

Phytochemical profile, antioxidant, and antimicrobial activities of *Tinospora crispa* L. stem fractions

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Abstract. *Tinospora crispa* L. (brotowali) is a traditional medicinal plant used in Southeast Asia to treat fever, diabetes, and infections. This study evaluated the phytochemical composition, antioxidant potential, and antimicrobial activity of its stem fractions. Dried stems of simplicia were extracted with 70% ethanol and fractionated into n-hexane, chloroform, ethyl acetate, and aqueous fractions. Phytochemical screening, total flavonoid, phenolic, and tannin content determination, DPPH radical scavenging assay, antibacterial testing, and Liquid Chromatography–Mass Spectrometry (LC–MS) profiling were performed. The ethyl acetate fraction exhibited the strongest antioxidant activity ($IC_{50} = 24.47 \mu\text{g/mL}$), correlating with its highest flavonoid (89.76 mg QE/g), phenolic (41.14 mg GAE/g), and tannin (114.17 mg TAE/g) levels. LC–MS analysis tentatively identified 28 compounds, with siomenine as the major alkaloid. Antibacterial evaluation revealed moderate inhibition zones (4–11 mm) against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. However, all tested microorganisms were classified as resistant according to Clinical and Laboratory Standards Institute (CLSI) criteria. Overall, this study demonstrates that polarity-guided fractionation effectively enriches antioxidant constituents in *T. crispa* L. stems. Further optimization, including MIC/MBC determination and advanced structural confirmation, is required to enhance antibacterial efficacy and substantiate its potential for pharmaceutical or functional applications.

1 Introduction

The climbing plant Brotowali (*Tinospora crispa* L.) has long been utilized in traditional medicine across Southeast Asia, particularly in Indonesia. Empirically, various parts of this plant have been used to alleviate infections, treat diarrhea, reduce fever, and relieve inflammation, ailments often linked to pathogenic microorganisms such as *Escherichia coli* and *Staphylococcus aureus* [1]. Among the different plant parts, the stem of *T. crispa* L. is the most frequently utilized in traditional preparations and is often regarded as the primary

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source of its medicinal efficacy. Consequently, recent studies have increasingly focused on the stem as a promising reservoir of bioactive compounds. The therapeutic potential of *T. crisper* L. stems is attributed to originate from its rich content of secondary metabolites such as alkaloids, flavonoids, and diterpenoids, which have been reported to exhibit potent antioxidant activity [2].

Previous studies on *T. crisper* L. have predominantly examined its biological activities in a fragmented manner, with most investigations focusing either on antioxidant potential or antimicrobial activity against a limited number of microorganisms. For instance, a study by [3] reported that the 80% ethanolic stem extract exhibited the strongest antioxidant activity, with an IC₅₀ value of 6.46 mg/mL as determined by the DPPH assay; however, antimicrobial activity was not evaluated in that work. In contrast, more advanced chemical-biological correlations have been reported for related species. Research on *Tinospora cordifolia* showed that its methanolic stem extract inhibited *Pseudomonas aeruginosa* biofilm formation in a concentration-dependent manner [4]. In that study, the 100% methanol extract contained the highest total phenolic and flavonoid contents, supported by LC-MS/MS characterization that identified several phenolic alkaloids, including calopiptin, lirioferine, moupinamide, piperanine, and yuanhunine. Furthermore, a subsequent investigation [5] of *T. cordifolia* using methanol-water stems extract demonstrated significant antioxidant and antimicrobial activity against *P. aeruginosa*. Nevertheless, efficacy against other Gram-negative bacteria, such as *E. coli*, was not examined.

Collectively, these studies highlight important limitations in the existing literature, particularly for *T. crisper* L. Most previous work has emphasized either antioxidant or antimicrobial activity in isolation, employed a narrow spectrum of test microorganisms, or relied primarily on crude methanolic extracts without systematic polarity-based fractionation. Moreover, while advanced LC-MS-based compound identification has been applied to *T. cordifolia*, comparable chemical profiling linked to bioactivity remains scarce for *T. crisper* L., especially at the fraction level.

