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PHYTOCHEMICAL PROFILING, GC/GC-MS ANALYSIS, AND ANTIBACTERIAL ACTIVITY OF DICHLOROMETHANE EXTRACT OF THE AERIAL PARTS OF *TINOSPORA CRISPA*

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ABSTRACT

Tinospora crispa is a medicinal plant, traditionally used for a wide range of pharmacological activities. This study conducted phytochemical screening, both qualitative and quantitative, of the non-polar dichloromethane (DCM) extract from the aerial parts of *T. crispa*, using gas chromatography-mass spectrometry (GC-MS) and antibacterial activity against *Escherichia coli* and *Salmonella typhimurium* through agar well diffusion method. The analysis confirmed the presence of steroids, terpenoids, fatty acids, tannins, saponins, coumarins, carbohydrates, resins, alkaloids, glycosides, cardiac glycosides, and proteins. However, anthocyanins, leucoanthocyanins, and emodins were absent. The extract comprised a complex mixture of compounds, including oxygenated monoterpenes (e.g., 2-cyclohexen-1-one, 4-(3-hydroxybutyl)-3,5,5-trimethyl), long-chain ketones (e.g., 2-pentadecanone, 6,10,14-trimethyl), fatty acids (n-hexadecanoic and octadecanoic acids), and a variety of sterols and triterpenoids such as ethyl iso-allocholate, stigmasterol, α-amyrin, and betulin. Notably, a high relative abundance (43.85% area sum) of 6a,14a-methanopicene, perhydro-1,2,4a,6b,9,9,12a-heptamethyl-10-hydroxy was observed, suggesting that triterpenoids may contribute significantly to the pharmacological effects of the extract. The antibacterial activity of the DCM extract was determined against *Escherichia coli* and *Salmonella typhimurium.* Statistical analysis indicated that extract concentration significantly influenced antibacterial activity ($p<0.005$), with a concentration-dependent increase against both pathogenic bacteria. The extract produced inhibition zones of 15.6 ± 0.3 mm (6 µl) and 11.6 ± 0.3 mm (3µl) against *S. typhimurium,* whereas inhibited zones of 9.2 ± 0.0.1 mm (6 µl) and 3.9 ± 0.05 mm (3 µl) were observed against *E. coli.* These findings provide a comprehensive chemical characterization of *T. crispa* DCM extract and highlight its potential as a source of bioactive compounds with antibacterial activity, supporting its traditional medical application and source of novel therapeutic agents.

Keywords: Antibacterial activity, Dichloromethane extract; Phytochemical profiling; *Tinospora crispa*; GC/GCMS; Aerial plant

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INTRODUCTION

Medicinal plants have long been recognized as a cornerstone source of bioactive phytochemicals, particularly utilized in herbal medicine and the pharmaceutical industry for the development of therapeutic nutraceutical products (Ramya, 2022). Plant-based medicines derived from crude extracts contain complex secondary metabolites and are used to treat diverse health conditions. The chromatograph application for screening provides significant insights into the pharmacological potential of the plants and suggests identifying medicinal properties. By facilitating the accurate identification of bioactive ingredients, the use of these techniques for medicinal plants (Satapute *et al.,* 2019).

In Pakistan, plant-derived natural products continue to grow due to their therapeutic potential and perceived good safety profile. Approximately 80% of the world population relies on traditional plant-based remedies for basic health care problems. This reliance underscores the importance of medicinal herbs, especially in areas where access to conventional medicine may be

unaffordable or restricted (World Health Organization, 1993).

Tinospora crispa (L.) Hook. f. & Thomson (Menispermaceae), a deciduous climbing plant, is commonly known in Urdu language "Gilo Boti", found in the tropical and subtropical areas of Asia, Africa, and the East Pacific. It has significant long ethnomedicinal history for its different part like, stems, seeds, rhizomes, and roots (Zulkefli *et al.,* 2013; Bajpai *et al.,* 2016), traditionally used to treat different health conditions such as typhoid fever, gastrointestinal disorders, loss of appetite, diarrhea, jaundice, diabetes, hypertension, rheumatism, malaria, inflammation, scabies, headaches, pharyngitis, skin disorders, and urine disorders (Adnan *et al.,* 2019; Warsinah *et al.,* 2020). *In vitro* and *in vivo s*tudies showed the antibacterial, antioxidant, antidiabetic, immunomodulatory, antimalarial, anti-inflammatory, and cytotoxic activities. *T. crispa* has been supported by significant traditional substantiated, by considerable scientific research (Llamasares-Castillo *et al.,* 2024).

The 167 rich phytoconstituents identified in *T. crispa* have a therapeutic potential and belong to several chemical classes, including alkaloids, terpenes, glycosides, Clerodane-type furanoditerpenoids, picoretoside, tinocrisposide, berberine, palmatine, columbine, picrotoxin, and polysaccharides, among other compounds that are bioactive (Anup Poudel, 2023; Parvathy *et al.,* 2023). While extensive research has been done on its bioactivity, limited studies have explored its non-polar phytochemical profile, especially using organic solvents such as dichloromethane (DCM) of *T. crispa.* The non-polar extracts of *T. crispa* contain unique classes of bioactive compounds such as terpenoids furanoditerpenes, lactones, steroids, flavonoids, and lignans, that may contain significant therapeutic properties (Rahman *et al.,* 2020; Mailander *et al.,* 2022).

The complex bioactive compounds found by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) determine polar and semi-polar bioactive compounds in the plant extracts, both qualitative and quantitative information (Hipol *et al.,* 2012; Płonka *et al.,* 2025). To identify new bioactive compounds for the development of research and pharmaceutical applications. Therefore, *T. crispa,* considering its therapeutic properties and chemical complexity (Shree and Krishnaveni, 2022b). The presence of bioactive compounds in the ethanolic stem extract of *T. crispa,* as determined by GC-MS analysis, indicated the presence of diverse phytochemical profile. The major constituents of *T. crispa* identified were Diacetin (1,2,3-propanetriol diacetate), 3-Tetradecene, and 4-Penten-1-ol, trimethylsilyl ether. The extract also contained various phthalate derivatives such as diethyl phthalate, bis(2-ethylhexyl) phthalate, and 1,2-benzene dicarboxylic acid, diethyl ester. Aromatic esters, including benzyl benzoate. Furthermore, several fatty acids were identified, notably 9-octadecenoic acid and 9,12,15-octadecatrienoic acid. Other notable compounds included phosphonic acid, dioctadecyl ester, 9-Eicosene, and 3-Furylmethanol (Shree and Krishnaveni, 2022b). The studies demonstrate that the antimicrobial activity of *T. crispa* is against multiple pathogenic bacteria. The crude extract of *T. crispa* showed significant antibacterial activity. The zones of inhibition from 12 to 18 mm against the tested strains, *Escherichia coli* and *Staphylococcus aureus,* respectively (Shree and Krishnaveni, 2022a). An *in vitro* disk diffusion assay was carried out to evaluate the antimicrobial activity of the aqueous, ethanol, and chloroform extracts of *T. crispa* (25, 50, 75, and 100%) against various gram-positive (*Staphylococcus aureus, Listeria monocytogenes, Streptococcus pneumonia*) and gram-negative (*Shigella flexneri, Salmonella typhimurium, Escherichia coli*, *Klebsiella pneumonia,* and *Proteus vulgaris*). All extracts show efficiency against both gram-positive and gram-negative bacteria (Ahmad *et al*., 2016). Additionally, the aqueous extract of *T. crispa* showed a moderate inhibitory effect against *S. aureus* and *E. coli* using an agar diffusion method. The ethanolic extract showed largest antibacterial zone on *Streptococcus pneumoniae* and *Escherichia coli*, followed by extracts with ethanol, MeOH, and water. In addition, chloroform, petroleum ether, and methanolic extracts of the plant exhibited effective antibacterial properties against some gram-positive and gram-negative bacteria by using the disc diffusion (Pham and Nguyen, 2020).

