Chemical Composition, Antioxidant, and Antimicrobial Activities of Bark and Wood Extracts from *Taxodium distichum* L.

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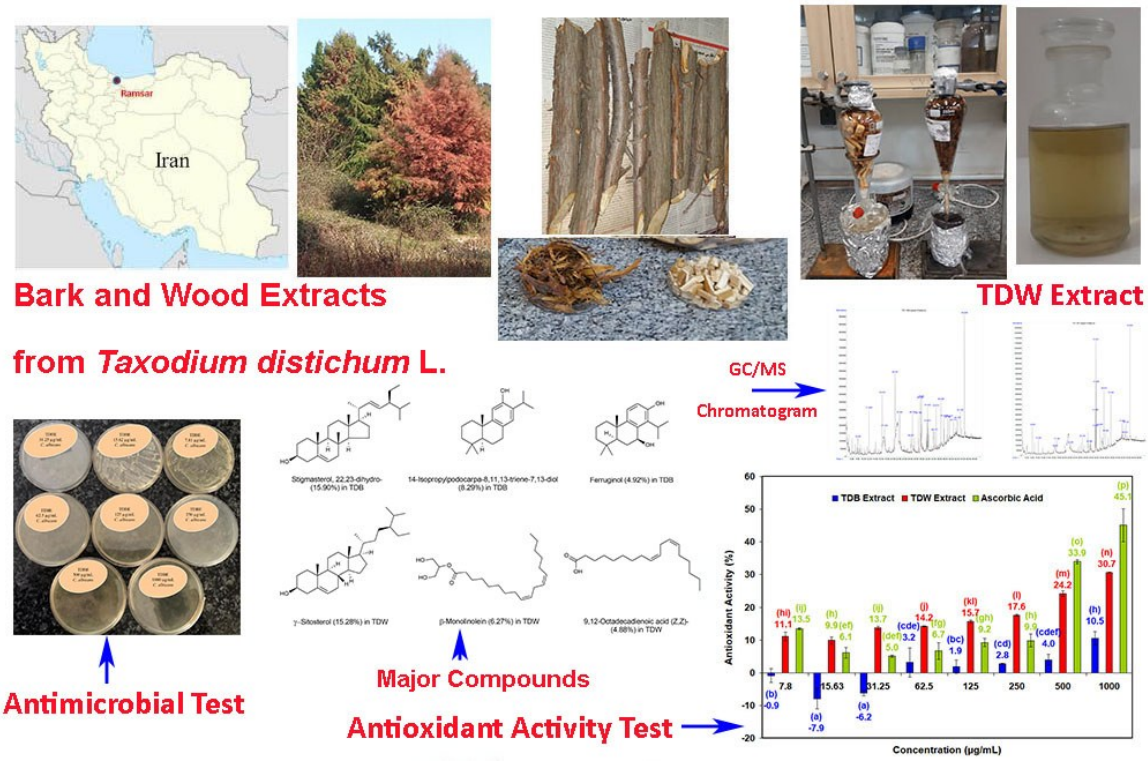
GRAPHICAL ABSTRACT



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The chemical composition and bioactivities were studied for hydroethanolic extracts from *Taxodium distichum* L. bark (TDB) and wood (TDW) samples harvested in Ramsar, Northern Iran. The TDW extract showed higher antioxidant activity (30.7%) compared to the TDB extract (10.5%) at the same concentration (1000 µg/mL), indicating a richer redox-active profile in wood tissues. The TDB extracts exhibited higher efficacy in antimicrobial tests, with minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) values against *Staphylococcus aureus* of 500 and 1000 µg/mL, respectively. Surprisingly, the MIC and minimum fungicidal concentration (MFC) of TDB extract against *Candida albicans* were 7.81 and 31.2 µg/mL, respectively. The TDW extract had no antibacterial activity but showed fungicidal activity at 7.81 µg/mL against *C. albicans*. These differences indicate that the TDB extract is more potent against microbial pathogens, while the TDW extract offers superior antioxidant potential. Gas chromatography-mass spectrometry (GC-MS) analysis revealed the presence of 22,23-dihydro-stigmasterol (15.9%) in TDB extract and γ-sitosterol (15.3%) in TDW extract as the major compounds. The combined findings underscore the therapeutic relevance of *T. distichum* bark and wood extracts as natural sources of bioactive compounds with possible applications in antimicrobial and antioxidant formulations for pharmaceutical, food, and cosmetic industries.

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Keywords: Taxodium distichum; Bark and wood extracts; Antimicrobial activity; Chemical composition; GC/MS

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INTRODUCTION

Natural products derived from plant sources have historically played a crucial role in the development of therapeutic agents, particularly exhibiting diverse chemical structures. Several compounds have shown excellent and novel antifungal, antibacterial, and antioxidant activities (HosseiniHashemi *et al*. 2016a,b; Unnam *et al*. 2025; Swain *et al*. 2025). Recently, resistance towards multiple antibacterial drugs has been reported, and this alarming situation has left only limited choices for antibacterial therapy (Aiesh *et al*. 2023). For this reason, there has been growing interest among natural and pharmaceutical

scientists in exploring medicinal plants as potential sources of antimicrobial agents (El-Saadony *et al*. 2025). Among various plant parts, bark and wood are often overlooked despite being rich sources of phenolics, terpenoids, flavonoids, and other secondary metabolites with potent biological activities (Salem *et al.* 2016; Barzegari *et al.* 2023; HosseiniHashemi *et al*. 2025).

T. distichum L. (Cupressaceae), commonly known as bald cypress, is a deciduous conifer native to the southeastern United States but cultivated in various parts of the world, including Iran (Mostafanejad and Sadati 2008). It has traditionally been valued for its durable timber, resistance to decay, and ecological significance in wetland ecosystems (Myers *et al.* 1995). Also, the plant grows in wetlands and areas frequently submerged, which exposes its tissues to prolonged microbial and fungal challenges. Such environmental conditions likely select for the accumulation of bioactive extractives, including phenolics and diterpenoids, which provide natural defense against microbial degradation (Kusumoto *et al*. 2010; Simoneit *et al*. 2019). Therefore, the chemical composition of bark and wood may be partially shaped by the need for enhanced resistance to water-borne pathogens.