To address these gaps, the present study integrates antioxidant evaluation using the DPPH assay, broad-spectrum antimicrobial testing against Gram-positive, Gram-negative, and fungal pathogens, sequential solvent fractionation based on polarity, and LC-MS profiling of bioactive fractions, specifically focusing on *T. crisper* L. stems. This integrated strategy constitutes a key novelty of the work, as it enables direct correlation between extraction polarity, chemical composition, and biological activity within a single experimental framework. By doing so, this study provides a more comprehensive and mechanistically informed understanding of the antioxidant and antimicrobial potential of *T. crisper* L.

2 Materials and Methods

2.1 Materials

Dried *T. crisper* L. stems were collected from Kejayan, Pasuruan, East Java, Indonesia. The solvents used for extraction and fractionation included 70% ethanol (analytical grade), n-hexane, chloroform, ethyl acetate, and distilled water. All reagents and chemicals employed for phytochemical screening and quantitative analyses were of analytical grade and used without further purification.

2.2 Extraction of *T. crisper* stem

A total of 2000 g of *T. crisper* L. stem simplicia was macerated in 10 L of 70% ethanol at room temperature until the residue became colorless. The filtrate was collected, and the

residue was further refluxed with a fresh portion of solvent to ensure complete extraction. The combined filtrates were concentrated under reduced pressure using a rotary evaporator at 45°C to yield a thick crude ethanolic extract. The extraction yield was calculated using the following equation:

$$\text{Extraction yield (\%)} = \left(\frac{\text{Weight of dried crude extract}}{\text{Weight of dried stem simplicia}} \right) \times 100\% \quad (1)$$

2.3 Fractionation

Fractionation of the crude extract was performed using a modified liquid–liquid partition method. Approximately 25 g of the ethanolic extract was dissolved in 100 mL of distilled water and sequentially partitioned with n-hexane, chloroform, and ethyl acetate in a separatory funnel. Each solvent was added, gently shaken for 5 minutes, and allowed to separate before the aqueous layer was collected for the next step. The extraction was repeated until the solvent layer became colorless. All obtained solvent layers, n-hexane, chloroform, ethyl acetate, and the remaining aqueous fraction, were evaporated under reduced pressure to yield four distinct fractions. Complete removal of residual water from the aqueous fraction was confirmed by drying to constant weight under reduced pressure, followed by the absence of further mass change upon additional drying, indicating that no residual moisture remained. For solvent fractionation, the yield of each fraction was calculated as follows:

$$\text{Fraction yield (\%)} = \left(\frac{\text{Weight of dried fraction}}{\text{Weight of dried crude extract}} \right) \times 100\% \quad (2)$$

2.4 Phytochemical Screening

Preliminary phytochemical screening of the crude extract and its fractions was carried out using standard qualitative methods [6]. Flavonoids were tested using the magnesium–hydrochloric acid method (formation of orange to red color), tannins with 1% FeCl₃ (blue-black coloration), and saponins by the frothing test. Terpenoids were detected using Bouchardat’s reagent (reddish-brown coloration), while alkaloids were confirmed using Mayer’s, Dragendorff’s, and Wagner’s reagents, indicated by the formation of white, orange, or reddish-brown precipitates, respectively.

2.5 Quantitative Phytochemical Analysis

Quantitative analysis of total flavonoids, phenolics, and tannins was conducted following the procedures described in the Indonesian Herbal Pharmacopoeia [7]. All assays were performed using UV–Vis spectrophotometry, and results were expressed in terms of standard equivalents.

2.5.1 Total Flavonoid Content (TFC)

The total flavonoid content was determined using quercetin as the reference standard. Standard quercetin solutions (25–100 µg/mL) were prepared in ethanol. A 0.5 mL aliquot of the sample or standard was mixed with 0.1 mL of 10% aluminum chloride (AlCl₃), 0.1 mL of 1 M sodium acetate, 1.5 mL of ethanol, and 0.8 mL of distilled water. The mixture was homogenized and incubated for 30 minutes at room temperature, and the absorbance was measured at 435 nm. The TFC value was expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g).

