5 g of plant material with 5 mL of 96% ethanol using a Soxhlet apparatus, followed by vacuum evaporation at a constant temperature of 40 °C. Phytochemical characterization was conducted using solvents of different polarities, with specific tests to identify compounds such as saponins, alkaloids, polyphenols, flavonoids, tannins, reducing compounds, terpenoids, quinones, and coumarins. Precise assays of total polyphenols and total flavonoids were performed, and antioxidant activity was assessed using the DPPH and FRAP methods. Antibacterial activity was tested against four bacterial strains, and quantitative analysis by LC-MS/MS was conducted to characterize the phenolic compounds in the crude extract of *T. polium*. This comprehensive methodological approach provides a thorough understanding of the phytochemical composition and the antioxidant and antibacterial properties of this plant.

This study introduces a groundbreaking methodological paradigm for the investigation of *T. polium* aerial parts collected in the Wilaya of Bouira, Algeria. The innovation is manifest in the meticulous curation of plant material, executed with precision during the flowering season, followed by a judicious air-drying period and storage regimen to uphold

material integrity. The extraction process, utilizing a Soxhlet apparatus and subsequent vacuum evaporation at a controlled temperature, underscores a commitment to methodological excellence. Phytochemical characterization, employing solvents of diverse polarities and employing specific tests, offers an exhaustive analysis of a spectrum of compounds, including saponins, alkaloids, polyphenols, flavonoids, tannins, reducing compounds, terpenoids, quinones, and coumarins. The study's quantitative dimension, through assays for total polyphenols and total flavonoids, coupled with the meticulous evaluation of antioxidant potential using DPPH and FRAP methods, contributes to a nuanced understanding of the plant's chemical composition and antioxidative capabilities. Antibacterial assays against four bacterial strains and the inclusion of quantitative LC-MS/MS analysis further elevate the study, unraveling the antibacterial efficacy and intricate phenolic compound profile of *T. polium*. In essence, this pioneering methodological approach establishes a new benchmark in unraveling the phytochemical intricacies of *T. polium*, advancing our comprehension of its potential applications in antioxidant and antibacterial domains.

2. Materials and Methods

2.1. The Harvest and the Preparation of the Plant

The aerial parts were harvested in the Wilaya of Bouira during the flowering season, which occurs from May to June 2020, at an altitude of 525 m (36°22′00″ N, 3°53′00″ E). Samples were identified and authenticated at the botanical laboratory of the agriculture department (University of Blida 1, Algeria), in collaboration with the Herbarium of National Higher Agronomic School, El-Harrach, Algiers.

Afterwards, the collected plant material underwent a drying process in open air.It was placed in the shade for 15 days to protect it from humidity and then kept at ambient temperature for an additional two weeks. Following the drying period, the aerial parts of the plant were finely ground using a knife grinder. The resulting plant powder was carefully stored in clean bags in a dark environment at room temperature (25 °C ± 2 °C) until it was needed.

2.2. Preparation of the Ethanolic Extract

The ethanolic extract is obtained by subjecting 0.5 g of the plant material to extraction with 5 mL of 96% ethanol using a Soxhlet apparatus. The resulting extract is then subjected to vacuum evaporation using a Heidolph type rotary evaporator, with the temperature kept at 40 °C throughout the process. This is similar to the method described by Chirane et al. [11], with minor modifications.

2.3. Phyto-Chemical Screening

To identify the main chemical groups (secondary metabolites) in the plant, we used solvents with different polarities, namely, water and ethanol. The method either formed insoluble complexes through precipitation reactions or produced colored complexes through specific color reactions [12]. Table 1 shows the procedure for the qualitative determination of phytochemicals present in plant samples.

Table 1. Methods of phytochemical screening of the aerial part of *T. polium*.

Compounds	Processes	Comments	References
Saponosides	2 mL of the aqueous extract + 5 drops of distilled water under vigorous agitation	Formation of a stable, persistent foam (height greater than 1 cm)	[13]

Table 1. *Cont.*

Compounds	Processes	Comments	References
Alkaloids	1 mL of 10% sulfuric acid extract + 3–5 drops of Mayer's reagent	A yellowish-white precipitate	[12,14,15]
	1 mL of 10% sulfuric acid extract + 2–5 drops of Bouchardat's reagent	A brown precipitate	
	1 mL of 10% sulfuric acid extract + 5 drops of Dragendorff's reagent	An orange-red precipitate	
Polyphenols	2 mL of aqueous extract plus 1 drop of 2% ferric chloride solution in alcohol	Dark blue-blackish or green color	[12]
Flavonoids	10 mL of the 1% HCl extract, make it basic with NaOH	A yellow-orange color	[14]
Free flavonoids (genin)	5 mL of 1% HCl extract + 2.5 mL of amyl alcohol (pentanol)	The alcoholic phase is colored yellow	[13]
Anthocyanins	Sulfuric acid solution + 5 mL of aqueous extract + 5 drops of 25% NH_4OH ammonium hydroxide	Red color in an acid medium and purplish-blue in a basic medium	[13,14]
Tannins catechists Gallic tannins	1 mL of the aqueous extract + 0.5 mL of Stiasny's reagent + heating in a water bath at 90 °C for 15 min	A light pink precipitate	[13]
	Saturation of 0.5 mL of the filtrate with 10 mL of 1% sodium acetate, + 100 µL of a 1% $FeCl_3$ solution	A blue-black hue	
Reducing compounds	5 mL of aqueous extract + 5 mL of Fehling's liquor. + Heat in a bain-marie at 70 °C for 2–3 min	A brick red precipitate	[15]
Free anthraquinones	5 mL of a 20% ammonia (NH_4 OH) solution + 10 mL of the ethanolic extract + stirring	More or less a red color	[12]
Terpenoids	1 mL of ethanolic extract + 0.4 mL of chloroform ($CHCl_3$) + 0.6 mL of concentrated H_2SO_4	Appearance of a brown ring at the interphase	[16]
Quinones	7 mL petroleum ether extract + a few drops of 10% NaOH	A yellow, red, or purple color	[13]
Coumarins	2 mL of the aqueous extract + 3 mL of 10% NaOH with stirring	A yellow color	[13]