Phytochemical studies on *T. distichum* have revealed the presence of diverse bioactive compounds, particularly abietane-type diterpenes, such as taxodione, ferruginol, and sugiol, as well as volatile terpenes, including α-pinene and limonene (Ahmed and Ei-Emary 1999; Simoneit *et al.* 2019; HosseiniHashemi *et al.* 2023; HosseiniHashemi *et al.* 2024; Shyu *et al.* 2024; HosseiniHashemi *et al*. 2025). Metabolomic profiling and *in silico* studies on *T. distichum* have recently highlighted compounds, such as gallic acid, amentoflavone, and hinokiflavone, as key contributors to bioactivity, with relevance for drug development against inflammation, oxidative stress, and infections (Selim *et al.* 2025). Recent studies have highlighted the bioactivity of *T. distichum* diterpenoids and sterols, particularly in metabolomic and pharmacological analyses (Simoneit *et al*. 2019; Shyu *et al*. 2024; Selim *et al*. 2025). These findings, along with increasing concerns regarding antimicrobial resistance (Ventola 2015), emphasize the importance of profiling non-volatile polar extracts of *T. distichum* bark and wood and investigating their antimicrobial properties.

Previous studies have reported promising antibacterial, antifungal, anti-inflammatory, and cytotoxic activities of *T. distichum* essential oils and cone extracts (Ogunwande *et al.* 2007; Al-Sayed 2018). While most of the research has focused on volatile oils or cone-derived compounds and their activity, there is limited knowledge about the non-volatile constituents in the bark and wood extracts, particularly those obtained using polar solvents such as ethanol. Ethanol is considered a safe and effective solvent for extracting phenolic compounds, which are often responsible for strong antioxidant and antimicrobial effects (Dai and Mumper 2010). Yet, to date, there has been limited empirical evidence on the chemical makeup and biological properties of ethanolic bark and wood extracts of this species. Therefore, the present study aimed to characterize the chemical composition and evaluate the antioxidant and antimicrobial activities of 96% aqueous ethanol extracts from the bark (TDB) and wood (TDW) of *T. distichum* L. This work not only expands the phytochemical understanding of this underexplored species but also explores its potential applications in pharmaceutical and preservative formulations.

EXPERIMENTAL

Plant Material

The bark and wood of *T. distichum* were collected from a cultivated forest in Ramsar, Iran, in December 2022. A map of the sampling location is provided in Fig. A1 (Appendix). The Ramsar wetlands are located in Mazandaran Province, northern Iran, at coordinates 36°53′11.68″ N and 50°34′11.68″ E. This wetland spans approximately 68.6 hectares with an average altitude of 20 m above sea level. The region experiences rainy and snowy weather during late autumn and winter. According to meteorological data from the Ramsar synoptic station, the recorded maximum and minimum temperatures are 32.6 °C and -3 °C, respectively, with an average annual precipitation of 1107 mm (Saeb *et al.* 2011). The sampling location was situated at an altitude of 20 m above sea level.

The collected plant materials (TDB, TDW) were shade-dried at ambient temperature (23 ± 2 °C). The TDB and TDW were cut into smaller fragments, with individual pieces measuring approximately 2 to 5 cm in length and width (Fig. 1). The botanical identity of the plant was confirmed by the author. A voucher specimen (No. 5242) was deposited in the Herbarium of the College of Agriculture and Natural Resources, Ka.C., Islamic Azad University, Karaj, Iran.

Preparation of Hydroethanolic Extracts

For the extraction, a small piece of cotton was placed at the outlet of a 250-mL separatory funnel. Approximately 75 g of each prepared plant part was loaded into the extraction funnel, followed by 160 mL of 96% aqueous ethanol (Fig. 1).

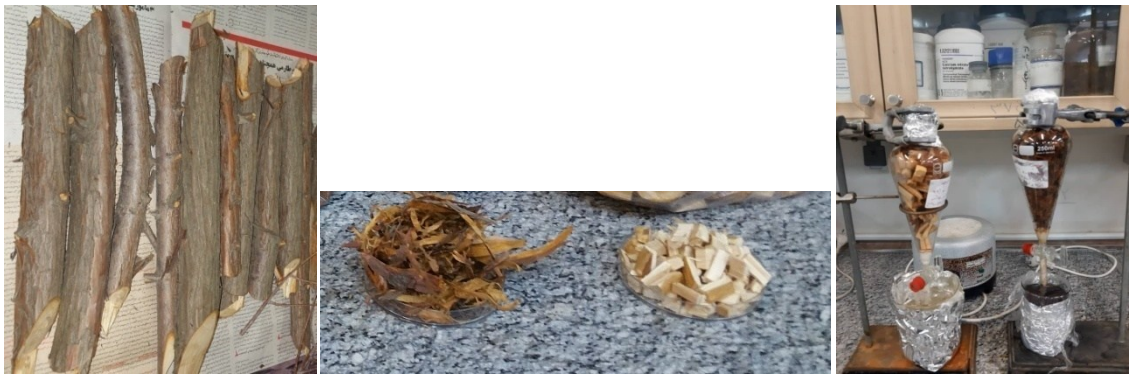

Fig. 1. The collected plant materials of TDB and TDW, their smaller fragments, and extraction funnels

The mixture was macerated at laboratory temperature for 48 h. After extraction, the outlet was opened to allow the extract to drip slowly. The filtrate was clarified, transferred into Petri dishes, and evaporated to dryness at room temperature (25 ± 5 °C) under a laminar flow hood to prevent chemical degradation of bioactive compounds.

The hydroethanolic extract obtained (approximately 70 mL from each plant part) (Fig. A2 (Appendix)) after evaporation was first filtered to remove any particulates and then dried over anhydrous sodium sulfate to eliminate residual moisture. To determine the dry weight content, a 5 mL aliquot of each extract was taken and oven-dried at 103 ± 2 °C for 24 h. The resulting dry weights were 1.3 g for bark and 0.9 g for wood. The remaining extracts were stored in 2-mL dark glass vials at 4 °C for further analysis by Gas

chromatography-mass spectrometry (GC-MS). This protocol was adapted from previously research work by Sabzikar *et al*. (2020) and Barzegari *et al*. (2023).

GC-MS Analysis

For the GC-MS analysis of the 96% aqueous ethanol extracts of TDB and TDW, the dried extracts (1 mg) obtained from each sample were dissolved in 100 mL of methanol to prepare stock solutions. Then, 100 µL of these stock solutions was further diluted with 900 µL of methanol before analysis. The analysis was performed using an Agilent 7890A gas chromatograph coupled with a 5975C mass spectrometer (Agilent Technologies, Palo Alto, CA, USA), fitted with an HP-5MS cross-linked capillary column (30 m × 0.25 mm, film thickness: 0.25 µm). Helium was used as the carrier gas at a flow rate of 1 mL/min. The column temperature during the GC part of the analysis was 60 °C.