2.5.2 Total Phenolic Content (TPC)

The total phenolic content was analyzed using the Folin–Ciocalteu method with gallic acid as the standard. Standard solutions of gallic acid (25–100 µg/mL) were prepared in ethanol. A 0.2 mL aliquot of each sample or standard was mixed with 1 mL of 10% (v/v) Folin–Ciocalteu reagent, incubated for 8 minutes, and followed by the addition of 1 mL of 7.5% sodium acetate solution. The mixture was incubated for 60 minutes in the dark, and absorbance was measured at 725 nm. The TPC was expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

2.5.3 Total Tannin Content (TTC)

The total tannin content was determined using tannic acid as the standard following the same procedure as the TPC assay. A 0.2 mL aliquot of the sample or standard was reacted with 1 mL of 10% Folin–Ciocalteu reagent and 1 mL of 7.5% sodium acetate solution, then incubated for 60 minutes in the dark. The absorbance was measured at 725 nm, and the TTC was expressed as milligrams of tannic acid equivalent per gram of extract (mg TAE/g).

2.6 Antioxidant Activity

The antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method as described by [11] with slight modifications. Test solutions of the crude extract and each fraction were prepared at concentrations ranging from 1.953 to 1000 ppm. Ascorbic acid (1–5 ppm) served as the positive control. A mixture of 1 mL of sample, 1 mL of 30 ppm DPPH, and 1 mL of ethanol was incubated in the dark at room temperature for 60 minutes. The absorbance was measured at 517 nm using a Visible spectrophotometer.

2.7 Antimicrobial Activity

The fraction with the strongest antioxidant activity was evaluated for antimicrobial potential against selected microorganisms, including Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and yeast (*Candida albicans*). The assay was performed using the standard disk diffusion method. Sterile paper disks (6 mm) impregnated with 10 µL of the test fraction were placed on Mueller–Hinton agar plates (for bacteria) or Sabouraud Dextrose Agar plates (for *C. albicans*) inoculated with standardized microbial suspensions (10⁶ CFU/mL). Plates were incubated at 37°C for 18–24 hours, and antimicrobial activity was determined by measuring the diameter of the inhibition zones (mm).

2.8 LC-MS Analysis

The most active fraction based on DPPH assay results was subjected to chemical profiling using Liquid Chromatography–Mass Spectrometry (LC–MS). Approximately 0.5 g of the fraction was dissolved in ethyl acetate. Compound separation was achieved on a Hypersil Gold C18 column (150 mm × 2.1 mm; particle size 3 µm). Mass spectrometric detection was carried out using Electrospray Ionization (ESI) operated in both positive and negative ion modes, depending on the ionization properties of the analytes. Tentative compound identification was performed by comparing the obtained spectra with reference data from the MassBank and NIST databases using MestReNova or MassLynx software.

3 Results and Discussion

3.1 Extraction and Fractionation

The maceration of *T. crispa* L. stem powder with 70% ethanol produced a greenish filtrate, which, upon solvent removal under reduced pressure at 45 °C, yielded a brownish-green, viscous crude extract with a characteristic odor, with a yield of 10.14%. This extraction yield is comparable to those reported for *T. crispa* L. and related *Tinospora* species extracted using hydroethanolic solvents, where yields typically range from 10–30 % depending on solvent composition, extraction time, and plant matrix characteristics [3]. From a mechanistic perspective, ethanol acts as a polar protic solvent, disrupting hydrogen bonding within the plant cell wall matrix and enhancing the mass transfer of intracellular constituents into the solvent phase. The presence of water further increases solvent polarity, favoring the extraction of polar compounds such as phenolics, flavonoids, and glycosylated metabolites, while still permitting partial solubilization of moderately nonpolar constituents. Similar observations have been reported in phytochemical extraction studies, where hydroethanolic systems consistently outperform absolute solvents in terms of total extractable metabolites [2].

Subsequent liquid–liquid partitioning of 25.01 g of crude extract yielded four fractions with distinct polarity profiles. The aqueous fraction accounted for the largest proportion of the extract (20.25 g, 81.00%), followed by the ethyl acetate (0.79 g, 3.16%), n-hexane (0.77 g, 3.08%), and chloroform (0.64 g, 2.56%) fractions. This distribution pattern indicates that the majority of extractable constituents in *T. crispa* L. stems are highly polar. Compounds commonly enriched in aqueous fractions include sugars, glycosides, flavonoid glycosides, tannins, and other hydrophilic phenolic compounds, which exhibit strong affinity for polar solvents due to multiple hydroxyl and heteroatom functionalities.