2.4. Assay of Total Polyphenols

The quantification of polyphenols, utilizing the Folin–Ciocalteu method pioneered by Slinkard and Singleton [17], is a robust approach to assess the total polyphenolic content in a given sample [18]. This method relies on the reduction of the phosphotungstic (WO_4^{2-}) phosphomolybdic (MoO_4^{2-}) mixture in Folin's reagent in an alkaline medium, facilitated by the oxidizable groups of phenolic compounds. This chemical reaction results in the formation of reduction products characterized by a distinctive blue color [19].

To quantify total polyphenols, we employed a modified version of a previously described method by Chirane et al. [11]. In brief, 0.25 mL of the ethanolic extract from the aerial part was mixed with 1.25 mL of 10% (*v*/*v*) Folin–Ciocalteau reagent. After a 3 min reaction, 1 mL of a sodium carbonate solution (Na_2CO_3) with a concentration of 75 g/L was added. Following a 30 min incubation in the dark at a temperature of 25 °C ± 2 °C, absorbance was measured at 765 nm using a UV spectrophotometer (Hitachi U 5100, Hitachi, Japan).

Polyphenol concentrations were determined by referencing the calibration curve, which was established using a standard gallic acid polyphenol at concentrations ranging

from 0 µg/mL to 125 µg/mL. The results are expressed in milligrams of gallic acid equivalent per gram of dry weight extract (mg Eq GAE/g DW). To ensure the results' reliability, the tests were performed three times for reproducibility.

2.5. Assay of Total Flavonoids

The determination of the total flavonoid content in the ethanolic extract of the aerial parts involved using the aluminum trichloride (AlCl3) method [20].The quantification of total flavonoids followed a method described by Hayat et al. [21], with minor modifications. Specifically, 2 mL of both the extract and standard were combined with 0.1 mL of AlCl3 solution (10%) (*m*/*v*), 0.1 mL of sodium acetate (1 M), and 2.8 mL of distilled water. The resulting mixture was stirred and incubated for 1 h in the dark at 25 °C ± 2 °C. Following incubation, absorbance was measured at 415 nm using a UV spectrophotometer (Hitachi U 5100, Hitachi, Japan).

Flavonoid concentrations were determined based on the calibration curve established with a standard flavonoid (quercetin) at concentrations ranging from 0 µg/mL to 20 µg/mL. The results are expressed in milligrams equivalent to quercetin per gram of dry weight extract (mg Eq QE/g DW). To ensure the reproducibility of the results, the tests were conducted three times.

2.6. Antioxidant Activity

2.6.1. DPPH Method

The assessment of antioxidant activity focused on the ethanolic extract of *T. polium* and the standard antioxidant, ascorbic acid, against the DPPH radical (diphenyl picrylhydrazyl). This analysis utilized a spectrophotometer at 517 nm, monitoring the reduction of the radical and its transition from violet (DPPH•) to yellow (DPPH-H). The intensity of this color change is inversely proportional to the ability of antioxidants in the medium to donate protons [22,23]. The reduction capacity was determined by tracking the decrease in the free radical concentration and absorbance during the reaction until the hydrogen-donating antioxidant was depleted [18,19]. To assess the anti-radical activity of various concentrations of the ethanolic extract from *T. polium*, we employed a method using DPPH as a relatively stable free radical, following a documented protocol described by Meriga et al. [24] with minor adjustments. Specifically, 200 µL of the ethanolic extract solution at various concentrations (0 µg/mL, 20 µg/mL, 40 µg/mL, 50 µg/mL, 70 µg/mL, 90 µg/mL, and 100 µg/mL) was added to 800 µL of the DPPH solution (0.004% prepared in methanol).

Simultaneously, a negative control was prepared by combining 200 µL of methanol with 800 µL of the methanolic DPPH solution. Following a 30 min incubation in the dark at 25 °C ± 2 °C, the absorbance reading was taken against a blank prepared for each concentration at 517 nm. The positive control involved a solution of a standard antioxidant, ascorbic acid, with absorbance measured under the same conditions as the samples. For each concentration, the percentage inhibition was calculated using Equation (1):

$$\text{PI (\%)} = ((\text{A control} - \text{A sample}))/(\text{A control}) \times 100 \quad (1)$$

2.6.2. FRAP Method

The ferric reducing antioxidant power (FRAP) assay is a crucial method for assessing antioxidant capacity, as the reducing power of an extract is closely correlated with its ability to counteract free radicals. This method measures the extract's capacity to convert ferric iron (Fe^{3+}) within the $K_3Fe(CN)_6$ complex to ferrous iron (Fe^{2+}) [25]. The reduction is quantified by the enhancement in the intensity of the blue color in the reaction medium at 700 nm [26].

The determination of the FRAP in the tested extracts followed a method described by El Atki et al. [27], with certain adjustments. Specifically, 200 µL of the extract was combined with 500 µL of phosphate buffer (0.2 M at pH 6.6) and 500 µL of potassium ferricyanide [$K_3Fe(CN)_6$] at a concentration of 1%. After incubating the solution at 50 °C for 20 min,

it was acidified with 500 µL of 10% trichloroacetic acid (TCA) and then centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 500 µL of distilled water and 100 µL of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. BHT was used as the standard. An increase in absorbance corresponds to an elevation in the reducing power of the tested extract [11].