The GC-MS parameters were as follows: injector temperature 260 °C; transfer line temperature 270 °C; oven temperature program: 60 °C (4 min), increased 3 °C/min to 100 °C (held for 2 min), then increased 4 °C/min to 250 °C (held for 5 min). The ionization energy was 70 eV, with a split ratio of 50:1 and column flow of 1 mL/min. The total run time was approximately 52 min as previously described (Sabzikar *et al.* 2020; Barzegari *et al.* 2023). Components were identified by comparing mass spectra and retention times with those in commercial libraries (Wiley 275 L, 1998; NIST-05) and published data (Joulain and Konig 1988; Adams 2001). The overall experimental workflow, from sample extraction to GC-MS analysis, is summarized schematically in Fig. A3 (Appendix).

Antioxidant Activity

The antioxidant activity of TDB and TDW extracts was assessed through the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, following established protocols (Halliwell 1997; Kim *et al.* 2002; Emami *et al.* 2010). Briefly, 2.5 mL of a 0.1 mM DPPH solution prepared in 70% methanol was combined with 100 µL of various concentrations of the extracts and ascorbic acid (AA) as a positive control. Serial dilutions of the stock solution and working solutions of TDB and TDW extracts were made to yield concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62, and 7.8 µg/mL. The working solutions were prepared as per previous methods (HosseiniHashemi and Aghajani 2017; Barzegari *et al.* 2023). Negative controls consisted of the DPPH solution mixed with an equal volume of solvent without any test substances or reference standards (Pillai *et al.* 2019; Alam *et al.* 2021). Methanol (Sigma-Aldrich, Darmstadt, Germany) was used to prepare control samples. Before conducting the assay, a UV scanning spectrophotometer (JEN WAY 6320D, Standford, UK) was calibrated using 70% methanol. The reaction mixtures were shaken briefly for 10 s and incubated in the dark at room temperature for 30 min. Absorbance was read at 517 nm with the UV spectrophotometer (HosseiniHashemi *et al.* 2017). All antioxidant assays were performed in four replicates (n=4) and repeated in two independent experiments.

Preparation of Culture Media and Microbial Cultivation

Müller-Hinton agar (MHA) and potato dextrose agar (PDA) were prepared by dissolving powders in distilled water, sterilized by autoclaving at 121 °C and 1.2 atm for 20 min, then poured into Petri dishes under aseptic conditions (Bauer *et al.* 1966; Pitt and Hocking 2009). Microbial strains, *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), and *Candida albicans* (*C. albicans*) were obtained from the Iran Fungal and Bacterial Culture Collection (Bauer *et al.* 1966; CLSI 2020). Bacteria were cultured for 24 h at 37 °C; fungus for 48 h at 25 °C.

At first, each concentration was tested in triplicate by inoculating it into three test tubes containing the culture medium. After observing the color changes, the same concentrations were subsequently plated in triplicate onto three separate Petri dishes. In this study, no positive control was used. Negative controls consisted of culture media containing only water as solvent. All antimicrobial assays were performed in triplicate (n=3) and repeated in two independent experiments.

Antifungal and Antibacterial Activities of TDB and TDW Extracts

The serial dilution method, a widely used technique in microbiology and related fields, estimates microbial growth by culturing microorganisms on suitable media and counting colonies (Taswell 1984; Hollinger 1993; APHA 2005; Ben-David and Davidson 2014). Bacterial strains were grown on Müller-Hinton agar (MHA) plates and suspended in Müller-Hinton broth. The minimum inhibitory concentration (MIC) was determined using the broth dilution method following Ericsson and Sherris (1971). Briefly, bacterial inoculum was adjusted to 0.5, McFarland standard was added (20 µL) to the broth containing filtered TDB and TDW extracts at serial eightfold dilutions (7.81 to 1000 µg/mL), with nutrient broth plus extract as a control. Cultures were incubated aerobically at 37 °C for 24 h, and MIC was defined as the lowest concentration inhibiting visible growth. To refine results, twofold dilutions between active and inactive concentrations were tested.

Minimum bactericidal concentration (MBC) was evaluated by streaking aliquots from turbid tubes onto MHA and incubating for 24 h at 37 °C; absence of growth indicated MBC, which reflects a 99.9% bacterial reduction (Ericcson and Sherris 1971).

Antifungal activity against *C. albicans* cultured on PDA was assessed using the standardized broth dilution method as described in the previous studies (Torres-Rodriguez *et al.* 1993; Basile *et al.* 2010). The MIC corresponded to the lowest extract concentration, preventing visible fungal growth.

Statistical Analysis

The results of antioxidant activity are given in mean values with their standard deviations. Statistical analysis was performed using the SPSS program, version 24.0 (International Business Machines (IBM) Corp., Armonk, NY, USA). Univariate and One-way analysis of variance (ANOVA) were conducted to determine the significance of differences between analytical results at a $p< 0.05$ significance level.

RESULTS AND DISCUSSION

Chemical Composition of TDB and TDW Extracts

Hydroethanolic extraction was performed for TDB and TDW extracts using 96% aqueous ethanol (analytical grade), which was selected based on preliminary extraction trials and literature precedent indicating that such mixtures effectively solubilize a wide range of polar and semi-polar secondary metabolites from coniferous bark and wood (Dai and Mumper 2010; Kusumoto *et al*. 2010; Đapić and Ristić 2017). This solvent system enables efficient extraction of phenolic acids, flavonoids, and moderately non-polar compounds such as abietane diterpenoids, thereby providing a comprehensive chemical profile suitable for evaluating antimicrobial and antioxidant activities. The extraction yields were 1.7% for TDB and 1.2% for TDW, calculated as the dry weight of extract

relative to the initial dried plant material. Bark (TDB) generally provided a higher yield due to higher content of extractable diterpenoids and phenolics.

The GC-MS analysis of TDB extract identified 17 compounds, representing 47.1% of the total, primarily fatty acid esters, fatty acids, oxygenated diterpenoids, monoterpene hydrocarbons, sesquiterpene hydrocarbons, and sterols (Table 1). Figure A4 (Appendix) shows the chromatogram of the extract of *T. distichum* bark analyzed in the HP-5MS column. The dominant compound was 22,23-dihydro-stigmasterol (15.9%), a phytosterol with strong anti-inflammatory, cholesterol-lowering, and membrane-stabilizing properties (Moghadasian 2000; Lagarda *et al.* 2006). Oxygenated diterpenoids, such as 14-isopropylpodocarpa-8,11,13-triene-7,13-diol (8.29%) and ferruginol (4.92%), were also abundant.