In contrast, the relatively low yields of the n-hexane and chloroform fractions suggest a limited abundance of nonpolar and weakly polar metabolites, such as sterols, triterpenoids, lipophilic alkaloids, and fatty acid derivatives. Ethyl acetate, with intermediate polarity, selectively extracted semi-polar compounds, including aglycone flavonoids and certain alkaloids, which are often associated with pronounced antioxidant and antimicrobial activities. Comparable fractionation trends have been reported for *T. crispa* L. and *T. cordifolia*, where aqueous fractions dominate in mass yield, while ethyl acetate fractions, despite their smaller mass, exhibit higher specific bioactivity [8].

3.2 Phytochemical Screening

Phytochemical screening of the ethanolic extract and its solvent fractions of *T. crispa* L. stems confirmed the presence of major secondary metabolite classes, including flavonoids, tannins, saponins, steroids, and alkaloids, as shown in Table 1. These results are consistent with previous reports describing *T. crispa* L. as a phenolic- and alkaloid-rich medicinal plant [1-3]. Strong positive reactions for phenolic/tannin compounds across all fractions, particularly in the ethanolic, ethyl acetate, and aqueous fractions, align with earlier studies highlighting the dominance of phenolic constituents in hydroethanolic extracts of *T. crispa* L., which are closely associated with antioxidant activity.

Flavonoids were detected in all fractions with moderate intensity, suggesting the presence of both polar flavonoid glycosides and less polar aglycones. Similar distribution patterns have been reported for *Tinospora* species, where flavonoids partition across multiple solvent fractions due to overlapping polarity ranges. Saponins were predominantly observed in the aqueous fraction, in agreement with their highly polar nature and previous findings indicating that water-based extracts of *T. crispa* L. are enriched in saponin-type compounds [2][9].

The alkaloid screening results showed some variability among Mayer's, Wagner's, and Dragendorff's reagents. This inconsistency is commonly reported and reflects differences in reagent sensitivity and alkaloid structural specificity. Mayer's reagent is generally regarded as the most sensitive for a broad range of alkaloids, whereas Dragendorff's reagent is more selective and may fail to detect alkaloids present at low concentrations [10]. Accordingly, the consistent positive response with Mayer's reagent across samples is considered the most reliable indicator of alkaloid presence in this study. Overall, the phytochemical screening supports previous reports on *T. crispa* L. stems and provides a qualitative basis for interpreting the antioxidant and antimicrobial activities observed, while underscoring the importance of LC–MS analysis for more precise chemical differentiation among fractions.

Table 1. Phytochemical Screening Data

| Phytochemical test | Ethanollic extract | <i>n</i> -hexane fraction | Chloroform fraction | Ethyl Acetate fraction | Aqueous fraction |
|----------------------|--------------------|---------------------------|---------------------|------------------------|------------------|
| Saponin | - | - | - | - | ++ |
| Phenol/Tannin | +++ | ++ | ++ | +++ | +++ |
| Flavonoid | + | + | ++ | ++ | + |
| Triterpenoid | - | - | - | - | - |
| Steroid | + | + | + | + | + |
| Alkaloid Mayer | +++ | + | + | + | + |
| Alkaloid Dragendorff | + | - | - | - | ++ |
| Alkaloid Wagner | - | + | + | + | + |

Information: not detected (-), detected (+), moderately detected (++), strongly detected (+++)

3.3 Quantitative Phytochemical Analysis

The quantitative analysis of flavonoids, total phenolics, and tannins in the crude ethanolic extract and its solvent fractions is summarized in Table 2. Overall, a consistent trend was observed across all three parameters, with the ethyl acetate fraction exhibiting the highest total flavonoid content (TFC), total phenolic content (TPC), and total tannin content (TTC), followed by the chloroform fraction, while the aqueous and *n*-hexane fractions showed comparatively lower values.

Table 2. TFC, TPC, and TTC of Ethanolic Extract and Fractions

| Sample | TFC (mg QE/g) | TPC (mg GAE/g) | TTC (mg TAE/g) |
|---------------------------|---------------|----------------|----------------|
| Ethanolic extract | 33.88 | 10.94 | 25.13 |
| <i>n</i> -hexane fraction | 25.38 | 5.68 | 12.19 |
| Chloroform fraction | 43.19 | 35.97 | 86.71 |
| Ethyl Acetate fraction | 89.76 | 41.14 | 114.17 |
| Aqueous fraction | 17.58 | 21.04 | 49.97 |

The predominance of flavonoids, phenolics, and tannins in the ethyl acetate fraction can be rationalized by solvent polarity and compound chemistry. Ethyl acetate is a semi-polar solvent with strong affinity for aromatic compounds bearing hydroxyl groups, such as flavonoid aglycones, low-molecular-weight phenolic acids, and condensed tannins. These compound classes are poorly extracted by nonpolar solvents like *n*-hexane and only partially recovered in highly polar aqueous systems, where polymeric phenolics and glycosides tend to dominate.

