Exploring Polyphenols Extraction from *Thuja occidentalis* L. Bark: Optimization, Phytochemical Profiling, and Biological Evaluation

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Polyphenols were extracted from *Thuja occidentalis* L. bark and characterized in terms of phytochemical composition and biological activity. The optimization process investigated the impacts of extraction duration, ultrasound amplitude, and ethanol concentration on the total phenolic content in the extract. The antioxidant capacity was examined using DPPH and ABTS assays, and anti-enzyme activity against alpha-glucosidase, alpha-amylase, lipase, acetylcholinesterase, and tyrosinase was determined. The experimental model revealed optimal extraction parameters: a hydroethanolic solvent with 44% *v:v* ethanol, a 15-minute extraction time, and a 40% ultrasound amplitude. These parameters were validated and confirmed by reaching the target Total Phenolic Content (TPC) predicted by *MODDE* software. The resulting lyophilized extract exhibited a high polyphenolic content (161.84 ± 23.48 mg GAE/g dry extract) and demonstrated strong antioxidant properties. Notably, it showed significant inhibition of alpha-glucosidase and alpha-amylase, suggesting potential antidiabetic effects, though its inhibition of tyrosinase activity was relatively weak. These findings provide a foundation for further investigations to confirm the qualitative and quantitative presence of specific polyphenolic metabolites in the extract and elucidate the mechanisms underlying its bioactive properties.

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INTRODUCTION

Thuja occidentalis (*T. occidentalis*) is a tree commonly known as the tree of life, arborvitae, or white cedar. It is native to eastern North America and is cultivated in Europe as an ornamental tree under various climatic and soil conditions. It is supposed that the species was first identified as a natural remedy by native populations in Canada, as the first

reports regarding its therapeutic use in this area were mentioned during a 16th-century expedition (Pîrvu 2000). This tree contains numerous tannins, flavonoids, coumarins, and proanthocyanidins. However, it's worth noting that the chemical profile of products and phytopharmaceuticals derived from *T. occidentalis* varies depending on whether fresh or dried plant material is used, both qualitatively and quantitatively (Caruntu *et al*. 2020; Monica *et al*. 2021). In the essential oil obtained from fresh leaves, the main constituents identified are primarily monoterpenes, including 65% thujone, 8% fenchone, and 2% α-pinene. Other monoterpenes such as carvotanacetone, origanol, myrcene, and camphene were also reported but in smaller amounts. The volatile oil extracted from dried plant material was found to make up approximately 4%, with major constituents being borneol, camphene, fenchone, limonene, α-terpinene, terpineol, thujone (Jasuja *et al*. 2015; Qureshi *et al*. 2016; Caruntu *et al*. 2020).

The oil from *Thuja* leaves has been used both internally and externally. Internally, it was used for its diuretic, antifungal, and abortive properties, while externally, it was applied to alleviate joint pain, and rheumatic pain, treat various fungal skin infections, and heal warts (Bhan 2016). Among the species that have shown high sensitivity to various extracts of *T. occidentalis* are *Salmonella* species, *Staphylococcus*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Candida albicans*, *Proteus vulgaris*, and *Enterococcus faecalis* (Caruntu *et al*. 2020; Khan n.d.).

The antiviral activity of *Thuja occidentalis* L. extracts has been tested in several studies, considering the empirical results obtained from the traditional use of leaf tinctures in the treatment of papillomas and warts. It has been primarily tested against various strains of the human immunodeficiency virus (HIV) and human papillomavirus (HPV) (Caruntu *et al*. 2020; Srivastava *et al*. 2023).

The benefits of using extracts of *Thuja occidentalis* L. in adjunctive cancer treatment have been recognized and exploited in various aspects of phytotherapy and homeopathy, but the mechanisms of action involved are not yet fully elucidated. Studies conducted to date associate the anticancer action of preparations of *Thuja occidentalis* L. with the *in vitro* cytotoxic effects induced by extracts, extract fractions, or individual components (*e.g*., thujone) isolated from the plant on malignant cell lines (Caruntu *et al*. 2020; Torres *et al*. 2016). A recent study led by Stan *et al.* (2019) investigated the beneficial effects of a homeopathic tincture of *Thuja occidentalis* on ulcerative colitis in rats. The tincture reduced inflammation in the colon in a dose-dependent manner.

Considering the potential of this species in particular, the aim of this study was to optimize the extraction of polyphenolic compounds from *Thuja occidentalis* L. bark, as well as to assess the developed optimal extract in terms of phytochemical composition and biological activity. The optimization process has been developed through experimental design, having as aim the potential maximization of the total phenolic compounds (TPC), and the value of this parameter represents the dependent variable of the process.

EXPERIMENTAL

Chemicals and Reagents

For the total polyphenol content, 95% ethanol (purchased from Girelli Alcool Srl, Zibido, San Giacomo, Italy) was used to obtain a 70% concentration solution, decahydrated Na_2CO_3 (purchased from Reactivul Srl, Râmnicu Vâlcea, Romania), and Folin–Ciocâlteu

reagent (purchased from Merck KGaA, Darmstadt, Germany) were used. For antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were used and obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). For enzymatic activity, α-glucosidase (G5003), α-amylase (A3176), lipase (L3126), acetylcholinesterase (C3389), and tyrosinase (T3824) were used, and these enzymes were purchased from Sigma-Aldrich, Saint Louis, MO, USA.