Table 1. Composition of *T. distichum* Bark Extract (TDB) Harvested in Winter (December 2022)

No.	Compound	Group	Retention Time (min)	KI^{exp}	KI^{lit}	Area (%)
1	dl-Limonene	MH	8.04	1069	1024	2.75
2	Octanoic acid, ethyl ester	FAE	11.82	1229	1188	0.51
3	*trans*-Caryophyllene	SH	17.11	1470	1419	1.01
4	Pentadecanoic acid, 14-methyl-, methyl ester	FAE	26.23	1954	1884	0.87
4	Palmitic acid	FA	26.76	2004	1929	1.63
6	Ethyl palmitate	FAE	27.34	2043	1998	0.54
7	Octadecanoic acid, methyl ester	FAE	29.37	2177	2177	1.29
8	9,12-Octadecadienoic acid, ethyl ester	FAE	29.80	2206	-	0.49
9	Octadecanoic acid	FA	29.86	2210	2188	0.37
10	Ethyl stearate	FAE	30.37	2246	2202	0.28
11	14-Isopropylpodocarpa-8,11,13-triene-7,13-diol	OD	31.82	2341	2302	8.29
12	Ferruginol	OD	31.92	2358	2315	4.92
13	Linoleic acid, butyl ester	FAE	33.87	2504	-	2.54
14	Sugiol	OD	35.49	2634	2659	1.56
15	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	FAE	36.46	2713	-	1.85
16	Ergost-7-en-3-ol, (3.beta.)-	Ste	43.11	3275	-	2.27
17	Stigmasterol, 22,23-dihydro-	Ste	44.53	3362	-	15.90

Monoterpene hydrocarbons: MH; sesquiterpene hydrocarbons: SH; oxygenated diterpenoids: OD; fatty acid esters: FAE; fatty acids: FA; sterols: Ste

The high levels of oxygenated diterpenoids, including ferruginol, sugiol (1.56%), and 14-isopropylpodocarpa-8,11,13-triene-7,13-diol, highlight the bark extract's potential antibacterial, antifungal, and antioxidant activities. Ferruginol has demonstrated cytotoxic effects on tumor cells and strong antifungal activity against *C. albicans* and other pathogens (Li *et al.* 2008; Saijo *et al.* 2015; Búfalo *et al.* 2016; Rengarajan *et al.* 2019).

The TDB extract also contained several fatty acids and their esters, such as palmitic acid (1.63%), stearic acid (0.37%), and various methyl/ethyl esters of linoleic and stearic acids, which contribute to membrane fluidity, wound healing, and mild antimicrobial effects (Das 2006). Additionally, volatile terpenes like dl-limonene (2.75%) and *trans*-caryophyllene (1.01%) add fragrance and antimicrobial properties commonly found in essential oils. Limonene has shown antioxidant and anticancer potential (Burt 2004; Sun 2007).

In contrast, GC-MS analysis of the 96% aqueous ethanol extract of TDW revealed a diverse profile of bioactive lipophilic compounds with potential pharmacological

significance (Table 2). In total, 13 compounds representing 49.8% of the total were identified in the TDW extract.

Table 2. Composition of *T. distichum* Wood Extract (TDW) Harvested in Winter (December 2022)

No.	Compound	Group	Retention Time (min)	KI^{exp}	KI ^{lit}	Area (%)
1	dl-Limonene	MH	8.02	1068	1024	1.78
2	Ethyl caprylate	FAE	11.83	1229	1196	2.78
3	Decanoic acid, ethyl ester	FAE	16.32	1432	1397	2.86
4	Ethyl laurate	FAE	20.36	1635	1585	0.88
5	Methyl palmitate	FAE	26.23	1954	1926	1.53
6	*n*-Hexadecanoic acid	FA	26.76	2005	1977	3.61
7	Methyl linoleate	FAE	28.79	2138	2100	1.89
8	9,12-Octadecadienoic acid (Z,Z)-	FA	29.32	2174	2170	4.88
9	Linoleic acid ethyl ester	FAE	29.80	2206	2177	1.85
10	Ferruginol	OD	31.92	2358	2315	3.30
11	*β*-Monolinolein	MG	36.46	2713	-	6.27
12	5-Cholestene-3-ol, 24-methyl-	Ste	43.11	3275	-	2.86
13	*γ*-Sitosterol	Ste	44.53	3362	-	15.28

Monoterpene hydrocarbons: MH; oxygenated diterpenes: OD; fatty acid esters: FAE; fatty acids: FA; MG: monoglyceride; sterols: Ste

Figure A5 (Appendix) shows the chromatogram of the extract of *T. distichum* wood analyzed in the HP-5MS column. The most abundant compound in the TDW extract was *γ*-sitosterol (15.3%), a phytosterol known for its anti-inflammatory, antioxidant, antimicrobial, and cholesterol-lowering activities (Lagarda *et al.* 2006). Another notable constituent was *β*-monolinolein (6.27%), a monoglyceride derived from linoleic acid, exhibiting antibacterial activity especially against Gram-positive bacteria by disrupting microbial membranes (Shahidi and Ambigaipalan 2015).

Structures of dominant compounds found in TDB and TDW extracts are provided in Fig. 2.

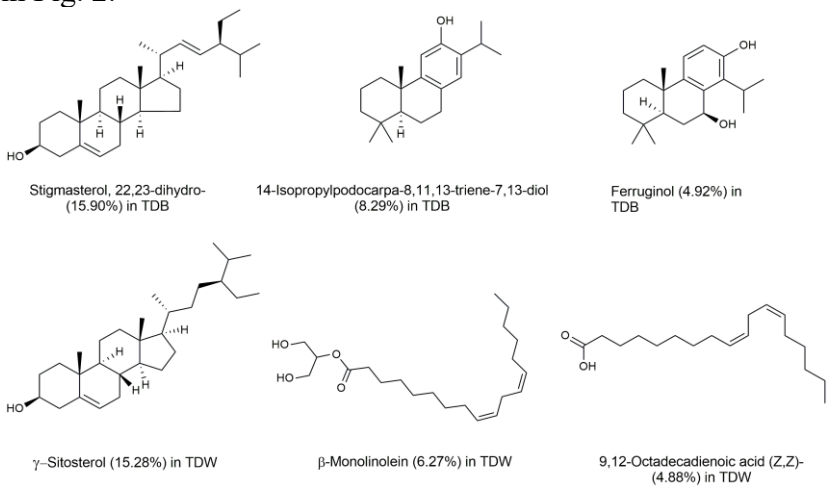

Fig. 2. Structure of the dominant compounds identified in the GC-MS analysis

These diterpenoids are derived from the abietane biosynthetic pathway, originating from geranylgeranyl diphosphate *via* cyclization and oxidation steps, and belong mainly to phenolic diterpenes known for antimicrobial activity.