Importantly, the TFC, TPC, and TTC of the ethyl acetate fraction were markedly higher than those of the crude ethanolic extract, clearly demonstrating that polarity-guided fractionation effectively concentrates flavonoids rather than merely redistributing them. The high TFC value observed in the ethyl acetate fraction is comparable to, or exceeds, previously reported values for *T. crispata* L. stem extracts obtained using ethanolic or semi-polar systems, suggesting that flavonoids in this species are predominantly present in aglycone or weakly glycosylated forms [3].

Similarly, the elevated total phenolic and tannin contents in the ethyl acetate fraction are consistent with reports identifying proanthocyanidins and other condensed tannins as major contributors to antioxidant activity in *T. crispata* L. stems. In contrast, although the aqueous fraction accounted for the highest mass yield, its lower specific flavonoid and phenolic contents indicate substantial dilution by highly polar, non-bioactive constituents such as sugars and inorganic salts. Overall, the consistent enrichment of TFC, TPC, and TTC in the ethyl acetate fraction underscores the effectiveness of polarity-guided fractionation in selectively concentrating bioactive phenolic compounds.

3.4 Antioxidant Activity

The antioxidant activity of *T. crispata* L. stem extracts, evaluated using the DPPH assay, was expressed as IC₅₀ values and classified according to established criteria, where IC₅₀ < 50 µg/mL indicates very strong and 50–100 µg/mL indicates strong antioxidant activity (Table 3). The ethyl acetate fraction exhibited the highest activity (IC₅₀ = 24.47 µg/mL, very strong), followed by the crude ethanolic extract (IC₅₀ = 37.23 µg/mL), while the chloroform, aqueous, and n-hexane fractions showed strong activity with IC₅₀ values between 56.48 and 77.52 µg/mL. This polarity-dependent trend is consistent with previous reports indicating that semi-polar solvents preferentially extract phenolic antioxidants.

Table 3. IC₅₀ Values of Ethanolic Extract, Fractions, and Ascorbic Acid

| Sample | IC ₅₀ (µg/mL) | Antioxidant Strength |
|--------------------------|--------------------------|----------------------|
| Ethanolic extract | 37.23 | Very strong |
| n-hexane fraction | 77.52 | Strong |
| Chloroform fraction | 56.48 | Strong |
| Ethyl Acetate fraction | 24.47 | Very strong |
| Aqueous fraction | 67.94 | Strong |
| Ascorbic acid (standard) | 0.462 | Very strong |

The superior antioxidant performance of the ethyl acetate fraction correlates well with its highest TFC, TPC, and TTC, confirming that these compounds are the primary contributors to DPPH radical scavenging through hydrogen or electron donation. In contrast, the lower activity of the aqueous, chloroform, and n-hexane fractions reflects dilution by highly polar non-phenolic constituents and limited solubility of phenolics in nonpolar media, respectively. Zulkefli (2013) reported that the stem extract of *T. crispata* L. possessed the highest DPPH inhibition with IC₅₀ 0.118 mg/mL. The high antioxidant activity of the stem extract of *T. crispata* L. is most probably due to the presence of apigenin and magnoflorine, as it has a hydroxyl group that donates the electron to reduce the DPPH radicals [11]. Overall, the strong agreement between IC₅₀ values and quantitative phytochemical data supports the conclusion that enrichment of semi-polar phenolic compounds underlies the potent antioxidant activity of the ethyl acetate fraction of *T. crispata* L. stems.