The present study identified the phytochemical constituents of the aerial parts of *T. crispa* non-polar (DCM extract) and its antibacterial potential against pathogenic bacteria isolated from the intestine of broilers. The objective is to perform detailed phytochemical profiling of the aerial parts of *T. crispa* dichloromethane (DCM) extracts using GC and GC-MS techniques and to determine the antibacterial activity of the TC DCM fraction.

MATERIALS AND METHODS

Collection of plant material: Fresh aerial parts of the *Tinospora crispa* were collected from the Nursery of Sindh Agriculture University, Tandojam, in September 2024. The plant was identified and authenticated by Dr. Niaz Ahmed Wahocho, Professor in the Department of Horticulture, Faculty of Crop Production, Sindh Agriculture University, Tandojam. A voucher specimen was deposited under the reference number. HOR. 407.

Extraction and fractionation: The freshly collected ten kg of aerial parts of *T. crispa* were washed thoroughly with distilled water to remove debris particles. The TC plant material was air-dried at room temperature (30℃)

for 12 hours to remove surface moisture. Once dried, the material was cut into small pieces using a clean cutter.

Extraction was performed by soaking 10 kg of the aerial *T. crispa* plant material in 10 liters of Dichloromethane (DCM) (Merck KGaA, Germany) (99% purity) (Alabri *et al.,* 2014). DCM was selected due to its efficacy in extracting bioactive compounds from *T. crispa.* The extraction was carried out in laboratory-grade glass jars at room temperature, with the material allowed to soak in the solvent for 48 hours. All the samples were processed twice, repeating the above procedure. After completing the soaking period, the mixtures were filtered using filter paper No. 1 (11µm particle retention size, Ahlstrom-Munksjo) with the help of a glass filter funnel. The extract was concentrated in a rotary vacuum evaporator (Buchi-Rotavapor-R 200) at 40 °C using DCM, and the dried extract material was stored in a refrigerator (BDW-40L320, China) at 20°C for further use in phytochemical analysis. Five micrograms of the dried DCM extract were further diluted in 5 mL of deionized water as a stock solution for assessing antibacterial activity.

Preliminary phytochemical screening of *Tinospora crispa*: Qualitative phytochemical tests for the identification of steroids, terpenoids, fatty acids, tannins, saponins, anthocyanins, leucoanthocyanins, coumarins, emodins, carbohydrates, resins, alkaloids, glycosides, cardiac glycosides, and proteins were carried out for *T. crispa,* as described by (Yadav and Agarwala, 2011; Bhandari *et al.,* 2013; Kumari *et al.,* 2017; Joshi and Awasthi, 2022). Each test was performed in triplicate with appropriate positive and negative controls. Observations were based on color changes and the formation of precipitates.

Analysis of Dichloromethane soluble fraction by Gas chromatography (GC): Gas chromatography analysis of the dichloromethane fraction was carried out using a Shimadzu GC-17-A instrument equipped with a capillary column ZB-5 (30 m × 0.32 mm internal diameter × 0.25 µm film thickness). The system utilized a flame ionization detector (FID), with the detector temperature set at 260 °C and the injector temperature at 240 °C. helium (99.9 % purity) carrier gas) was used at a constant flow rate of 4.8835 mL/min. The oven temperature was initially set at 50 ºC and held for 5 minutes, then increased at a rate of 5 ºC per minute to 235 ºC and finally held at 235 ºC for 35 minutes. The separated compounds were then subjected to mass spectrometric detection, and the identified constituents are described in the results section.

Analysis of the Dichloromethane soluble fraction by GC-MS: The chemical composition of the *T. crispa* DCM extract was analyzed using a GC-MSTQ system and ZB-5MS (30 m × 250µm × 0.25 µm film thickness). The injector was operated in split mode with a 10:1 split ratio. The injection volume was 2 µl using a 10 µl syringe at an injection speed of 50 µL/s. The injection port was maintained at 250 ºC, with a total carrier gas (helium) flow of 16.2 mL/min and column flow of 1.2 mL/min (pressure: 9.79 psi). The septum purge flow was 3 mL/min, and gas saver mode (20 mL/min) was activated after 2 minutes. The oven temperature was programmed as follows: initially held at 50 ºC for 5 minutes, then ramped at 7 ºC/min to 200 ºC (held for 20 minutes), followed by a second ramp of 7 ºC/min to 300 ºC (held for 20 minutes). The total running time was 80.714 minutes. The transfer line temperature was maintained at 260 ºC, and both the helium quench gas and nitrogen collision gas for the QQQ collision cell were turned off. The instrument was operated under a vacuum for detection.

Identification of components through GC/GC-MS: GC and GC-MS were used to identify chemical compounds in each extract and fractionation. The Automated Mass Spectral Deconvolution and Identification System (AMDIS, version 2.69) was used to compare the acquired mass spectra with the National Institute of Standards and Technology (NIST) database in order to identify the compounds. This was followed by comparing peak retention times and spectra with those of standard compounds, using the eight-peak index method (Petrovick *et al.,* 1997). The identification process also involved matching the data with the NIST database and examining the characteristic fragmentation patterns specific to the mass spectra of a particular class of compounds.

Tested pathogenic microorganisms: The pathogenic organisms (*Escherichia coli* and *Salmonella typhimurium*) were collected from the microbiology laboratory at the Sindh Institute of Animal Health (SIAH), Karachi. The pathogenic organism was isolated from the gastrointestinal tract of infected broilers. Sick or diseased, freshly dead broiler birds were collected from different poultry farms, and individual cases subjected to clinical examination. A history of diarrhea, depression, and reduced growth, severe enteritis, general congestion of the internal abdominal viscera, as well as perihepatitis, pericarditis, and peritonitis. The samples were inoculated into Eosin Methylene Blue agar, MacConkey agar (Sigma, UK), XLD, BSA agar, and Salmonella-Shigella agar (Sigma, UK) and incubated aerobically at 37 °C for 24 h. After 24 hours, plates without growth were incubated for an additional 24 hours. The growth of microorganisms was identified by examining colony morphology, followed by biochemical identification.

Antibacterial activity assay (agar well diffusion method): The antibacterial activity of dichloromethane *T. crispa* extracts against pathogenic bacteria (*E. coli* and *S. typhimurium*) was evaluated using the agar well diffusion

method (Yeni and Sari, 2025). Bacterial suspensions were prepared in sterile normal saline and adjusted to a 0.5 McFarland standard. Tryptic soya agar (TSA) plates (Sigma, UK) were seeded with 100 µL of the bacterial inoculum. For each test compound, 3 µL and 6 µL were spotted into wells. Standard antibiotics (Enrofloxacin and Ciprofloxacin) were used as positive controls, while dimethyl sulfoxide (DMSO) served as a negative control. The plates were incubated aerobically at 37°C for 24 hours. After incubation, the diameter of the inhibition zones was measured in millimeters. The antibacterial activity of DCM TC extracts, standard antibiotics, and DMSO) was compared. Each experiment was performed in triplicate (n=3) following CRD design and results were expressed as the mean zone of inhibition (mm ± SEM).

Preparation of antibiotic stock solutions: Ciprofloxacin (2 mg/ml) and enrofloxacin (20% solution; 200 mg/ml) were used as positive controls. For each antibiotic, a 100 µg/ml solution was prepared. For enrofloxacin, 5 µl of the stock solution was diluted with 9.995 ml of sterile distilled water. In contrast, for ciprofloxacin, 0.5 ml of the stock solution was diluted with 9.5 ml of sterile distilled water. In the antibacterial assay, 6 µL (0.6 µg) of each working solution was dispensed into wells.