2.7. Antibacterial Activity

2.7.1. Bacterial Material

To evaluate the antibacterial potential of the ethanolic extract from *T. polium*, we used four bacterial strains commonly associated with various human pathologies. Among the Gram-negative strains were *Pseudomonas aeruginosa ATCC 9027* and *Escherichia coli ATCC 8739*,whereas the Gram-positive strains included *Staphylococcus aureus ATCC 6538* and *Bacillus subtilis ATCC 6633*. These bacterial strains, purified, identified, and referenced, were provided by the hygiene laboratory.

2.7.2. Sensitivity Test

The sensitivity testing of bacteria exposed to our plant extract utilized the disc diffusion method on Mueller Hinton agar medium [28]. Sterile Mueller Hinton medium, 15 mL per dish, was poured into sterile Petri dishes. After cooling, 1 mL of an 18 h bacterial inoculum, adjusted to 0.5 McFarland (optical density, OD, ranging from 0.08 to 0.10; measured at a wavelength of 625 nm, corresponding to 10^8 CFU/mL, colony-forming units), was evenly spread across the entire surface of the Mueller Hinton medium [29,30].

Sterile 6 mm diameter Whatman N°1 paper disks were impregnated with the crude extract and various dilutions of the same ethanolic extract within the range (25 mg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL, 150 mg/mL, 200 mg/mL, 300 mg/mL, and 400 mg/mL). Using sterile forceps, the disks were gently placed on the surface of the Mueller Hinton agar medium. Following a 30 min pre-diffusion at 25 °C ± 2 °C, the Petri dishes were incubated at 37 °C for 24 h. The extract is considered active if it induces a zone of inhibition greater than 10 mm around the impregnated disc [3]. Each experiment was replicated three times under the same conditions. Additionally, under identical conditions, two reference antibiotics, gentamycin and tetracycline (100 mg), selected for their broad spectrum of action, and discs impregnated with a dimethyl sulfoxide solution (negative control) were placed in separate boxes.

2.8. LC-MS/MS Analysis

Quantitative analysis of phenolics of the *T. polium* crude extract was carried out by LC-MS/MS (Agilent Technologies 1260 Infinity II, 6460 Triple Quad Mass spectrometer). A Poroshell 120 SB-C18 (3.0 × 100 mm, I.D., 2.7 µm) column was employed following Erenler et al. [31]. In this process, 50 mg samples were transferred into 2 mL Eppendorf tubes, and 2 mL of ethanol were added to the resulting solution, which was then stirred. The mixture underwent extraction using hexane, followed by centrifugation at 9000 rpm for 10 min. From the methanol phase in the resulting solution, a 100 µL sample was extracted and diluted to 900 µL using a mixture of 450 parts water and 450 parts methanol.

The resulting diluted sample was filtered and analyzed by LC/MS-MS with a 5.12 mL injection volume, 0.400 mL/min flow rate, 30 min processing time, formic acid (0.1%) and ammonium formate (5.0 mM) in water A. Formic acid (0.1%) and ammonium formate (5.0 mM) in methanol B were used for the mobile phase. The gradient program for the B mobile phase was adjusted as 25% for 1–3 min, 50% for 4–12 min, 90% for 13–21 min, and 3% for 22–25 min The column temperature was 40 °C. The capillary voltage was 4000 V, the nebulizing gas (N2) flow was 11 L/min, the pressure was 15 psi, and the gas temperature was 300 °C.

3. Results

3.1. Phyto-Chemical Screening

The identification of chemical compounds was achieved through a careful examination of coloration reactions, which involved noticeable shifts in color, and precipitation reactions induced by reagents specifically designed for each group of compounds. Our comprehensive phytochemical screening revealed the prevalence of major chemical compound families within the plant tissues of *T. polium*, and a detailed breakdown is provided in Table 2.

Table 2. Results of phyto-chemical screening of the aerial part of *T. polium*.

Phytochemicals			Results
Saponosides			+ presence
Alkaloids			+ presence
Polyphenols			+ presence
Flavonoids	Flavonoids		+ presence
	Free flavonoids		+ presence
Anthocyanins			- absence
Tannins		Gallic tannins	+ presence
		Catechic tannins	+ presence
Reducing compounds			+ presence
Free anthraquinones			- absence
Terpenoids			+ presence
Quinones			+ presence
Coumarins			+ presence

This extensive screening revealed the presence of various compounds, including saponins, alkaloids, polyphenols, flavonoids, tannins, reducing compounds, terpenoids, quinines, and coumarins. These results align with previous investigations conducted by Hammoudi et al. [32] and Abdollahi et al. [5] and are also in agreement with those of Ansari et al. [33], Asghari et al. [1], Malki et al. [34], and Venditti et al. [35]. Importantly, the test also confirmed the absence of specific bioactive substances such as anthocyanins and free anthraquinones.

From these comprehensive findings, we infer that the studied plant possesses a rich reservoir of secondary metabolites. The specific components identified in its extract play a pivotal role in shaping therapeutic and biological properties [36], particularly in terms of antioxidant and antibacterial activities. This recognition has fueled increased interest and attention from researchers dedicated to unraveling the potential of this plant.

3.2. Assay of Total Polyphenols

The assay results emphasize the abundance of polyphenols in the ethanolic extract derived from the aerial parts of the *T. polium* plant, reaching a content of approximately 227.43 mg Eq GAE/g DW extract. The results of the gallic acid calibration curve are depicted in Figure 1.