The wood extract also contained various fatty acids and their methyl and ethyl esters, including linoleic acid, palmitic acid (*n*-hexadecanoic acid), and ethyl caprylate, which play essential biological roles and demonstrate antimicrobial, anti-inflammatory, and emollient properties (Das 2006). Additionally, ferruginol (3.30%), a phenolic diterpenoid commonly found in coniferous plants, was identified and is known for potent antifungal, antibacterial, and anticancer effects (Chan *et al.* 2023). The presence of both free and esterified fatty acids suggests broad-spectrum bioactivity consistent with prior reports of *T. distichum*'s antimicrobial potential.

Seasonal factors, particularly cold exposure during winter, can upregulate biosynthesis of certain secondary metabolites, such as sterols and fatty acids, possibly explaining the richness of bioactivities observed (Soni *et al.* 2015). Winter-harvested tissues showed higher concentrations of secondary metabolites, likely due to cold stress-induced upregulation of phenolics, terpenoids, and resin biosynthesis, which protect plant tissues against oxidative stress and microbial attack (Dudareva *et al*. 2004; Gauthier *et al*. 2010). Overall, the chemical composition supports both traditional and emerging therapeutic uses of *T. distichum* wood as a natural source of antimicrobial and anti-inflammatory agents.

Comparing the winter-harvested TDB and TDW extracts revealed distinct yet overlapping phytochemical profiles, both enriched with bioactive constituents, suggesting diverse therapeutic potentials. Both tissues contain sterols as the major components; however, γ-sitosterol (15.3%) predominated in wood, while stigmasterol (15.9%) was more abundant in bark. These phytosterols are well known for their anti-inflammatory and cholesterol-lowering properties (Piironen *et al.* 2000), implying potential cardiovascular and metabolic health benefits, though their relative abundance may influence potency.

Ferruginol, an oxygenated diterpenoid with known antimicrobial and anticancer effects (Shao *et al.* 2023), was present in both extracts but at slightly higher levels in the TDB (4.92%) extract compared to the TDW (3.30%) extract, indicating a shared antimicrobial capacity. Winter harvesting likely contributes to this chemical profile, as cold stress is known to increase sterol and secondary metabolite contents in plants, potentially enhancing bioactivity (Demuner *et al.* 2011; NndWammbi *et al.* 2018; Sultan *et al.* 2018; Tlhapi *et al.* 2024).

Antioxidant Activity of TDB and TDW Extracts

The antioxidant activity (AOA, %) of 96% aqueous ethanol extracts from TDB and TDW of *T. distichum*, along with ascorbic acid as the standard reference, was evaluated across a concentration range of 7.8 to 1000 µg/mL (Fig. 3).

All experiments were analyzed using Univariate and One-way ANOVA, followed by Duncan's multiple range test (DMRT). Significance is indicated as $p< 0.05$. Detailed statistics are included in Tables A1-A2 (Appendix). Negative DPPH values at low concentrations likely reflect assay variability near the detection limit or possible pro-oxidant behavior of certain phenolic constituents (Halliwell 1997). The data indicate a concentration-dependent response, but with statistically significant differences in potency between the extract samples and ascorbic acid.

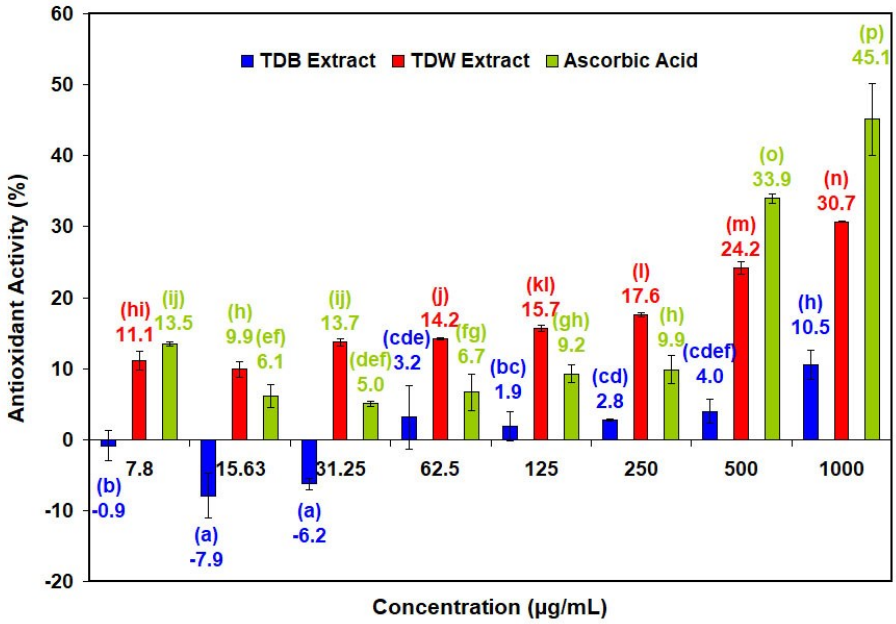

Fig. 3. Antioxidant activities of *T. distichum* bark (TDB) and wood (TDW) extracts at different concentrations (µg/mL)

The TDB extract exhibited low antioxidant activity, with DPPH radical scavenging values ranging from -0.9% to 10.54% across the tested concentration range (7.8 to 1000 µg/mL), indicating either pro-oxidant behavior or assay variability at sub-threshold levels. This suggests that the TDB extract contains limited amounts of antioxidant compounds or that such compounds are less extractable in ethanol under the applied conditions.

In contrast, the TDW extract showed consistently higher antioxidant activity, increasing from 11.1% at 7.8 µg/mL to 30.7% at 1000 µg/mL. This pattern implies that the wood tissue contains considerable quantities of active phenolic or flavonoid compounds with redox potential. These findings align with previous reports identifying compounds, such as gallic acid and lignans, in wood matrices of *Cupressaceae* species (Donoso-Fierro *et al.* 2009; Ul'yanovskii *et al.* 2022; Selim *et al.* 2025).

As expected, ascorbic acid displayed the highest antioxidant activity, peaking at 45.1% at 1000 µg/mL, which confirms the reliability of the assay. Interestingly, the TDW extract at higher concentrations (500 to 1000 µg/mL) approached the antioxidant level of ascorbic acid, suggesting its potential as a natural antioxidant source, though still less potent overall.

The IC_{50} values for antioxidant activity could not be precisely determined within the tested concentration range (up to 1000 µg/mL) for TDB and TDW extracts, indicating relatively low radical scavenging potency compared to ascorbic acid. The higher antioxidant activity of TDB correlates with its elevated content of oxygenated diterpenoids (*e.g.*, sugiol, ferruginol) and phenolics, supporting previous reports that such compounds contribute significantly to radical scavenging capacity (Kusumoto *et al*. 2010).