Statistical analysis: Data were tabulated using Microsoft Excel 365, and Statistics version 8.1 SPSS software was used. Each experiment was conducted in triplicate (n = 3), and results are presented as mean ± standard error of the mean (SEM). Data was analyzed using and a T-test at $p<0.05$, which was considered statistically significant. Post-hoc comparisons were performed using Tukey's HSD test for all pairwise comparisons.

RESULTS

The Dichloromethane extract from the aerial parts of *T. crispa* contains secondary metabolites such as steroids, terpenoids, fatty acids, tannins, saponins, coumarins, carbohydrates, resins, alkaloids, cardiac glycosides, and proteins, as indicated by characteristic color changes or precipitate formation shown in Figure 1. Table 1 presents the phytoconstituents, types of tests performed, observations, and presence of constituents in *T. crispa*.

Table 1. The phytochemical constituents, type of test, and observations of the Dichloromethane extract of the aerial parts of the *T. crispa.*

S.no	Phytoconstituents	Type of test	Observations	Analysis
1.	Steroids	Salkowski test	The upper (chloroform) layer turns red and the acid layer shows a yellow color with green fluorescence	+
2	Terpenoids	Liebermann-Burchard test	blue and green rings indicate terpenoids	+
3	Fatty acids	Filter paper test	transparent residue (clear filter paper)	+
4	Tannins	Lead acetate test	A yellowish precipitate form	+
5	Saponins	Foam test	Foam formation	+
6	Anthocyanins	HCI ammonia test	An initial pink-red appearance that turns blue-violet	-
7	Leucoanthocyanins	Isoamyl alcohol test	The upper layer appears red	-
8	Coumarins	NaOH test	The formation of a yellow color	+
9	Emodins	Ammonia- benzene test	The appearance of a red color	-
10	Carbohydrates	Molisch's test	A violet ring at the interface indicates carbohydrates	+
11	Resins	Turbidity test	The appearance of turbidity (cloudiness)	+
12	Alkaloids	Mayer's test	A creamy white precipitate forms,	+
13	Glycosides	Wanger,s test	A reddish-brown precipitate forms,	+
14	Cardiac Glycosides	Keller-kiliani test	A green-blue color appears	+
15	Proteins	Biuret test	A violet/pink color develops	+

Indications: "+" means positive activity, "-" negative activity

The antibacterial activity of DCM TC extract against *E. coli* and *S. typhimurium* is presented in Table 4. Statistical analysis indicated that treatments had a highly significant ($p<0.0001$) effect on inhabitant zones for both pathogenic bacteria. The DCM extract of *T. crispa* exhibited moderate antibacterial activity in a concentration-dependent manner, *S. typhimurium* producing inhibition zones of 15.6 ± 0.3 mm (6µl) and 11.6 ± 0.3 mm (3µl) (Figure 6). In contrast, antibacterial activity against *E. coli* was comparatively lower, with zones of inhibition 9.*2* ± 0.0.1 mm (6 µl) and 3.9 ± 0.05 mm (3 µl) (Figure 7). Among the positive controls, ciprofloxacin showed the highest activity with mean inhibition zones of 27.8 ± 0.06 mm for *E. coli* (Figure 7) and 24.6 ± 0.3 mm for *S. typhimurium*, followed by enrofloxacin (25.6 ± 0.3 and 22.6 ± 0.3 mm, respectively) (Figure 6). DMSO (negative control) (Figures 6 and 7) did not show any inhibitory effect.

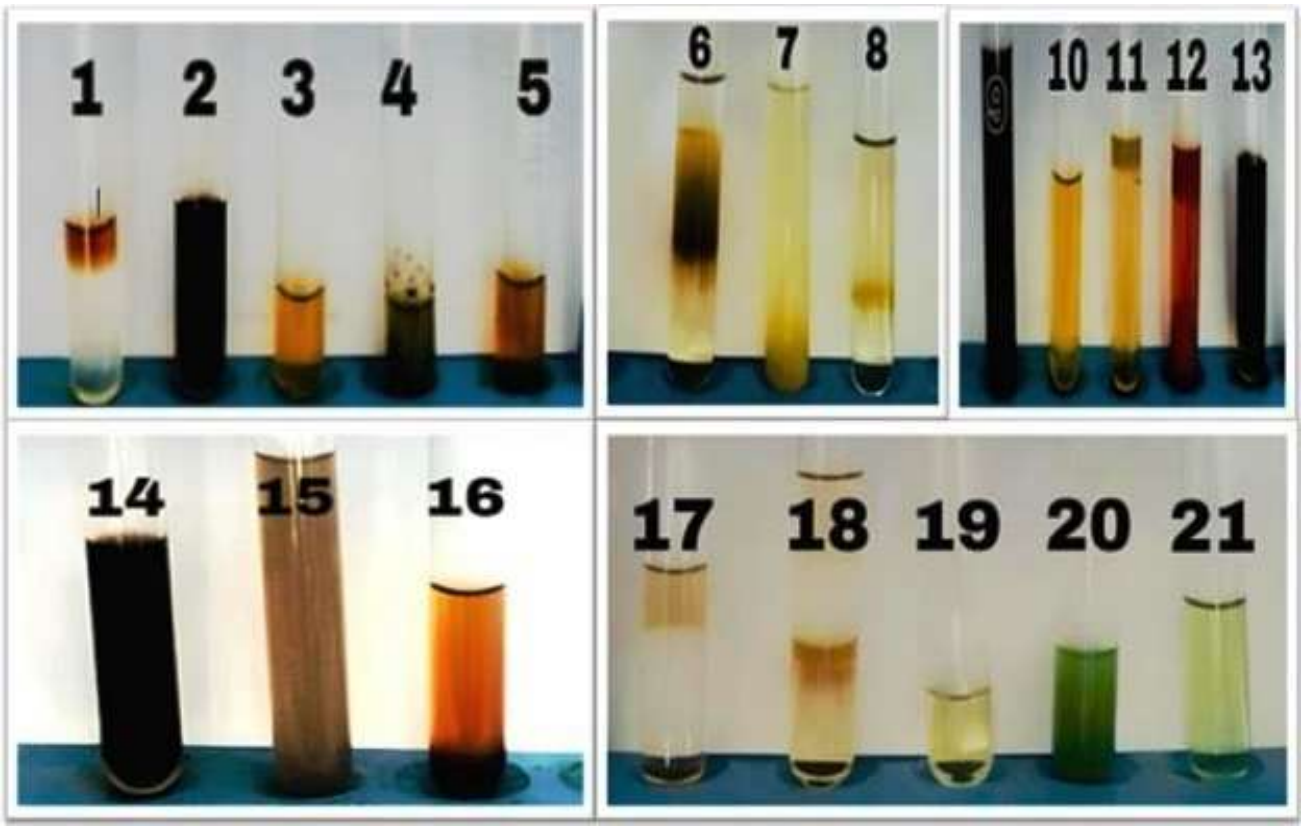
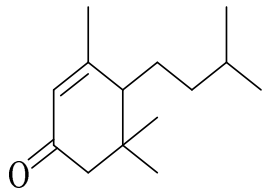
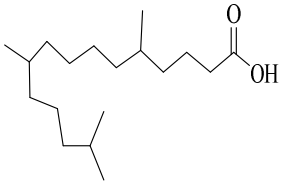
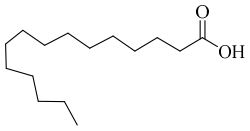
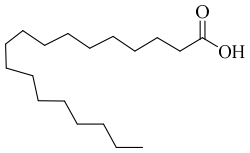
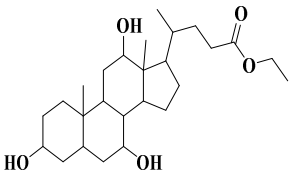
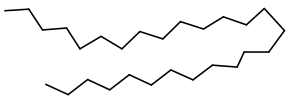
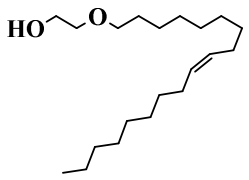
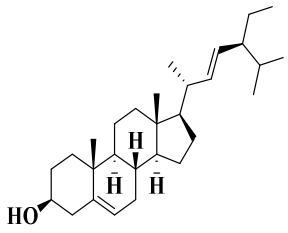
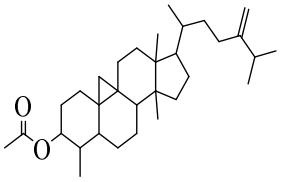
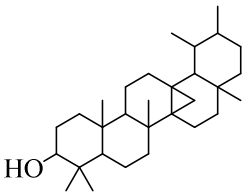
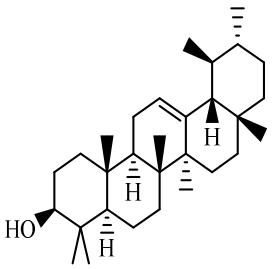
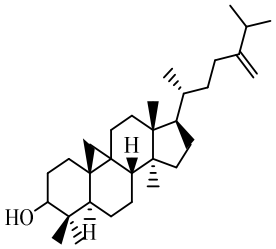
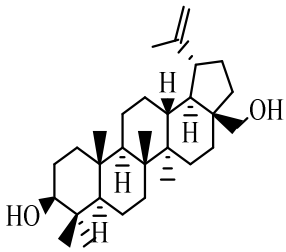