3.5 Antimicrobial Activity

The antimicrobial activity of the ethyl acetate fraction of *T. crisper* L. stems was evaluated against representative Gram-positive, Gram-negative, and fungal microorganisms as shown in Table 3-7. The focus on this ethyl acetate fraction was guided by its highest antioxidant activity and enrichment of phenolic and flavonoid compounds, as demonstrated by the quantitative phytochemical analysis. The ethyl acetate fraction produced measurable inhibition zones against all tested microorganisms. However, according to Clinical and Laboratory Standards Institute (CLSI M100) criteria, the observed inhibition diameters fell within the “resistant” category, indicating moderate rather than potent antimicrobial activity [12]. This finding is consistent with earlier reports describing *T. crisper* L. extracts as exhibiting partial or moderate antibacterial effects, particularly when tested at extract-level concentrations rather than purified compounds. Notably, the inhibition zone observed against *B. subtilis* (10.78 mm) and *E. coli* is comparable to or slightly higher than values reported in previous studies by Aminul (2011) using chloroform extracts of *T. crisper* L., while the lower activity against *S. aureus* and *C. albicans* aligns with their documented intrinsic resistance mechanisms [13].

The greater susceptibility of *B. subtilis* relative to Gram-negative bacteria can be attributed to differences in cell wall architecture. The absence of an outer lipopolysaccharide membrane in Gram-positive bacteria facilitates the penetration of phenolic and flavonoid compounds, whereas Gram-negative bacteria possess an additional permeability barrier that limits compound uptake. Similar patterns of selective inhibition favoring Gram-positive species have been widely reported for phenolic-rich plant extracts [4].

Importantly, the antimicrobial behavior of the ethyl acetate fraction correlates well with its phytochemical profile and antioxidant performance. The high TFC, TPC, and TTC of this fraction suggest that redox-active compounds are the primary contributors to both antioxidant and antimicrobial activities. These compounds exert antioxidant effects via electron or hydrogen donation and simultaneously inhibit microbial growth through mechanisms including membrane disruption, protein precipitation, enzyme inhibition, and interference with quorum-sensing pathways. Therefore, the strong antioxidant activity of the ethyl acetate fraction serves as an indirect indicator of its antimicrobial potential, reinforcing the integrated bioactivity profile of *T. crisper* L. stem extracts.

Table 3. Antimicrobial Activity of Ethyl Acetate Fraction of *T. crisper* L. Stem against *B. subtilis*

| Test | Replication | Diameter of Inhibition Zone (mm) | Average Diameter (mm) | Category | CLSI Category |
|--------------------|-------------|----------------------------------|-----------------------|----------|---------------|
| Sample | 1 | 10.20 | 10.78 ± 0.52 | Moderate | Resistant |
| | 2 | 11.20 | | | |
| | 3 | 10.95 | | | |
| Cloramphenicol (+) | 1 | 20.60 | 21.67 ± 0.92 | Strong | Susceptible |
| | 2 | 22.20 | | | |
| | 3 | 22.20 | | | |
| Aquadest (-) | 1 | 0 | 0 ± 0 | Weak | Resistant |
| | 2 | 0 | | | |
| | 3 | 0 | | | |

Table 4. Antimicrobial Activity of Ethyl Acetate Fraction of *T. crispa* L. Stem against *E. coli*

| Test | Replication | Diameter of Inhibition Zone (mm) | Average Diameter (mm) | Category | CLSI Category |
|--------------------|-------------|----------------------------------|-----------------------|----------|---------------|
| Sample | 1 | 8.65 | 8.93 ± 0.91 | Moderate | Resistant |
| | 2 | 8.20 | | | |
| | 3 | 9.95 | | | |
| Cloramphenicol (+) | 1 | 23.25 | 21.53 ± 1.50 | Strong | Susceptible |
| | 2 | 20.50 | | | |
| | 3 | 20.85 | | | |
| Aquadest (-) | 1 | 0 | 0 ± 0 | Weak | Resistant |
| | 2 | 0 | | | |
| | 3 | 0 | | | |

Table 5. Antimicrobial Activity of Ethyl Acetate Fraction of *T. crispa* L. Stem against *S. aureus*

| Test | Replication | Diameter of Inhibition Zone (mm) | Average Diameter (mm) | Category | CLSI Category |
|--------------------|-------------|----------------------------------|-----------------------|----------|---------------|
| Sample | 1 | 4.10 | 4.52 ± 0.43 | Moderate | Resistant |
| | 2 | 4.50 | | | |
| | 3 | 4.95 | | | |
| Cloramphenicol (+) | 1 | 28.25 | 27.71 ± 0.92 | Strong | Susceptible |
| | 2 | 28.24 | | | |
| | 3 | 26.65 | | | |
| Aquadest (-) | 1 | 0 | 0 ± 0 | Weak | Resistant |
| | 2 | 0 | | | |
| | 3 | 0 | | | |