The TDB extract showed weak antioxidant properties, which was likely due to a lower concentration of phenolic compounds or inefficient extraction. The TDW extract's antioxidant performance was both dose-dependent and statistically stable, indicating potential for functional food or cosmetic formulations.

Some measured antioxidant values were slightly negative, which may occur due to baseline correction, instrument noise, or minor systematic errors in the assay. Similar observations have been reported in other studies using DPPH or ABTS radical-scavenging assays, where low antioxidant activity or interference by sample matrix can lead to negative readings (Brand-Williams *et al*. 1995; Apak *et al*. 2013).

Antibacterial and Antifungal Activities of TDB and TDW Extracts

The antimicrobial activities of the TDB and TDW extracts were evaluated through their minimum fungicidal concentration (MFC) against *C. albicans* and minimum bactericidal concentration (MBC) against *S. aureus* (Table 3).

Table 3. Effect of TDB and TDW Extracts' Concentrations on MIC and MFC/MBC of Selected Fungal and Bacterial Strains

***Taxodium distichum* L.**	**Strain**	**MIC (µg/mL)**	**MFC/MBC (µg/mL)**
TDB Extract	*C. albicans*	7.81	31.25
	S. aureus	500	1000
	P. aeruginosa	-	-
	E. coli	-	-
TDW Extract	*C. albicans*	7.81	7.81
	S. aureus	-	-
	P. aeruginosa	-	-
	E. coli	-	-

The TDB extract showed antifungal activity against *C. albicans* with MIC and MFC values of 7.81 and 31.2 µg/mL, respectively. Additionally, bactericidal activity against *S. aureus* was observed at concentrations of 500 µg/mL (MIC) and 1000 µg/mL (MBC). Regarding the antibacterial activity, TDB extract demonstrated bactericidal effects against the Gram-positive *S. aureus* starting at concentrations of 500 µg/mL and above, effectively reducing viable bacterial colonies. These antimicrobial properties can be attributed to bioactive compounds, such as phenolics and terpenoids, including abietane-type diterpenes like taxodione and ferruginol, known for their strong antibacterial actions (El Tantawy *et al.* 1999; Djapic *et al.* 2022).

In contrast, no inhibitory effect was observed against the Gram-negative *P. aeruginosa* and *E. coli* at the tested concentrations. This lack of activity can be explained by the intrinsic resistance mechanisms of Gram-negative bacteria, including an outer membrane rich in lipopolysaccharides that restricts the penetration of hydrophobic phytochemicals, as well as efflux pumps and hydrolytic enzymes that neutralize many antimicrobial compounds (Cloete *et al*. 2003). These structural and functional defenses typically reduce the susceptibility of *P. aeruginosa* and *E. coli* to plant-derived extracts.

The TDW extract exhibited fungicidal activity against *C. albicans* with MIC and MFC values of 7.81 µg/mL, while no antibacterial activity was detected against *S. aureus*, *E. coli*, or *P. aeruginosa* at the tested concentrations. At lower concentrations (7.81 to 250 µg/mL), fungal inhibition was minimal or absent, whereas strong fungicidal effects were observed, particularly at 500 and 1000 µg/mL. This pattern suggests the presence of active non-volatile polar phytochemicals, such as phenolic acids, flavonoids, or lignans, which may disrupt fungal membrane integrity or enzymatic systems (Cowan 1999; Salem *et al.* 2016). Despite the TDB extract showing slightly higher potency in parallel tests, the TDW extract still displayed considerable antifungal potential at higher doses.

However, similar to the TDB extract, the TDW extract exhibited no detectable antibacterial activity against *S. aureus*, *P. aeruginosa*, or *E. coli* at the tested concentrations. The absence of activity against *S. aureus* may reflect the lower abundance of oxygenated diterpenoids in TDW compared to TDB, whereas the resistance of the Gram-negative strains is consistent with their outer-membrane-associated protection.

These findings corroborate earlier reports that heartwood and inner bark tissues of Cupressaceae, including *T. distichum*, contain antifungal agents like sugiol, hinokiflavone, and various diterpenes (Kusumoto *et al.* 2010; Simoneit *et al.* 2019; Zaher *et al.* 2020).

The TDW extract contains notable amounts of *β*-monolinolein (6.27%) and several methyl and ethyl esters of fatty acids, such as methyl linoleate and ethyl laurate, which may enhance its emollient, antioxidant, and lipid-rich character (Đapić and Ristić 2017). Conversely, the TDB extract shows higher concentrations of oxygenated diterpenoids such as 14-isopropylpodocarpa-8,11,13-triene-7,13-diol (8.29%) and sugiol (1.56%), linked to strong antifungal and antioxidant activities (Kusumoto *et al.* 2010). The presence of volatile terpenes, such as dl-limonene and *trans*-caryophyllene, in the bark further imparts antimicrobial and aromatic qualities largely absent in the wood.

While both extracts demonstrated multifunctional bioactivities, the TDW extract tended toward lipid-based antioxidant and skin-protective applications, whereas the TDB extract exhibited a stronger chemical profile for defense-related and antimicrobial uses. Both extracts demonstrated confirmed antifungal activity against *C. albicans*, although their phytochemical compositions suggest different contributing mechanisms. Antibacterial activity was strain-specific, with TDB showing measurable activity only against *S. aureus*, while neither extract inhibited the Gram-negative bacteria. These outcomes highlight the importance of bacterial cell-wall architecture and extract composition in determining susceptibility.

The TDB extract exhibited higher antimicrobial activity, which was likely due to its greater concentration of oxygenated diterpenoids and volatile terpenes, which possess stronger bactericidal and fungicidal properties compared to the lipid-rich TDW extract.

The combined findings emphasize the potential application of *T. distichum* bark and wood extracts in pharmaceutical, food-preservation, and cosmetic formulations, and support the need for further mechanistic and synergistic studies. These findings align with previous studies on Cupressaceae diterpenoids showing antifungal and antioxidant activities (Donoso-Fierro *et al*. 2009; Kusumoto *et al*. 2010; Simoneit *et al*. 2019).

CONCLUSIONS

1. This study on *T. distichum* bark and wood hydroethanolic extracts showed that they contain phytochemicals with antioxidant and antimicrobial properties, which could be useful in discovering new compounds with therapeutic potential.
2. The gas chromatography-mass spectrometry (GC-MS) analysis of the extracts revealed the presence of various bioactive phytochemicals, some of which are found in both bark and wood extracts, such as dl-Limonene and ferruginol.
3. The difference in antioxidant potential between bark and wood extracts indicates that the phytochemicals in wood extracts might have undiscovered antioxidant properties. Conversely, higher antimicrobial activity in the bark extract might be related to the presence of compounds already known for their antifungal and antibacterial properties.