Fig. 1. Qualitative phytochemical screening of the Dichloromethane extract of *T. crispa* aerial parts based on characteristic color changes or precipitate formation: test tubes, 1. Salkowski test, 2. Liebermann-Burchard test, 3. Filter paper test, 4. Lead acetate test, 5. Foam test, 6.HCl-Ammonia test, 7. Isoamyl alcohol test, 8. NaOH test, 9. Ammonia-Benzene test, 10. Molisch's test, 11. Turbidity test, 12. Mayer's test, 13. Wagner's test, 14. Keller-Kiliani test, 15. Biuret test, Test tubes 16-21 represent negative controls.

Table 2. GC-MS profile of DCM-PE soluble fraction of Dichloromethane extract of aerial parts of *T. crispa*

S-no	Name of compounds	Molecular formula	Structure	Mass fragments (% rel.)	Retention time (RT)	Area sum %	Match value %
1	2-Cyclohexen-1-one, 4-(3-hydroxybutyl)-3,5,5-trimethyl.	$C_{13}H_{22}O_2$)		41 (85.5), 43 (100), 45 (67.3), 69 (76.6), 95 (67.7), 109 (70.5), 135 (73.8), 150 (551).	24.2	0.33	88.5
2	2-Pentadecanone, 6,10,14-trimethyl.	$C_{18}H_{36}O$		43 (100), 58 (89.8), 71 (45.0), 57 (42.6), 59 (40.7), 41 (37.3), 55 (34.5), 69 (24.5), 85 (22.9), 95 (20.3).	26.3	0.61	93.6

3	*n*-Hexadecanoic acid.	$C_{16}H_{32}O_2$		60 (100), 73 (98.0), 57 (84.0), 43 (81.7), 55 (76.7), 41 (57.4), 129(43.5), 71 (37.3), 69 (35.1), 83 (26.7).	28.3	4.33	93.3
4	Octadecanoic acid.	$C_{18}H_{36}O_2$)		43 (100), 73 (94.2), 60 (88.1), 57 (87.5), 55 (27.0), 41 (68.8), 129 (50.0), 69 (43.2), 71 (42.9), 83 (31.7).	33.6	0.96	89.9
5	Ethyl iso-allocholate.	$C_{26}H_{44}O_5$		43 (100), 55 (91.4), 41 (86.7), 57 (797), 69 (609), 81 (547), 44 (492).	57.3	0.57	74
6	Heptacosane.	$C_{27}H_{56}$		57 (100), 43 (79.8), 71 (62.2), 85 (41.6), 55 (28.3), 41 (27.5), 69 (17.6), 99 (14.0), 56 (13.9), 29 (13.5).	58.3	1.6	90.4
7	Ethanol, 2-(9-octadecenyloxy)-, (*Z*).	$C_{20}H_{40}O_2$		55 (100), 82 (96.8), 69 (77.0), 96 (70.8), 83 (67.0), 67 (66.4), 81 (62.7), 41 (62.1), 43 (57.7), 95 (54.6).	60.5	11.81	80.3
8	Stigmasterol.	$C_{29}H_{48}O$		55 (100), 83 (86.7), 81 (73.7), 255 (63.4), 69 (63.1), 41 (4.55), 95 (44.7), 43 (44.5), 97 (42.7),	62.1	2.51	79.3

9	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3*β*,4*α*,5*α*).	$C_{32}H_{52}O_2$		133 (39.5). 43 (100), 55 (83.4), 69 (63.9), 95 (54.8), 81 (53.1), 41 (51.7), 107 (44.6), 93 (41.5), 105 (40.0), 109 (34.9).	62.7	8.42	80.3
10	6a,14a-Methanopicene, perhydro-1,2,4a,6b,9,9,12a-heptamethyl-10-hydroxy.	$C_{30}H_{50}O$		426 (100), 95 (72.6), 109 (54.8), 123 (49.3), 135 (48.2), 121 (47.7), 69 (47.1), 55 (44.8), 81 (42.2), 205 (38.1).	63.3	43.85	78.3
11	*α*-Amyrin.	$C_{30}H_{50}O$		218 (100), 44 (25.1), 219 (19.0), 43 (15.7), 55 (15.4), 203 (14.8), 69 13.7), 426 (13.5), 189 (13.0), 122 (11.8).	63.7	4.19	85.7
12	9,19-Cyclolanostan-3-ol, 24-methylene-, (3*β*).	$C_{31}H_{52}O$		55 (100), 95 (96.9), 81 (69.9), 107 (67.9), 69 (64.9), 109 (59.9), 93 (57.9), 121 (55.9), 43 (52.9).	63.9	4.37	95.6
13	Betulin.	$C_{30}H_{50}O_2$		189 (100), 95 (83.0), 135 (72.0), 81 (69.1), 203 (65.3), 55 (62.1), 93(60.9), 121 (60.6), 107 (60.1), 41 (59.8).	59.9	3.29	80.4