Table 6. Antimicrobial Activity of Ethyl Acetate Fraction of *T. crispa* L. Stem against *P. aeruginosa*

| Test | Replication | Diameter of Inhibition Zone (mm) | Average Diameter (mm) | Category | CLSI Category |
|--------------------|-------------|----------------------------------|-----------------------|----------|---------------|
| Sample | 1 | 5.95 | 5.77 ± 0.28 | Moderate | Resistant |
| | 2 | 5.90 | | | |
| | 3 | 5.45 | | | |
| Cloramphenicol (+) | 1 | 5.25 | 8.72 ± 3.05 | Strong | Resistant |
| | 2 | 11.00 | | | |
| | 3 | 9.90 | | | |
| Aquadest (-) | 1 | 0 | 0 ± 0 | Weak | Resistant |
| | 2 | 0 | | | |
| | 3 | 0 | | | |

Table 7. Antimicrobial Activity of Ethyl Acetate Fraction of *T. crispa* L. Stem against *C. albicans*

| Test | Replication | Diameter of Inhibition Zone (mm) | Average Diameter (mm) | Category | CLSI Category |
|--------------|-------------|----------------------------------|-----------------------|----------|---------------|
| Sample | 1 | 4.10 | 6.15 ± 2.28 | Moderate | Resistant |
| | 2 | 8.60 | | | |
| | 3 | 5.75 | | | |
| Nystatin (+) | 1 | 24.95 | 26.38 ± 2.70 | Strong | Susceptible |
| | 2 | 24.70 | | | |
| | 3 | 29.50 | | | |
| Aquadest (-) | 1 | 0 | | | |

| | | | | | |
|--|---|---|-------|------|-----------|
| | 2 | 0 | 0 ± 0 | Weak | Resistant |
| | 3 | 0 | | | |

3.6 LC-MS Chromatogram of Ethyl Acetate Fraction

The LC-MS analysis of the ethyl acetate fraction from *T. crisper* L. stem provided detailed insight into its chemical composition, as shown in the chromatogram below (Figure 1). The chromatographic profile revealed several major peaks corresponding to compounds tentatively identified through comparison of their retention times and mass-to-charge (m/z) values with reference spectra available in the MassBank database (Table 8).

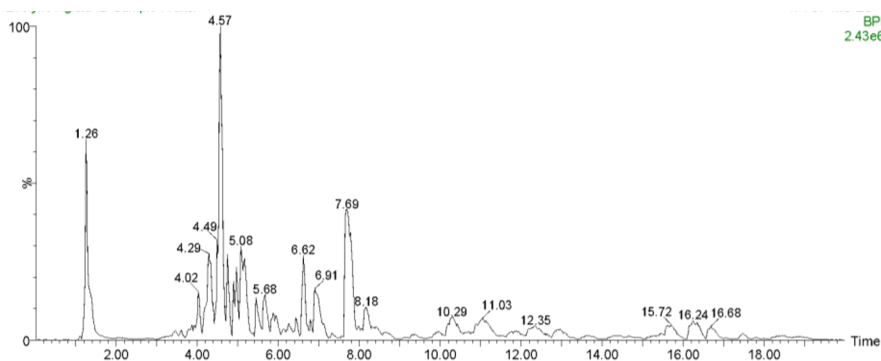


Fig. 1. LC-MS Chromatogram of the Ethyl Acetate Fraction

Table 8. Major compounds proposed from LC-MS peaks with the highest % area

| Rt (min) | % area | m/z | Proposed Compound | Chemical Class |
|----------|--------|----------|---|------------------------------|
| 4.57 | 18.75 | 330.1718 | Siomenine | Alkaloid |
| 7.73 | 13.43 | 568.2367 | (2S)-N-[[[1-[[[(2R,3S,4R,5R)-5-[[[(2R,3S,4S,5S)-5-[(2,4-dioxypyrimidin-1-yl)methyl]-3,4-dihydroxyoxolan-2-yl]methoxy]-3,4-dihydroxyoxolan-2-yl]methyl]triazol-4-yl]methyl]pyrrolidine-2-carboxamide | Nucleoside derivative |
| 1.28 | 9.96 | 136.0631 | Adenine | Purine/primary metabolite |
| 5.13 | 9.31 | 463.3063 | N-[2-amino-9-[(2R,4R)-2-(hydroxymethyl)-1,3-dioxolan-4-yl]purin-6-yl]tetradecanamide | Purine nucleoside analog |
| 4.31 | 5.52 | 166.0859 | N,N-dimethyl-[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6-amine | Triazole-triazine derivative |