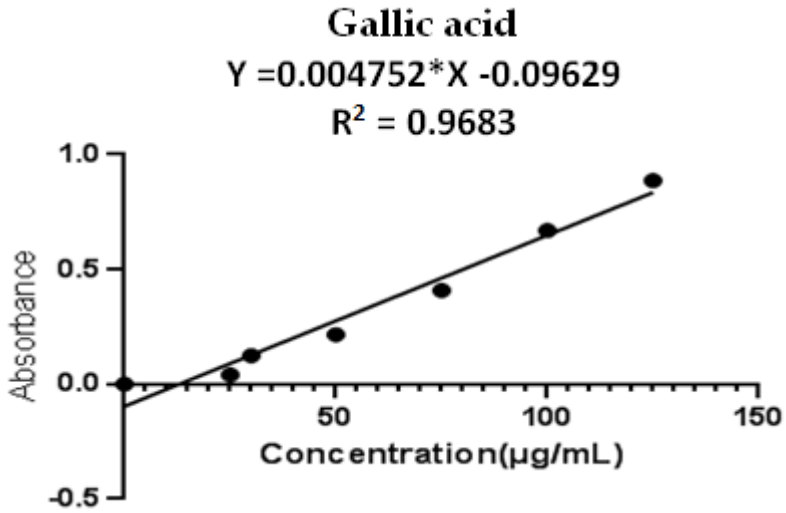

Figure 1. The gallic acid calibration curve.

This concentration of total phenols surpasses the values reported by Goulas et al. [37], i.e., 164 mg Eq FA/g of extract and 144 mg Eq FA/g of extract in aqueous and methanolic extracts, respectively. Similarly, the polyphenol assay values of the ethanolic extract measured by El Atki et al. [27] and by Ardestani et al. [38] was 112.27 ± 4.75 mg Eq FA/g of extract and 180.2 ± 7.5 mg Eq FA/g of extract, respectively, which are lower than those recorded in our study. However, the value measured in the present study significantly exceeds the contents noted by El Atki et al. [27] and Hammoudi et al. [32], 4.38 ± 0.12 mg Eq FA/g of extract and 89.05 ± 0.5 mg Eq FA/g of extract, respectively, in ethyl acetate extracts.

This research highlights the dynamic qualitative and quantitative variations in polyphenolic content among different plants [5]. These variations are influenced by factors such as the extraction method, the nature of the solvent [11], specifically the polarity of the solvent used in the extraction [38], and variables like the harvest period and the plant's developmental stage, all contributing to the nuanced estimation of total polyphenol content [25].

3.3. Assay of Total Flavonoids

The analysis revealed that the ethanolic extract of the aerial parts of the *T. polium* plant contains a total flavonoid content of 20.78 mg Eq QE/g DW extract. The results of the quercetin calibration curve are shown in Figure 2.

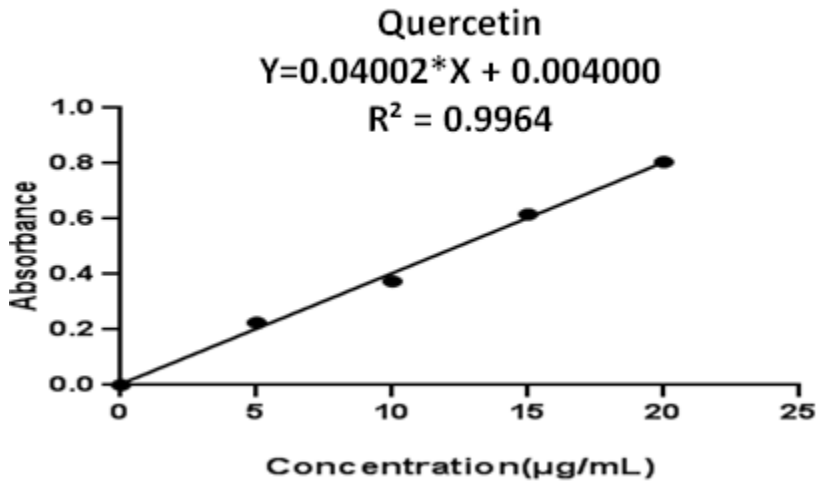

Figure 2. The quercetin calibration curve.

Our recorded flavonoid concentration was lower than that reported by El Atki et al. [27], indicating 174.54 mg Eq rutin/g of extract for the ethanolic extract of a Moroccan species. In contrast, Chabane et al. [39] found a content of around 24.43 ± 0. mg Eq QE/g DW extract for a methanolic extract of an Algerian species, which is higher compared to our result. Additionally, Aouadhi et al. [40], studying a similar extract but with a Tunisian species, reported a very low value of 2.67 mg eq catechin/mL.

As per existing literature, the concentration of flavonoids in plant extracts is influenced by various factors, including the polarity of the solvents used in the extraction process [41], the geographical region of origin [21], and the type of standard employed, potentially leading to variations in results (quercetin, catechin, and rutin) [38].

3.4. Antioxidant Activity

3.4.1. DPPH Method

In Table 3, the inhibition values indicated approximately 65 µg/mL for the ethanolic extract of *T. polium* and 12.5 µg/mL for ascorbic acid. Notably, the percentage inhibition of ascorbic acid surpassed that of the ethanolic extract across all concentrations. Antioxidant molecules, such as ascorbic acid, tocopherol, flavonoids, and tannins, have demonstrated their ability to reduce and decolorize DPPH due to hydrogen donation.

Table 3. Antioxidant activity expressed by the IC_{50} (µg/mL).