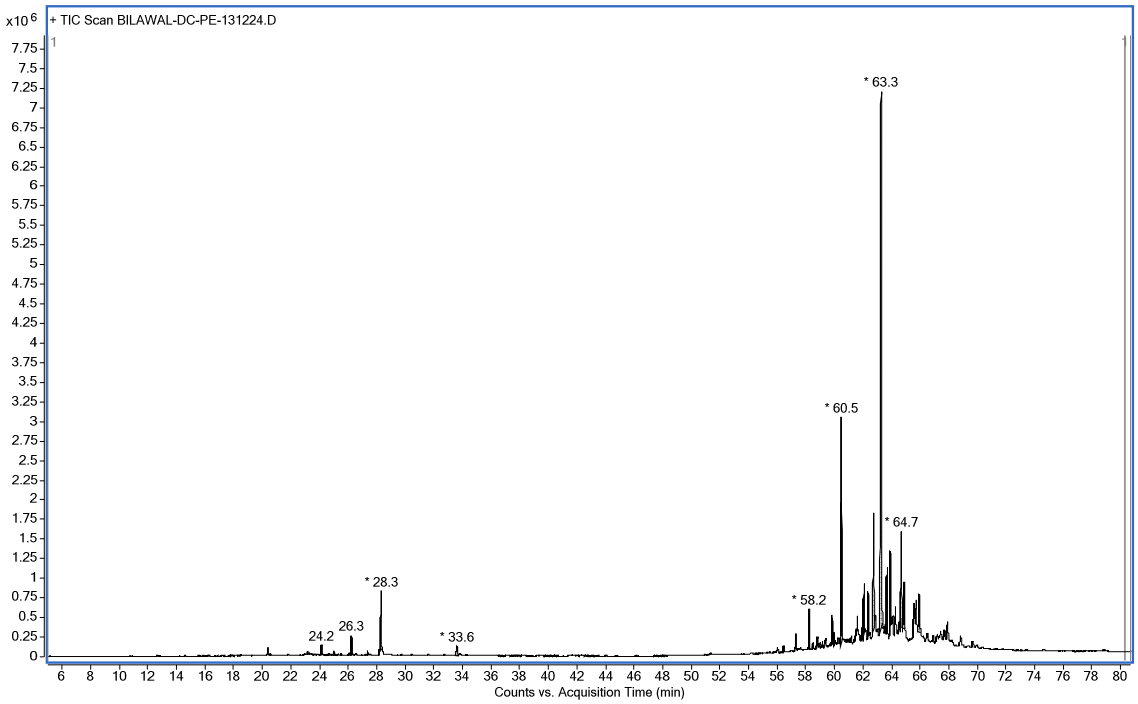


Fig. 2. GC-MS chromatogram of the DCM extract of *T. crispa* aerial parts. Major peaks were observed at retention times 24.2, 26.3, 28.3, 33.6, 58.2, 60.5, 63.3, and 64.7 minutes, indicating the presence of diverse phytochemical constituents.

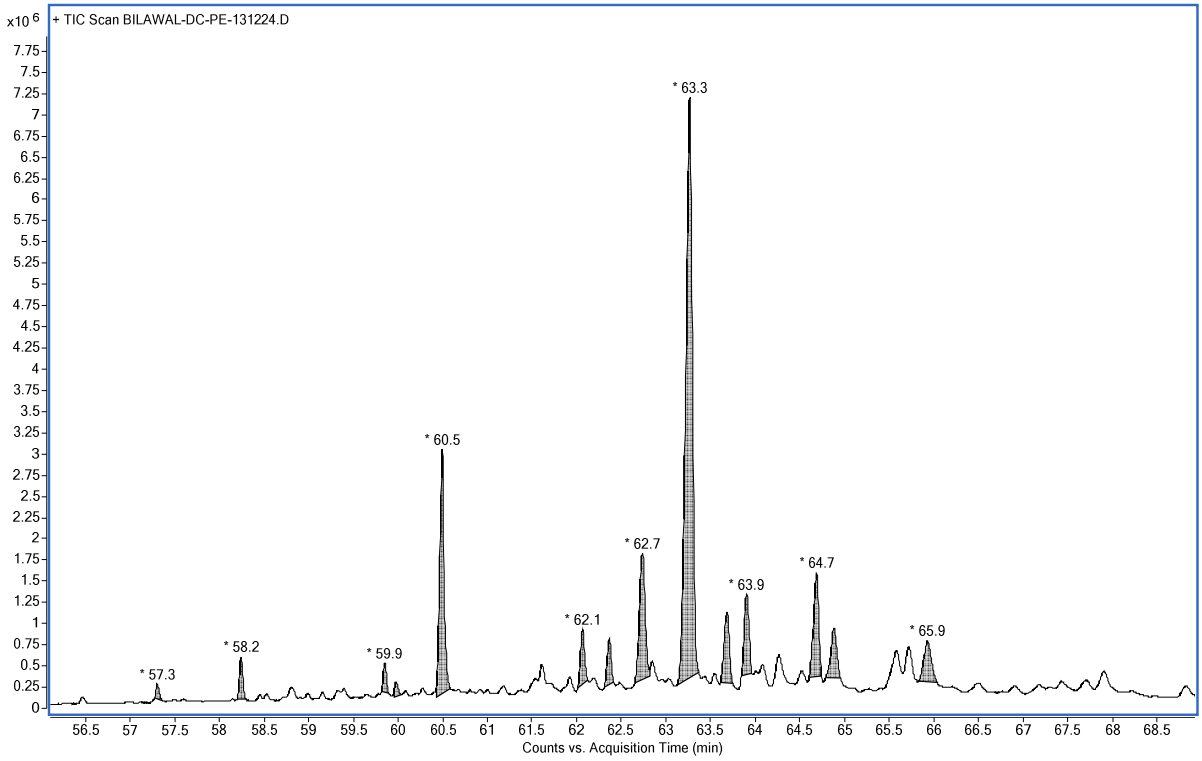
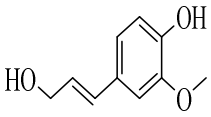
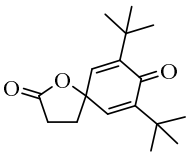
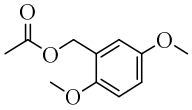
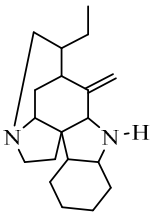
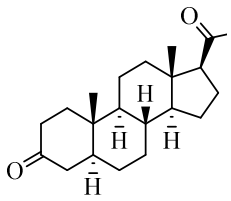
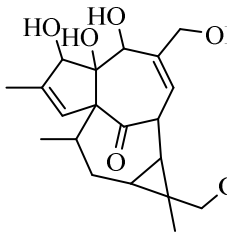
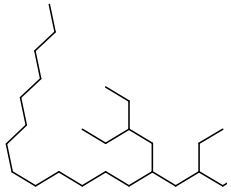
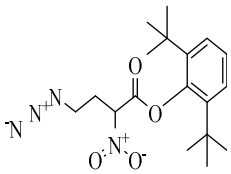
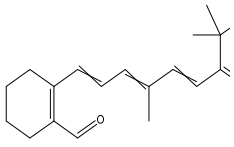
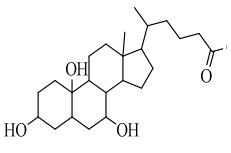
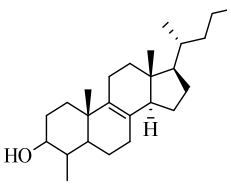
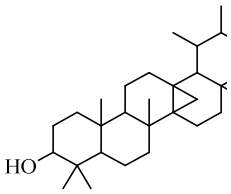
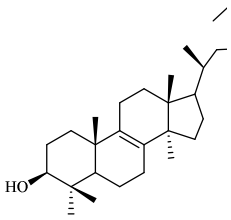
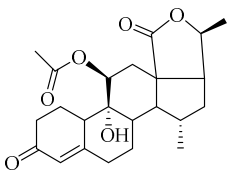


Fig. 3. GC-MS chromatogram of *T. crispa* DCM extract showing the dominant peaks between 57–66 minutes. Peaks at retention times 58.2, 60.5, 62.1, 62.7, 63.3, 63.9, 64.7, and 65.9 minutes suggest the presence of multiple phytochemicals.

Table 3. GC-MS profile of TC-DCM soluble fraction of Dichloromethane extract of aerial parts of *T. crispa*.