Among the detected constituents, a compound tentatively assigned as the alkaloid siomenine/sinomenine (Figure 2) was observed as a putative major peak, eluting at a retention time of 4.57 minutes and accounting for 18.75% of the total peak area. This assignment was based on close agreement between the observed m/z value and reported molecular ions of sinomenine, as well as comparable retention behavior reported for isoquinoline alkaloids under similar chromatographic conditions. However, it should be

emphasized that this identification remains tentative and requires further confirmation by co-injection with an authentic standard and/or detailed MS/MS fragmentation analysis.

Previous phytochemical studies on *Tinospora* species have documented the presence of structurally related isoquinoline and aporphine alkaloids, suggesting that the detection of a sinomenine-like compound in *T. crispa* L. is chemically plausible but should be interpreted with caution [14]. Therefore, sinomenine is herein presented as a tentatively identified major alkaloid rather than a definitively confirmed constituent. Despite this limitation, the prominence of a sinomenine-like alkaloid peak may partially explain the bioactivities observed in the ethyl acetate fraction. Alkaloids from the *Tinospora* genus have been widely reported to exhibit antioxidant, antimicrobial, and immunomodulatory properties. Alkaloids possess antioxidant activity through electron or hydrogen donation and modulation of oxidative stress pathways, as well as antimicrobial effects linked to membrane disruption, interference with nucleic acid synthesis, and inhibition of biofilm formation. Its well-documented anti-inflammatory and immunomodulatory activities, mediated through suppression of NF- κ B signaling and activation of the Nrf2/HO-1 pathway, further support the biological relevance of isoquinoline alkaloids detected in this fraction [15].

Collectively, the LC-MS findings suggest that isoquinoline-type alkaloids may contribute significantly to the antioxidant and antimicrobial activities of the ethyl acetate fraction of *T. crispa* L. stems. Nevertheless, definitive structural confirmation of sinomenine and related alkaloids through targeted MS/MS analysis and nuclear magnetic resonance (NMR) spectroscopy is required in future studies to substantiate their precise identity and contribution to bioactivity.

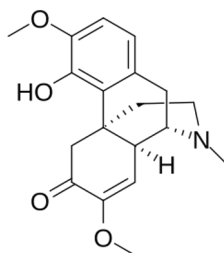


Fig. 2. Siomenine

4 Conclusion

Phytochemical analysis confirmed that the stem of *T. crispa* L. contains diverse classes of secondary metabolites, including flavonoids, phenols/tannins, alkaloids, terpenoids, and saponins, with compositional variations depending on solvent polarity. Among the tested fractions, the ethyl acetate fraction demonstrated the strongest antioxidant activity ($IC_{50} = 24.47 \mu\text{g/mL}$) and the highest total contents of flavonoids, phenols, and tannins. This fraction also exhibited moderate antimicrobial effects against *B. subtilis*, *E. coli*, *P. aeruginosa*, and *C. albicans*, though less potent than the positive control. LC-MS profiling tentatively identified siomenine, an isoquinoline alkaloid, as a putative major constituent of the ethyl acetate fraction based on spectral comparison with reference databases. While this tentative identification suggests that isoquinoline-type alkaloids may contribute to the observed antioxidant and antimicrobial activities, definitive structural confirmation and attribution of bioactivity require further targeted analysis. Overall, the ethyl acetate fraction of *T. crispa* L. stem is enriched with bioactive secondary metabolites and represents a promising source of

natural antioxidant and antimicrobial agents. Further investigations, including MIC/MBC determination, advanced structural elucidation, and evaluation against resistant microbial strains, are necessary to substantiate its pharmacological potential and support its prospective development for pharmaceutical or functional applications.

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