4. Ultimately, the presence of these valuable phytochemicals in the bark and wood extracts of *T. distichum* and their bioactivities demonstrated their therapeutic potential, which warrants future research to clarify the mechanisms of individual compounds in detail.
5. The limitations associated with this study include following the simple extraction procedure based on previous research and relying on a single method for measuring antioxidant activity without measuring total phenol or total flavonoid content analysis, which should be considered in future research. The use of liquid chromatography-mass spectrometry (LC-MS) for the profiling of more diverse compounds from the hydroethanolic extract is recommended for future research, which is also lacking in this study.

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APPENDIX

Fig. A1. A map of sampling location of Ramsar, Mazandaran province, Northern Iran

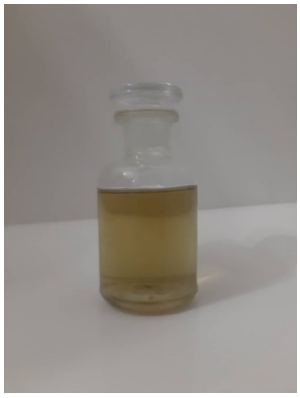

Fig. A2. The remaining extracts of TDW extract

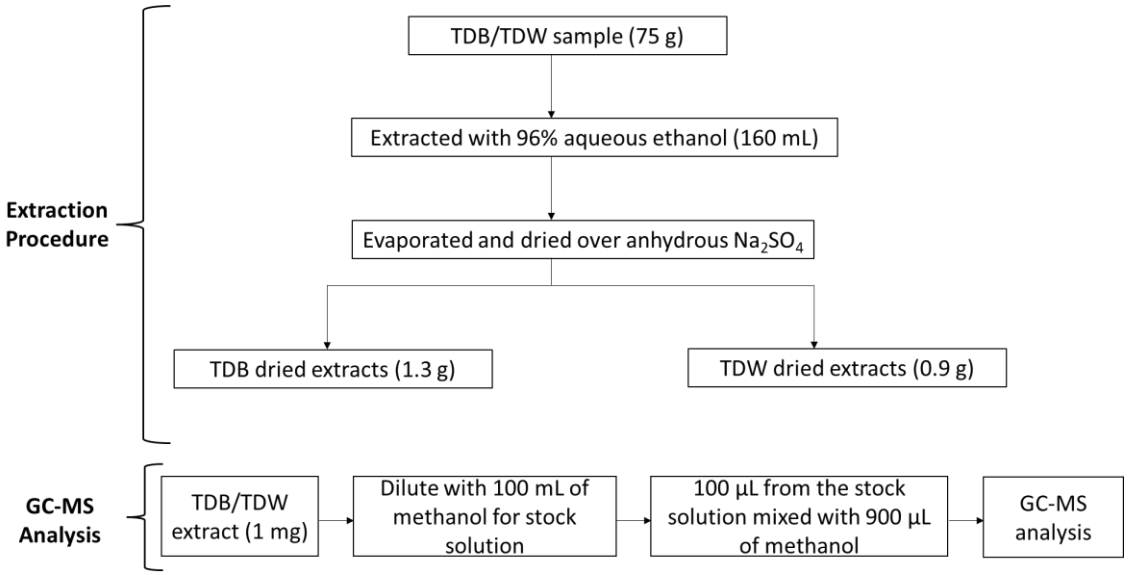

Fig. A3. Schematic representation of the experimental workflow showing the extraction of TBD/TDW samples and GC-MS analysis