S-no	Name of compounds	Molecular Formula	Structure	Mass fragments (% rel.)	RT	Area sum %	Match value %
1	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxypheno.l	$C_{10}H_{12}O_3$		137 (100), 180 (68.9), 124(59.1), 91 (39.5), 77 (29.1), 119 (28.6), 65 (21.4), 103 (20.8), 51 (20.0), 55 (19.7).	24.7	2.43	94.2
2	5,5,8a-Trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene.	$C_{12}H_{20}O$		124 (100), 96 (19.9), 83 (15.2), 41 (13.5), 82 (12.7), 125 (10.2), 55 (9.8), 111 (7.9), 39 (7.1), 54 (6.3).	25.5	0.37	76.4
3	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione.	$C_{17}H_{24}O_3$		57 (100), 205 (53.7), 55 (48.1), 175 (31.8), 217 (25.7), 177 (24.6), 189 (24.4), 220 (20.2), 91 (18.0), 109 (13.2).	27.2	0.29	92.1
4	Benzenemethanol, 2,5-dimethoxy-, acetate.	$C_{11}H_{14}O_4$		210 (100), 168 (71.4), 43 (46.4), 121 (46.3), 167 (46.3), 151 (35.6), 135 (28.1), 153 (26.3), 139 (25.7), 125 (24.9).	28.8	2.48	69.4
5	Curan, 16,17-didehydro-, (20.xi.).	$C_{19}H_{24}N_2$		55 (100), 280 (78.0), 43 (71.0), 41 (70.0), 144 (51.0), 182 (49.0), 57 (42.0), 196 (40.0), 69 (38.0), 197 (38.0).	51.6	0.52	62.1

6	3,20-Allopregnanedione.	$C_{21}H_{32}O_2$		43 (100), 84 (60.5), 316 (54.9), 55 (44.0), 81 (38.4), 231 (38.0), 41 (37.7), 67 (32.7), 95 (31.4), 298 (31.2).	55.1	0.47	84
7	1H-2,8a-Methanocyclopenta[a]s cyclopropa[e]cyclodecen-11-one, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a,6-trihydroxy-1,4-bis(hydroxymethyl)-1,7,9-trimethyl.	$C_{20}H_{28}O_6$		43 (100), 41 (81.8), 77 (71.8), 91 (71.1), 93 (53.6), 121 (53.6), 53 (48.3), 55 (44.2), 79 (44.2), 39 (43.6).	55.8	0.87	77.5
8	Octadecane, 3-ethyl-5-(2-ethylbutyl).	$C_{26}H_{54}$		43 (100), 57 (70.1), 71 (58.3), 85 (41.8), 55 (37.1), 41 (33.7), 69 (21.5), 70 (14.4), 99 (13.4), 83 (13.0).	56.5	0.68	75.6
9	4-Azido-2-nitrobutyric acid, 2,6-di-t-butyl-4-methoxyphenyl ester.	$C_{19}H_{28}N_4O_5$		43 (100), 57 (86.9), 236 (69.0), 235 (50.5), 73 (46.0), 179 (44.3), 69 (428), 221 (38.9), 41 (36.8), 55 (30.0).	57	10.09	70.5
10	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde.	$C_{23}H_{32}O$		43 (100), 41 (59.0), 55 (950.0), 91 (46.0), 135 (43.0), 69 (42.0), 79 (37.0), 105 (36.0), 95 (35.0), 81 (34.0).	57.9	0.49	79.3
11	Ethyl iso-allocholate.	$C_{26}H_{44}O_5$		43 (100), 55 (91.4), 41 (86.7), 57 (79.7), 69 (60.9), 81 (54.7),	58.2	0.64	79.8

12	Cholesta-8,24-dien-3-ol, 4-methyl-, (*3β,4α*).	$C_{28}H_{46}O$		44 (49.2), 29 (47.6), 17 (46.9), 83 (46.0), 41(100), 69 (66.0), 43(58.0), 55(58.0) 57(31.0), 29(28.0), 91(20.0), 105(20.0, 27(19.0), 39 (18.0).	58.8	1.14	77.8
13	6a,14a-Methanopicene, perhydro-1,2,4a,6b,9,9,12a-heptamethyl-10-hydroxy.	$C_{30}H_{50}O$		426 (100), 95 (72.6), 109 (54.8), 123 (49.3), 135 (48.2), 121 (47.7), 69 (47.1), 55 (44.8), 81 (42.2), 205 (38.1).	59.1	1.17	78
14	17-(1,5-Dimethyl-3-phenylthiohex-4-enyl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopent(a)phenanthren-3-ol.	$C_{36}H_{54}OS$		109(100), 81 (57.8), 43(56.8), 409 (56.8), 55(46.4), 69(42.6), 41 (39.3), 95 (37.2), 107 (3.4), 93 (30.9).	61.9	0.57	77.7
15	Pregn-4-en-18-oic acid, 11-(acetyloxy)-7,9,20-trihydroxy-3-oxo-, γ-lactone, (7*α*,11*α*,20R).	$C_{23}H_{30}O_7$		43 (100), 235 (65.2), 418 (36.7), 124 (36.3), 55 (31.4), 67(26.5), 79(24.0), 41 (22.8), 340 (20.6), 81 (17.3).	63.7	5	65.2

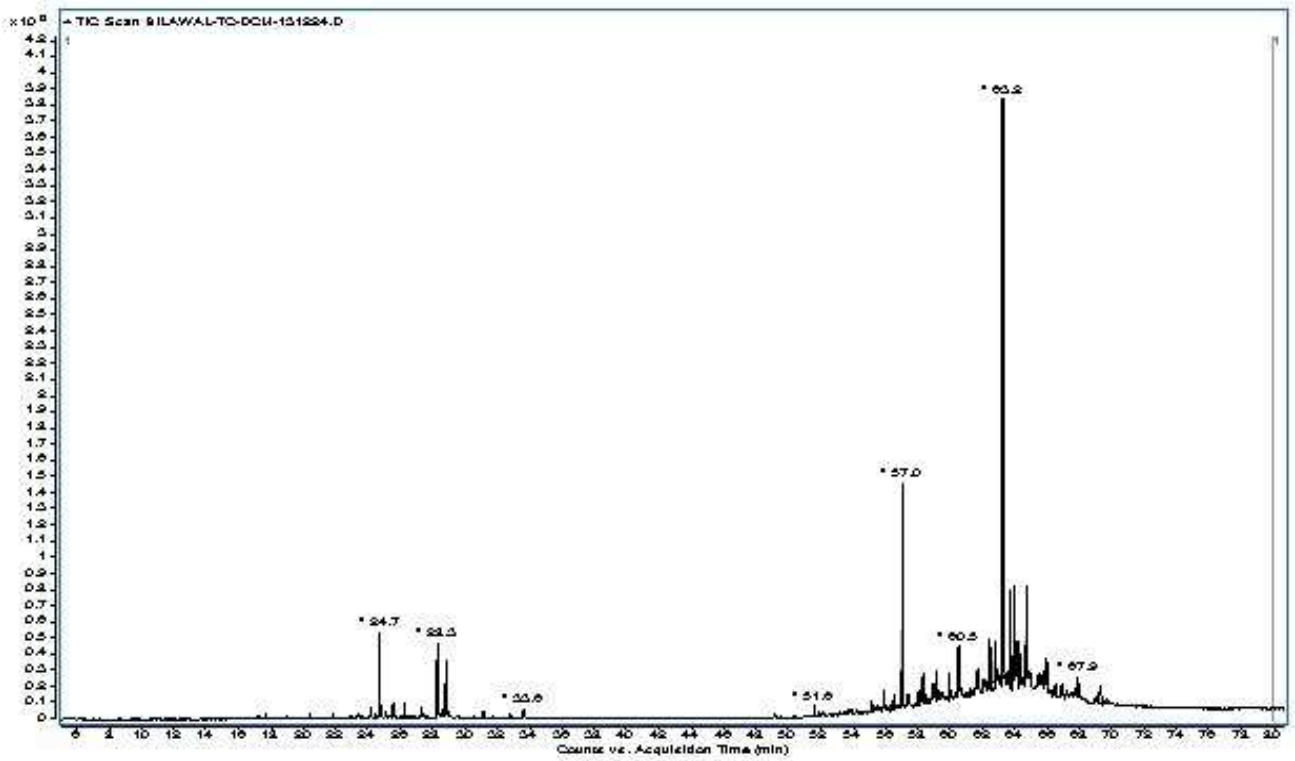


Fig. 4. GC-MS chromatogram of *T. crispa* DCM extract. This chromatogram displays a major peak at 63.2 minutes along with notable peaks at 24.7, 28.3, 33.6, 51.6, 57.5, and 67.9 minutes, revealing the presence of bioactive components in the extract.