Samples	IC_{50} (µg.mL^{-1})
Ethanolic extract	65
Ascorbic acid	12.5

Comparatively, the antioxidant activity against the DPPH radical in extracts from *T. polium* species with different geographical origins was lower than that of our species. Studies have reported the highest antioxidant activity in polar solvent extracts [27]. Research by Aouadhi et al. [40] and Sharififar et al. [42] on the species *T. polium* cultivated in Tunisia and Iran revealed IC_{50} values of around 21 ± 0.5 µg/mL and 20.1 ± 1.7 µg/mL, respectively, for the methanolic extract. Ardestani et al. [38] and Panovska et al. [43] found an IC_{50} value of 9.8 µg/mL and 10.0 mg/mL, respectively, while studying the antioxidant effect of the ethyl acetate extract of *T. polium* species from Iran and Macedonia. Additionally, El Atki et al. [27] and Ardestani et al. [38] reported IC_{50} values of 0.397 ± 0.042 mg/mL and 48.9 µg/mL, respectively, for the ethanolic extract of *T. polium* from Morocco and Iran. These studies suggest that extracts of *T. polium*, rich in phenolic compounds, are likely responsible for the antioxidant activity [19]. Furthermore, this activity is proportional to the results of assays for total polyphenols and flavonoids, indicating that extracts with higher polyphenolic content exhibit enhanced antiradical activity. This correlation is also influenced by the polarity of the extraction [21]. The studies of El Atki et al. [27] and Popovici et al. [44] have confirmed the association between the content of phenolic compounds and anti-radical activity.

3.4.2. FRAP Method

The obtained results, detailed in Table 4, reveal that the ethanolic extract of *T. polium* exhibits a notable ability to reduce ferric ions (Fe^{3+}), yielding an IC_{50} value of 21 mg/mL. However, it is noteworthy that this reducing power is comparatively lower than that of the synthetic antioxidant BHT, which exhibits a superior IC_{50} value of 0.108 mg/mL. Previous studies consistently affirm the robust reducing power of *T. polium* [45].

Table 4. Power reducing expressed by the IC_{50} (mg/mL).

Samples	IC_{50} (mg/mL)
Ethanolic extract	21
BHT	0.108

The IC_{50} values reported by El Atki et al. [16], 0.193 ± 0.006 mg/mL for the methanolic extract and 0.381 ± 0.009 mg/mL for the ethanolic extract of *T. polium* subsp aurum, underscore a higher reducing power than that observed in this study. Conversely, Khaled-Khouja et al. [45], through examining the methanolic extract of *T. polium*, report a lower IC_{50} value of 414.65 mg AAE/g extract, indicating reduced reducing power compared to our findings. Dridi et al. [46], in their assessment of the methanolic extract, report a significantly higher IC_{50} value of 32 µg/mL, denoting superior reducing power compared to our study.

Extracts demonstrating potent reducing power typically exhibit lower IC_{50} values, indicative of a high content of phenolic compounds [47]. The observed reducing power of *T. polium* likely stems from the presence of a hydroxyl group in the phenolic compounds, facilitating electron donation [26]. Therefore, we can infer from this analysis that polyphenols, particularly flavonoids, play a pivotal role in chelating transition metals involved in the Fenton reaction, which generates hydroxyl radicals through the reaction of iron with hydrogen peroxide [47].

3.5. Antibacterial Activity

The determination of the zone of inhibition is a critical aspect of evaluating the sensitivity or resistance of bacterial strains to the tested extract. After a 24 h incubation period, the diameters of the inhibition zones were measured precisely. The results, as detailed in Table 5 and Figure 3, highlight the considerable efficacy of the crude extract against Staphylococcus aureus and *Pseudomonas aeruginosa* (see Figure 3j,n), displaying substantial inhibition zones of 20 mm and 16 mm, respectively. This broad-spectrum inhibitory activity extended across both Gram negative and Gram-positive bacteria.

In contrast, the various concentrations of the extract exhibited diverse effects on the tested strains. Their actions varied, demonstrating significant effectiveness against Staphylococcus aureus with a 13 mm inhibition zone at 300 mg/mL, shown in Figure 3i, and intermediate effects on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (11 mm, 11 mm, and 10 mm, respectively) at 400 mg/mL, shown in Figure 3i,m,r. The antibacterial activity of *T. polium* has been mostly studied using the organic extracts of plant aerial parts [48]. Hammoudi et al. [32] found a significant and notable result from the butanolic extract of *T. polium* on seven strains studied. Similarly, Fettah et al. [28] found a high diameter of the inhibition zone for the butanolic extract of *T. polium*. Essawi and Srour [49] screened the antibacterial activity of aqueous and organic extracts of *T. polium* aerial parts against eight bacterial strains and showed the activity of both extracts against Gram-negative and Gram-positive bacteria. However, the aqueous extract showed less activity compared to the organic extract.

The variability in the extract's effectiveness may be attributed to its chemical composition, including the polarity of the bioactive substances. The impact of these substances appears to be influenced by factors such as the conditions of drying and grinding of the plant, the mode of extraction, and the concentration of active ingredients [50,51].

The antibacterial study of *T. polium* underscores its effectiveness against various bacterial strains, irrespective of their Gram classification (positive or negative). This effect is attributed to secondary metabolites, particularly phenolic compounds present in the extract. Furthermore, the presence of flavonoids is noteworthy, given their recognized antibacterial properties [32].

Table 5. Diameters (mm) of the zones of inhibition induced by the extract.

Bacterial Strains	Extract (mg/mL)								
	Crude Extract	400	300	200	150	100	75	50	25
Bacillus subtilis ATCC 6633	15	14	13	10	09	07	07	06	06
Staphylococcus aureus ATCC 6538	20	11	11	10	09	09	09	07	06
Pseudomonas aeruginosa ATCC 9027	16	11	10	09	09	09	06	06	06
Escherichia coli ATCC 8739	10	10	10	09	08	08	06	06	06