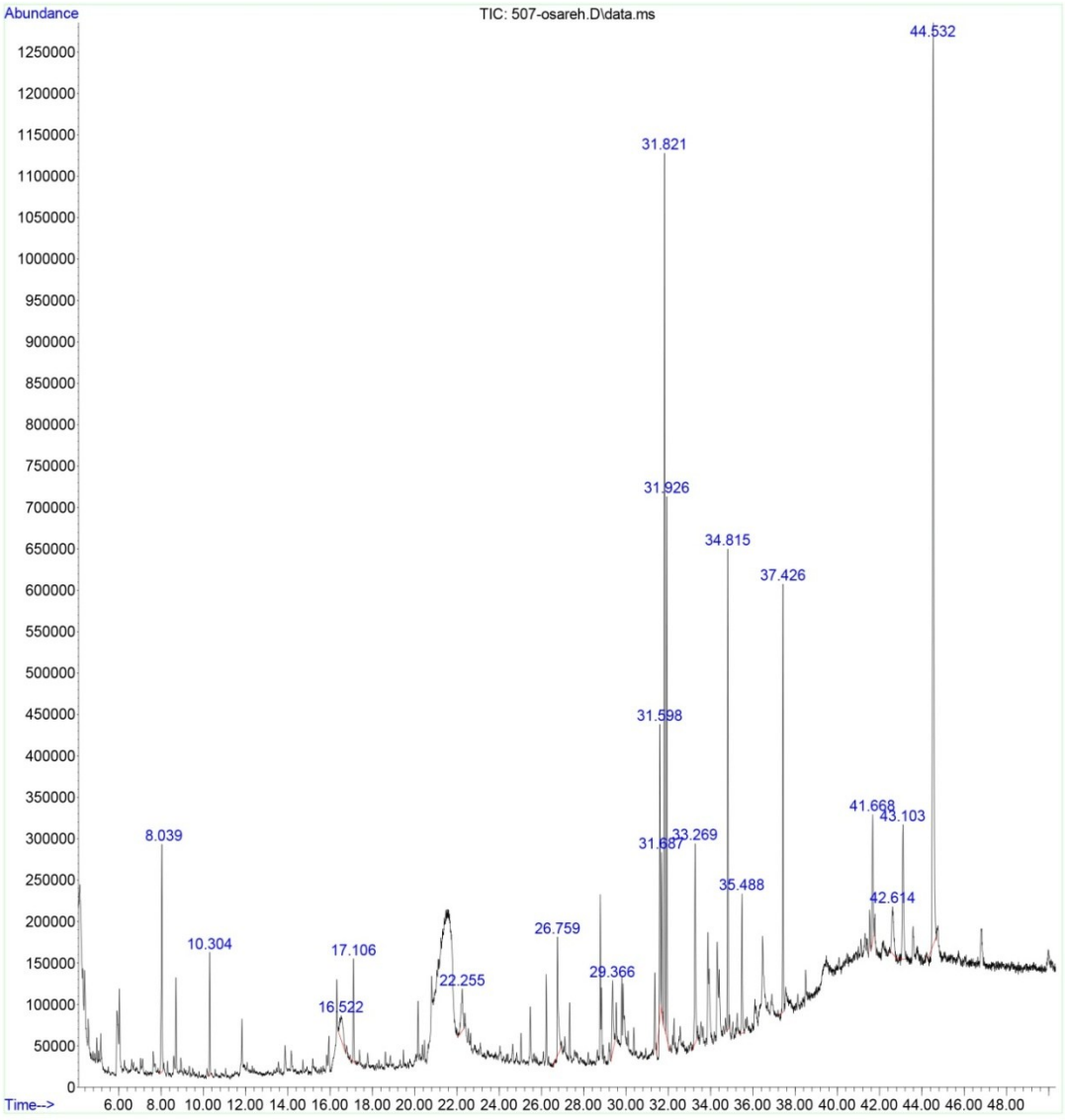

Fig. A4. Chromatogram of extract from *T. distichum* bark (TDB) harvested in winter (December 2022). Components: Stigmasterol, 22,23-dihydro- (44.532 min), 14-Isopropylpodocarpa-8,11,13-triene-7,13-diol (31.820 min), and ferruginol (31.924 min)

Table A1. Results of Univariate ANOVA (Analysis of Variance) Testing the Effect of TDB and TDW Extracts Concentrations on Antioxidant Activity of *T. distichum* Compared with AA

Source	Type III Sum of Squares	df	Mean Square	F	p-value
Corrected Model	13921.472	23	605.281	157.025	0.000
Intercept	12501.481	1	12501.481	3243.201	0.000
Treatment	5303.234	2	2651.617	687.897	0.000
Concentration	6583.618	7	940.517	243.994	0.000
Treatment × Concentration	2034.620	14	145.330	37.702	0.000
Error	277.537	72	3.855		
Total	26700.490	96			
Corrected Total	14199.009	95			

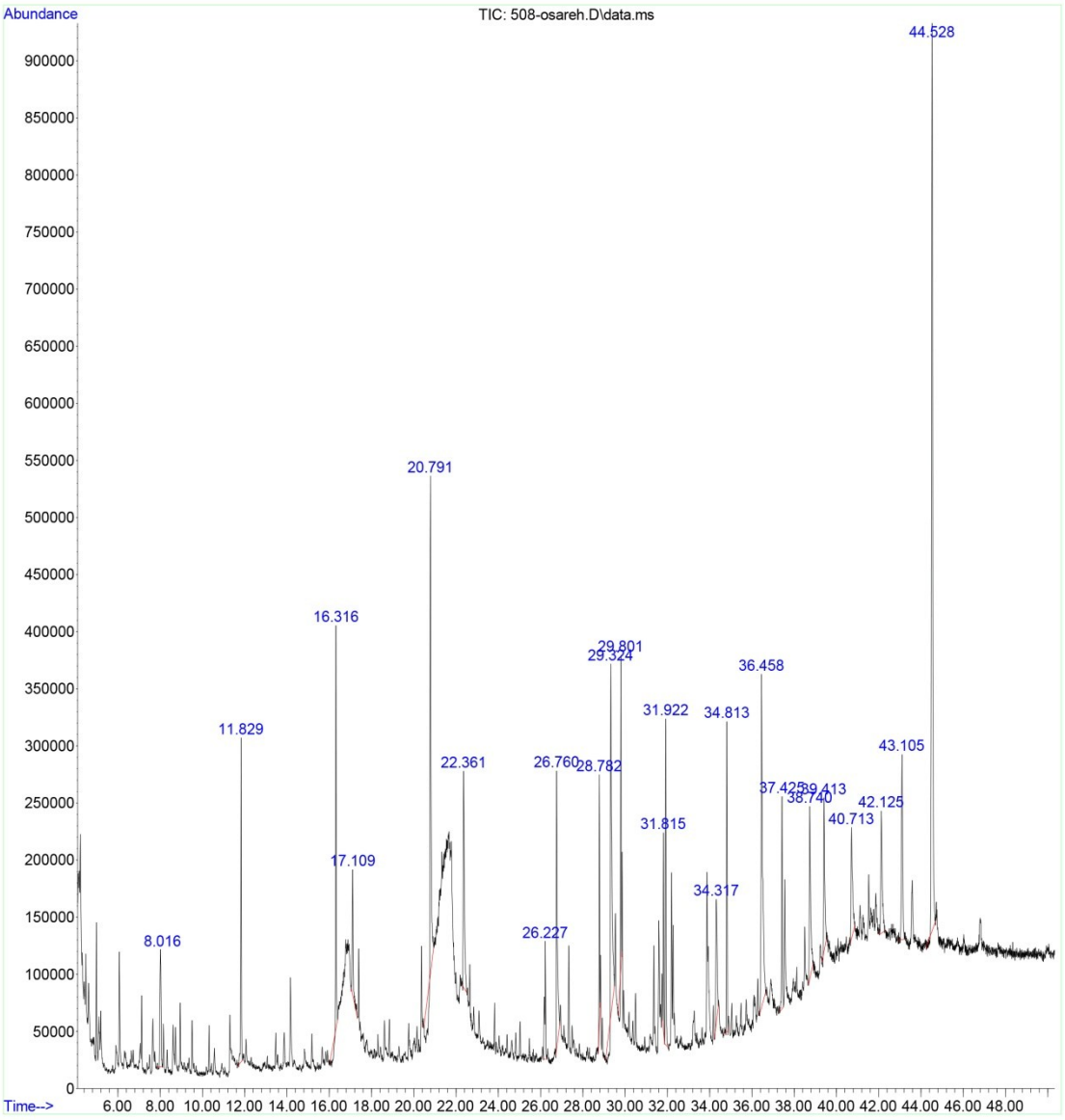

Fig. A5. Chromatogram of extract from *T. distichum* wood (TDW) harvested in winter (December 2022). Components: *γ*-Sitosterol (44.527 min), *β*-Monolinolein (36.459 min), 9,12-Octadecadienoic acid (Z,Z)- (29.324 min), and ferruginol (31.924 min)

Table A2. Results of One-way ANOVA Testing the Effect of TDB and TDW Extracts Concentration on Antioxidant Activity of *T. distichum* Compared with AA.

Source	Sum of Squares	Df	Mean Square	F	p-value
Between Groups	13921.472	23	605.281	157.025	0.000
Within Groups	277.537	72	3.855		
Total	14199.009	95			