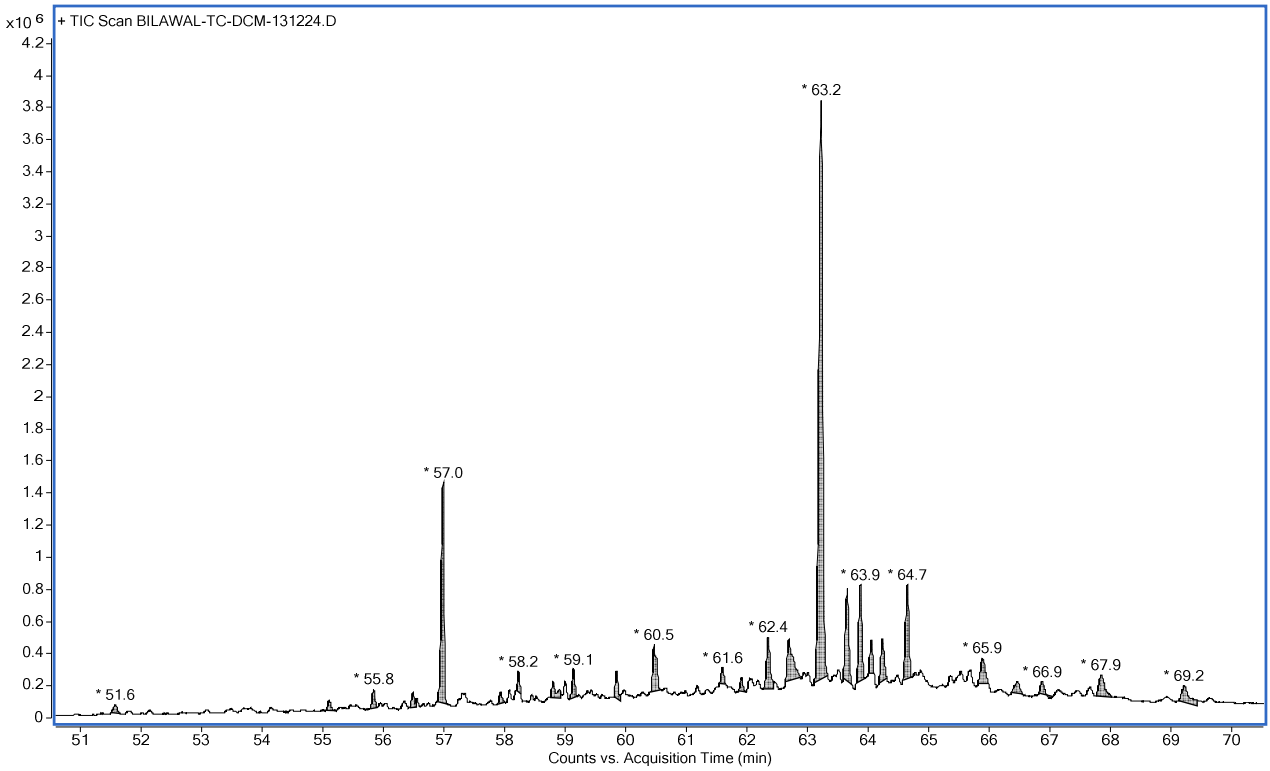


Fig. 5. Total ion chromatogram (TIC) of the DCM extract of *T. crispa* analyzed through GC-MS. Major peaks were observed between 57-64 min, indicating the presence of multiple volatile compounds.

Table 4. Mean inhibition zones (mm) of the pathogenic bacteria for the DCM *Tinospora crispa* extract.

Treatments	Zone of inhibition (mm)	
	Escherichia coli	*Salmonella typhimurium*
DCM TC extract 3µl	9.2±0.1[d]	11.6±0.3[d]
DCM TC extract 6µl	13.9±0.05[c]	15.6±0.3[c]
Enrofloxacin 6µl	25.6±0.3[b]	22.6±0.3[b]
Ciprofloxacin 6µl	27.8±0.06[a]	24.6±0.3[a]
DMSO	0.00±0.0[e]	0.00±0.0[e]

Dimethyl sulfoxide (DMSO), Means within the same column followed by different superscript letters differ significantly (Tukey's HSD, P<0.05). Overall treatment effect was highly significant for *E. coli* (P < 0.0001) and *S. typhimurium* (P<0.0001)

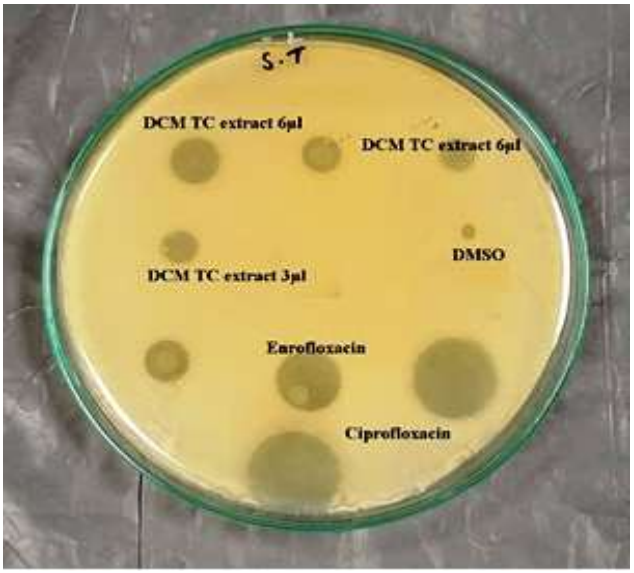


Fig. 6. Showing zone of inhibition, produced by DCM TC extract 3 and 6µl, standard antibiotics and DMSO (negative control) against *S. typhimurium*, on tryptic soya agar (TSA) plates.

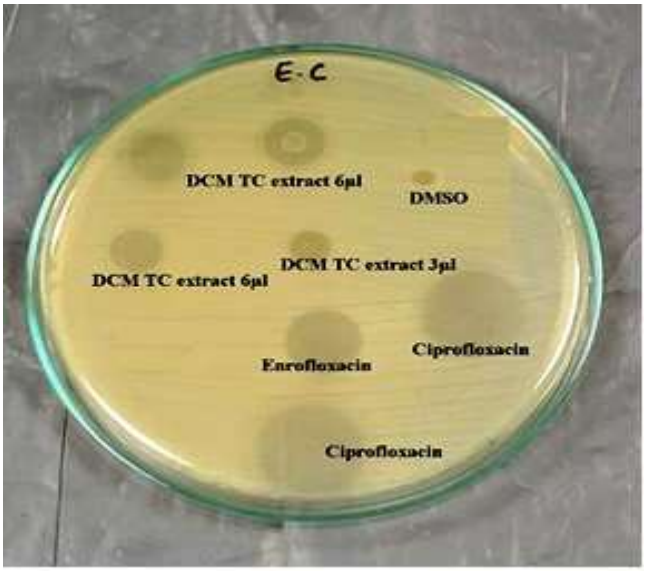


Fig. 7. Showing zone of inhibition, produced by DCM TC extract 3 and 6 µl, standard antibiotics and DMSO (negative control) against *E. coli,* on tryptic soya agar (TSA) plates.