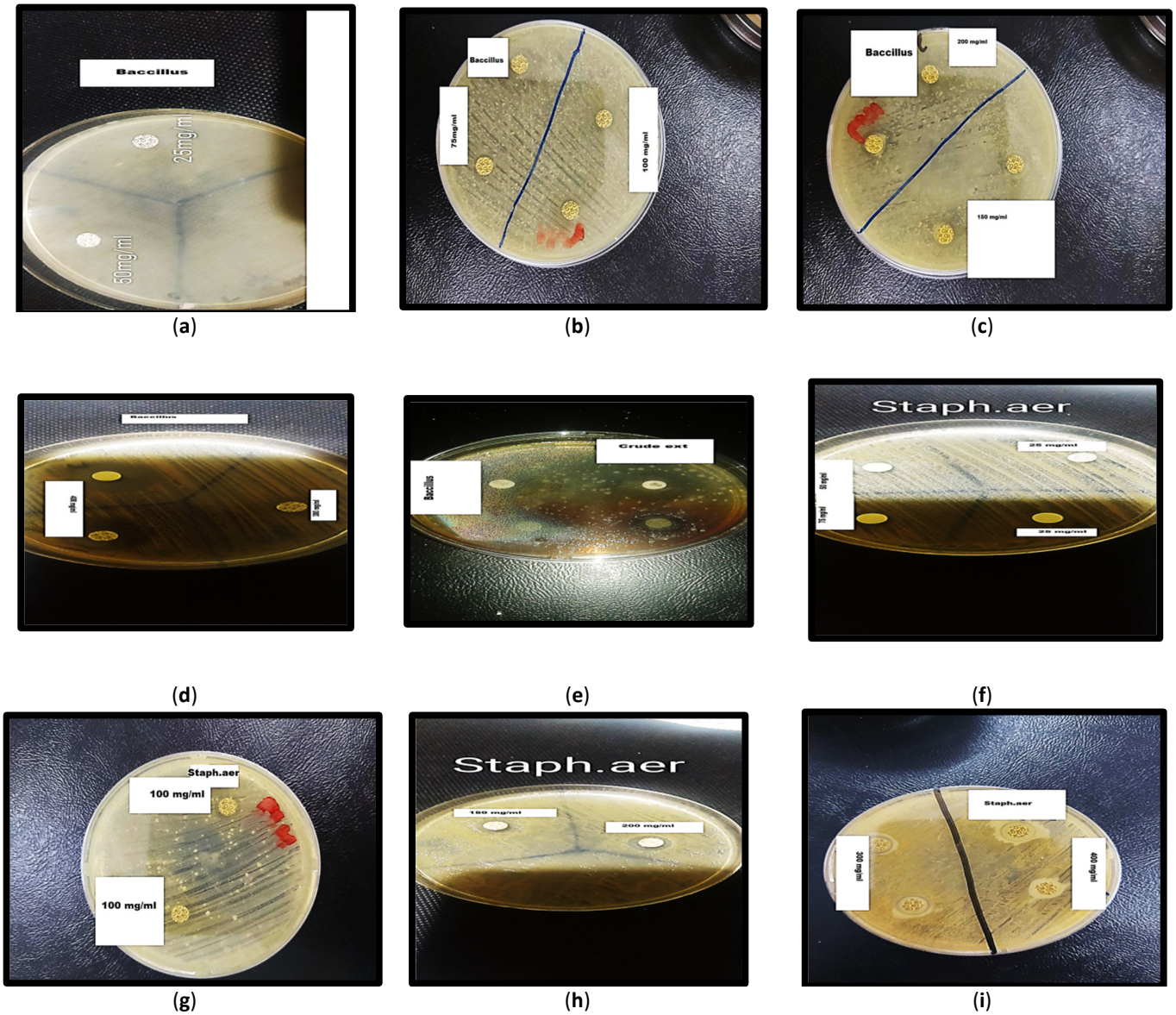

Figure 3. *Cont.*

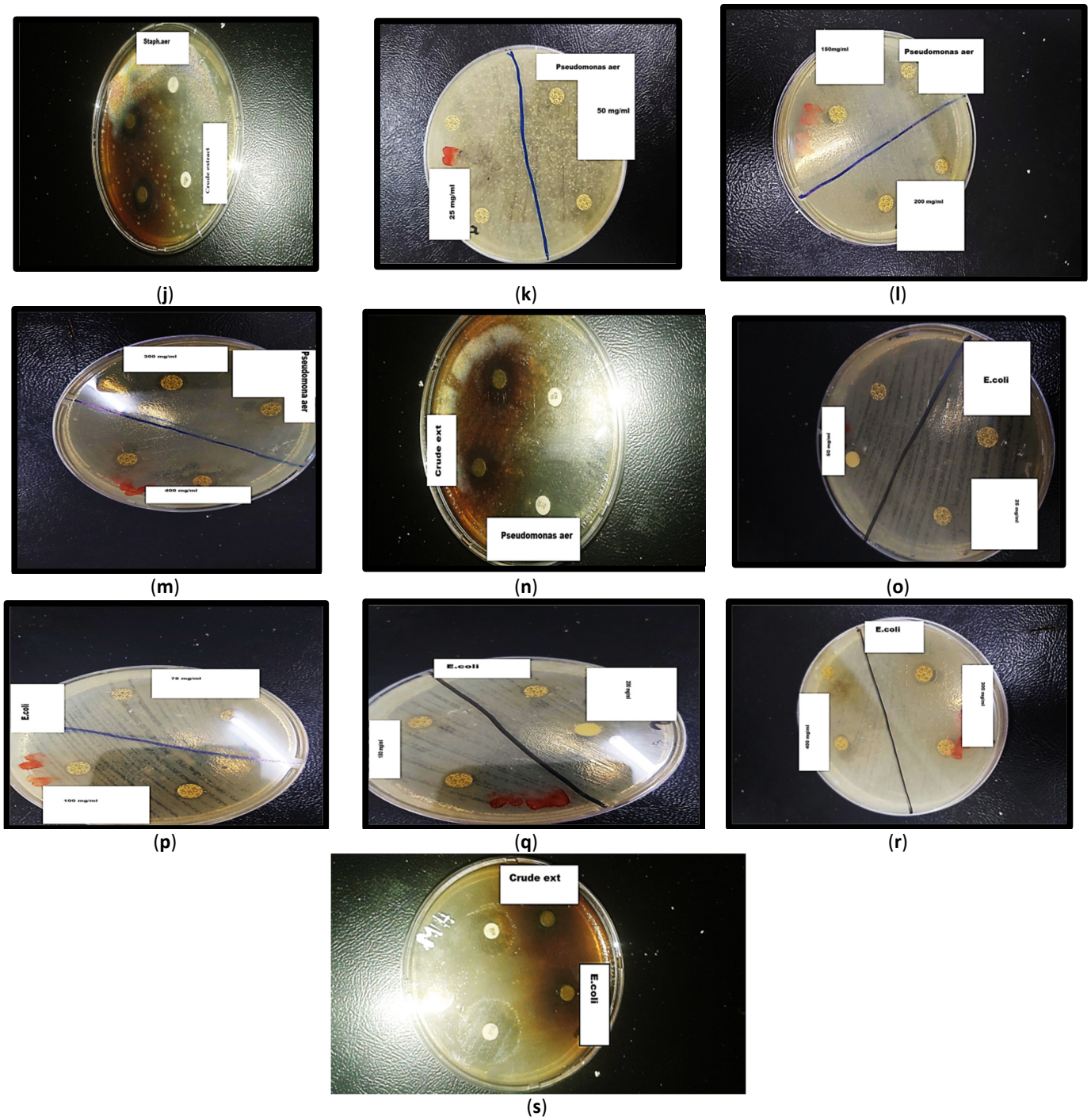

Figure 3. Antibacterial activity of the ethanolic extract on *Bacillus subtili*, (**a**) (25 mg/mL and 50 mg/mL), (**b**) (75 mg/mL and 100 mg/mL), (**c**) (150 mg/mL and 200 mg/mL), (**d**) (300 mg/mL and 400 mg/mL) and (**e**) (crude extract), on *Staphylococcus aureus*, (**f**) (25 mg/mL and 50 mg/mL and 75 mg/mL), (**g**) (100 mg/mL), (**h**) (150 mg/mL and 200 mg/mL), (**i**) (300 mg/mL and 400 mg/mL) and (**j**) (crude extract) on *Pseudomonas aeruginosa*, (**k**) (25 mg/mL and 50 mg/mL), (**l**) (150 mg/mL and 200 mg/mL), (**m**) (300 mg/mL and 400 mg/mL), (**n**) (crude extract) and on *Escherichia coli*, (**o**) (25 mg/mL and 50 mg/mL), (**p**) (75 mg/mL and 100 mg/mL), (**q**) (150 mg/mL and 200 mg/mL), (**r**) (300 mg/mL and 400 mg/mL), (**s**) (crude extract).

3.6. LC-MS/MS Chemical Profiling of T. polium Crude Extract

The crude extract of *T. polium* was analyzed using liquid chromatography coupled with mass spectrometry (LC-MS/MS) to emphasize its phytoconstituents (see Figure 4) and Table 6. The comprehensive analysis of phenolic compounds within the plant extract, conducted using this advanced technique, provides a captivating exploration into the molecular tapestry of remarkable richness and complexity. It unveils an in-depth panorama of metabolites present in this botanical resource.

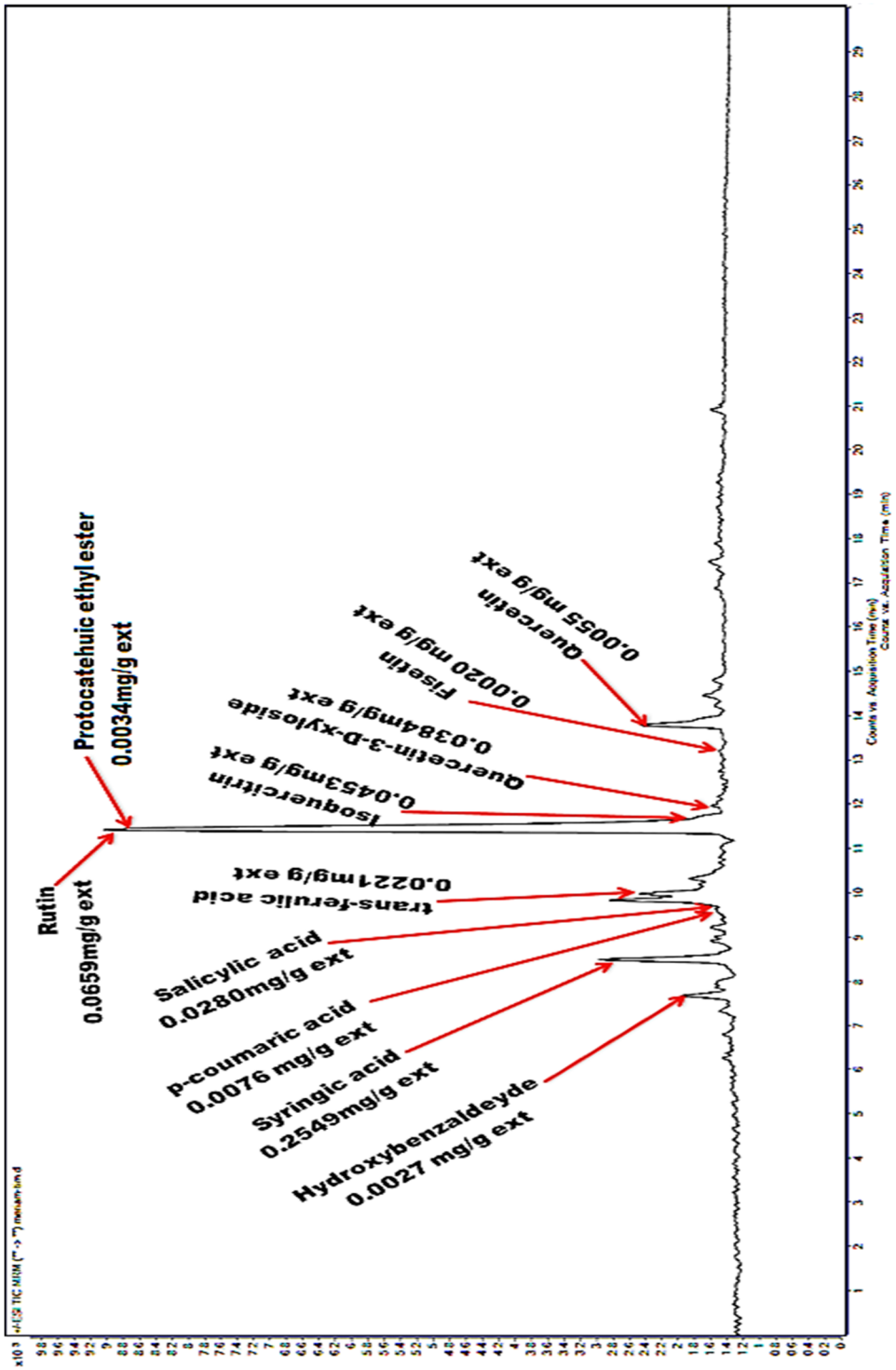

Figure 4. Chromatogram of ethanolic extract of *T. polium*.

Table 6. LC-MS/MS analysis of ethanolic extract of *T. polium* (mg/g extract).

Name	RT	Amount	
Hydroxybenzaldeyde	7.8004	0.002759139	mg/g extract
Syringic acid	8.5063	0.254928673	mg/g extract
P-coumaric acid	9.70736667	0.007627976	mg/g extract
Salicylic acid	9.82075	0.028086868	mg/g extract
Trans-ferulic acid	10.2212167	0.022191482	mg/g extract
Protocatehuicethyl ester	11.5255667	0.003452414	mg/g extract
Rutin	11.5043	0.065910668	mg/g extract
Isoquercitrin	11.5532833	0.045366072	mg/g extract
Quercetin-3-D-xyloside	12.0633333	0.03845436	mg/g extract
Fisetin	13.2768	0.002043481	mg/g extract
Quercetin	14.1836667	0.005570226	mg/g extract

Among the polyphenols present in Table 6, hydroxybenzaldehyde stands out with a significant concentration of 0.0027 mg/g of plant extract and a retention time (RT) of 7.8004 s, suggesting a potential role in protection against oxidative stress and a possible involvement in cellular defense mechanisms. Syringic acid (RT: 8.5063) distinguishes itself with a substantial concentration of 0.2549 mg/g, garnering particular interest due to its potential anti-inflammatory properties. Similarly, p-coumaric acid (RT: 9.7073) and salicylic acid (RT: 9.8207) exhibit detectable concentrations, implying their involvement in specific metabolic pathways of the plant. The detailed analysis also highlights trans-ferulic acid (RT: 10.2212) and Protocatehuic ethyl ester (RT: 11.5255) as distinct phenolic compounds, each contributing uniquely to the metabolic palette. Trans-ferulic acid, recognized for its protective properties against UV radiation, and Protocatehuic ethyl ester, with its distinctive structure, could potentially offer enhanced bioavailability, paving the way for applications in pharmacology.

The category of flavonoids adds an intriguing dimension to the composition, revealing variable concentrations ranging from 0.0020 mg/g to 0.0659 mg/g for compounds such as rutin (RT: 11.5043), isoquercitrin (RT: 11.5532), quercetin-3-D-xyloside (RT: 12.0633), fisetin (RT: 13.2768), and quercetin (RT: 14.18366667). This variability underscores the functional diversity of these compounds within the plant.

This LC-MS/MS analysis discriminates between polyphenols and flavonoids in the studied plant extract, offering a comprehensive perspective on its chemical complexity. Identified polyphenols, including hydroxybenzaldehyde, syringic acid, p-coumaric acid, salicylic acid, trans-ferulic acid, and Protocatehuic ethyl ester, contribute to enriching the biochemical complexity of the plant. On the other hand, flavonoids, encompassing rutin, isoquercitrin, quercetin-3-D-xyloside, fisetin, and quercetin, add an additional dimension by offering potential health benefits. These results shed light on the diversity of phenolic compounds in the studied plant, providing a solid foundation for further investigations into their bioactive properties and potential applications in fields such as medicine, nutrition, and pharmacology. The analytical approach used provides a robust methodology for the precise and detailed characterizationof the phenolic composition of plant extracts, opening up new perspectives for understanding bioactive compounds and their implications for human health.

4. Conclusions

This comprehensive study of *T. polium* has revealed a diversity of chemical compounds, including saponins, alkaloids, polyphenols, flavonoids, tannins, and terpenoids. Analyses confirmed the absence of anthocyanins and anthraquinones. The ethanolic extract exhibited a high content of polyphenols (227.43 mg Eq GAE/g DW extract) and flavonoids

(20.78 mg Eq QE/g DW extract). The evaluation of antioxidant activity showed significant results, albeit slightly lower than some studies. The correlation between antioxidant activity, polyphenols, and flavonoids was confirmed, emphasizing their key role in this activity. The extract demonstrated notable antibacterial efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, indicating broad-spectrum action. However, varying concentrations showed different effects on the tested bacterial strains. The integration of LC-MS/MS mass spectrometry for quantitative analysis of phenolic compounds represents a methodological novelty, offering detailed molecular insights. This quantitative analysis shows that the *T. polium* plantpresents a rich reservoir of bioactive compounds, justifying its therapeutic potential. Variations in chemical composition highlight the importance of an integrated approach in characterizing medicinal plants. These findings encourage further research into the therapeutic potential of this plant.

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