DISCUSSION

The qualitative screening of *T. crispa* dichloromethane (DCM) extract from the aerial parts revealed a complex profile of phytochemical compounds. The extract contained several secondary metabolites, including steroids, terpenoids, fatty acids, tannins, saponins, coumarins, carbohydrates, resins, alkaloids, glycosides, cardiac glycosides, and proteins; however, anthocyanins, leucoanthocyanins, and emodins were absent due to a lack of fruits and flowers in the DCM extract. Distinct color changes and precipitate formation during the tests confirmed the presence of various metabolites, such as the violet ring in Molisch's test for carbohydrates, the blue and green rings for terpenoids, and the red upper layer with green fluorescence in the steroid test (Harwoko and Warsinah, 2020) and support with recent findings on the chemical diversity of *T. crispa* extracts (Shree and Krishnaveni, 2022b; Jaya *et al.,* 2024). The red chloroform layer and the yellow acid layer with green fluorescence that shows the positive reaction for the presence of steroidal compounds correspond to their known anti-inflammatory and immunomodulatory properties. Terpenoid was confirmed by producing blue and green rings (Ahmad *et al.*, 2016; Haque *et al.,* 2017), supporting the use of *T. crispa* in ethnomedicine to treat inflammatory and infection-related disorders (Neneng *et al.,* 2025).

The presence of fatty acids in the extracts, which support their anti-inflammatory and membrane-stabilizing properties, is indicated by clear residue formation on filter papers. The reddish precipitate formation indicates the presence of tannin during the lead acetate test for tannins.

These are the polyphenolic compounds, known for their astringent and antioxidant properties, involved in modulating inflammatory responses (Wangchuk *et al.,* 2011; Haque *et al.,* 2020; Yuandani *et al.,* 2023). The

presence of the saponins is indicated by foam formation during the test. Saponins are compounds that have antibacterial and immunostimulatory activities. Moreover, yellow coloring indicated by the presence seen in the NaOH test points confirmed the presence of coumarins, which possess their antibacterial and anticoagulant properties in the plant extract (Shovon, 2012; Kumar *et al.,* 2013). The absence of pigmentation in the HCl-ammonia and isoamyl alcohol tests confirmed the lack of anthocyanins and leucoanthocyanins.

During photochemical screening, the violet ring indicated carbohydrates through Molisch test, the turbidity test (cloudiness) indicated resins, the Mayer test shows the creamy white precipitate of alkaloids, a reddish-brown form shows the presence of glycosides by wanger test, the green-blue color indicates the presence of cardiac glycosides by the Keller-Kiliani test, a vloet/pink color indicated the presence of protein by the biuret test. These metabolites have been associated with broad-spectrum pharmacological activity such as antimalarial, antimicrobial, antipyretic, antihypertensive, antioxidant, and antidiabetic properties, and cardiovascular therapy by modifying the activity of Na+/K+-ATPase. These findings collectively highlight the phytochemical diversity of *Tinospora crispa* and its relevance to ethnopharmacological practices (Chaudhary *et al.,* 2024).

The present study finds bioactive compounds in the DCM extract of *T. crispa* by using Gas Chromatography (GC) and Gas Chromatography with Mass Spectrometry (GC-MS). Major classes included oxygenated monoterpenes, fatty acids, long-chain ketones, alkanes, sterols, and triterpenoids. *T. crispa* plays an important role in the discovery of new bioactive compounds that can be used in pharmaceutical research and development. Specifically, 2-Cyclohexen-1-one, 4-(3-hydroxybutyl)-3,5,5-trimethyl was identified through GC-MS as an oxygenated monoterpene, known for its antimicrobial activities, despite being found in low concentrations (Haque *et al.,* 2023). Methylated ketones, such as 2-Pentadecanone, 6,10,14-trimethyl, have demonstrated anti-inflammatory properties of ketonic compounds (Okereke *et al.,* 2023). Additionally, several fatty acids, such as Octadecanoic acid and n-Hexadecanoic acid, also show antibacterial and anti-inflammatory properties, providing mechanistic support for the ethnobotanical use of the extract of plant (Haque *et al.,* 2023).

Steroidal and triterpenoid compounds were highly abundant Stigmasterol, α-Amyrin, and Betulin are known to have anti-inflammatory, antioxidant, and anticancer activities (Shahid *et al.,* 2009; Kumar *et al.,* 2013; Kumar *et al.,* 2020). Specifically, the compound 6a,14a-Methanopicene, perhydro-1,2,4a,6b,9,9,12a-heptamethyl-10-hydroxy, which appears to be a major contributor to the significant biological activities of *T. crispa*, along with other sterol derivatives such as 9,19-Cyclolanostan-3-ol, 24-methylene-, (3β), supports that triterpenoids play an important role in the therapeutic potential, in inflammatory and modulation of oxidative stress (Shree and Krishnaveni, 2022a; Haque *et al.,* 2023).

Among those key chemical compounds, 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol and 5,5,8a-Trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene were identified, contributing powerful biological activities, such as antioxidant and antimicrobial activities (Rakib *et al.* 2020). Aromatic compounds such as Benzenemethanol, 2,5-dimethoxy, and acetate improve the chemical diversity of the plant extract, contributing to its overall bioactivity (Jaya *et al.,* 2024). Furthermore, identification of 17-(1,5-Dimethyl-3-phenylthiohex-4-enyl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopent(a)phenanthren-3-ol and Pregn-4-en-18-oic acid, 11-(acetyloxy)-7,9,20-trihydro and Pregn-4-en-18-oic acid, 11-(acetyloxy)-7,9,20-trihydroxy-3-oxo-, γ-lactone, suggests the presence of modified steroids and lactones (Sinha *et al.,* 2017) Because these compounds can potentially, these compounds may have a significant role in cardioprotective and hormonal modulatory activities, which has been previously reported by (Shree and Krishnaveni, 2022b; Haque *et al.,* 2023). The *T. crispa* dichloromethane (DCM) aerial parts extracts show antibacterial activity against *E. coli* and *S. typhimurium* in a concentration-dependent manner, with a 9 to 16 mm zone of inhibition, respectively. In contrast, ciprofloxacin and enrofloxacin antibiotics were demonstrated markedly larger zones (22-28 mm), highlighting the higher potency of conventional antibiotics. Similar trends of concentration-dependent inhibition by *T. crispa* extracts (Yeni and Sari, 2025). Phytochemicals such as alkaloids, flavonoids, tannins, and terpenoids have antibacterial properties, which may be attributed to their action through mechanisms including cell wall rupture and enzyme inhibition (Ramli *et al.,* 2023). However, the activity of the crude extract remained lower, likely due to the low concentration of the TC extract's active compounds and the inherent resistance of gram-negative bacteria, which possess complex cell wall structures that restrict phytochemical penetration (Haque *et al.,* 2023).

Conclusion: Phytochemical screening of the DCM aerial parts of *T. crispa* revealed the presence of steroids, terpenoids, fatty acids, tannins, saponins, carbohydrates, resins, alkaloids, glycosides, cardiac glycosides, and proteins. GC-MS analysis identified twenty-eight compounds, including 2-Cyclohexen-1-one, 4-(3-hydroxybutyl)-3,5,5-trimethyl-, n-Hexadecanoic acid, Octadecanoic acid, Stigmasterol, Ethyl iso-allocholate, α-Amyrin, Betulin, known for its antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, antiviral, anticancer

properties. The DCM TC fraction shows significant antibacterial activity against *E. coli* and *S. typhimurium,* with inhibition zones increasing in a concentration-dependent manner. Based on the findings of this study, the aerial parts of *T. crispa* may represent a promising natural source of medicinal compounds, owing to the presence of diverse phytochemicals and bioactive constituents.

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Conflicts of interest: The author declares no conflict of interest.

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