Plant Material

The bark of *Thuja occidentalis* was collected in 2022 from the Medicinal Plant Garden of the "George Emil Palade" University of Medicine, Pharmacy, Science, and Technology (UMFST), Târgu Mureș, Romania, from a tree between 25 and 40 years old. The collection was carried out by Prof. Dr. Corneliu Tanase from the Department of Pharmaceutical Botany. The bark was manually decorticated and separated, followed by conditioning through air drying at room temperature, and protected from direct sunlight. The dried samples were subsequently stored intact in paper bags, and before extraction, they were ground using a household coffee grinder to achieve the optimal particle size.

Design of Experiment and Preparation for Extraction

The software *MODDE* v.12 for Design of Experiments (DOE) and optimization software (SartoriusStedim, Malmo, Sweden) were used to optimize the extraction conditions. The experimental plan generated had three levels, considering three independent variables (extraction parameters): exposure time (5, 10, and 15 min), alcohol concentration of the solvent (30%, 50%, and 70%), and the amplitude of the ultrasonic generator (30%, 40%, and 50%).

In practice, for each extraction, approximately 5 g of plant material was weighed into a round-bottom flask, to which 100 mL of solvent was added. The resulting mixture was subjected to ultrasonication. The extractions were carried out using an ultrasonic probe-type disruptor, Hielscher Ultrasonics UP 200St (200W, 26kHz), equipped with an immersion probe (sonotrode) of type S26d2 (*Ø* 2 mm, *L*= 120 mm, 2 to 50 mL). To minimize the thermal effect induced by ultrasonication, all extractions were performed in an ice bath. After extraction, the supernatant was separated by vacuum filtration. The dependent variable chosen was the polyphenolic content. In the case of the optimized extract, to facilitate handling and storage, the extract solution separated by vacuum filtration was concentrated under reduced pressure using a rotary evaporator and then lyophilized to obtain a dry extract, which was subsequently analyzed.

Total Phenolic Content (TPC)

The total phenolic content of the extracts was determined using the Folin-Ciocâlteu method adapted for a microplate reader. In a 96-well plate, 10 µL of each sample were pipetted, to which 100 µL of Folin-Ciocâlteu reagent, previously diluted 1:10 with distilled water, was added. The plate was stirred, and then 80 µL of a 1% aqueous solution of Na_2CO_3 was pipetted into the reaction mixture. The plate was subsequently incubated for 30 min at room temperature, protected from light, and after incubation, the absorbance of the samples was measured at 760 nm. The final results were expressed in milligrams of gallic acid equivalents (GAE) per gram of dried plant material for the determinations on the optimized freeze-dried extracts, respectively as GAE per mL of extract during the experimental runs of the optimization process, in order to assess the extractive yield in solution (Babotă *et al*. 2022).

Determination of the Antioxidant Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The method mentioned earlier (Coman *et al*. 2023) was used to evaluate the antiradical effect of the crude extract of *Thuja occidentalis* on the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). In this method, the optimized OpTh extract (50 µL) was mixed with a 0.1 mM DPPH methanolic solution and allowed to incubate for 30 min in the dark. After the incubation period, the absorbance of the samples was measured at a wavelength of 517 nm, and the IC_{50} value for OpTh was calculated based on these measurements.

2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) assay

A method based on the ABTS assay was used to evaluate the antiradical activity of the crude extract of *Thuja occidentalis*, as detailed earlier (Tanase *et al*. 2019a). In this context, OpTh (50 µL) was combined with a solution containing the radical of ABTS (200 µL), and the mixture was incubated for 10 min in the dark. After incubation, the absorbance of the samples was measured at a wavelength of 734 nm, and the results were processed to calculate and express the IC_{50} relative to the positive control.

Enzyme Inhibitory Assays

Determination of α-glucosidase and α-amylase inhibitory activity

The anti-diabetic property of the optimized extract OpTh was tested based on their in vitro inhibitory activity against α-amylase and α-glucosidase (Ștefănescu *et al*. 2022). For the α-amylase analysis, 25 µL of the optimized extract (previously dissolved in 20 mM phosphate buffer, pH = 6.9 supplemented with 5% DMSO, concentrations ranging from 0.002-4 mg/mL) were homogenized with 50 µL of enzymatic solution (0.05 mg/mL in phosphate buffer), and the mixture was pre-incubated for 10 min at 37 °C in the dark. Subsequently, 50 µL of 0.05% starch solution were added to the reaction mixture, and the plate was incubated for an additional 10 min at 37 °C in the dark. Finally, the catalytic reaction was stopped by adding 25 µL of 100 mM hydrochloric acid, and immediately, 100 µL of 5 mM IKI solution were added. The absorbance of the samples was read at a wavelength of 615 nm. For the α-glucosidase analysis, 50 µL of OpTh were mixed with glutathione (0.5 mg/mL) (50 µL), 10 mM PNPG (p-nitrophenyl-β-D-glucuronide) (50 µL), and α-glucosidase in PB (50 µL) and incubated for 15 min at 37 °C. The enzymatic reaction was stopped after the incubation period by adding 0.2 M sodium carbonate (50 µL), and the absorbance was measured at 400 nm. Acarbose (3.12 to 200 µg/mL) was used as the positive control in the α-amylase and α-glucosidase inhibition assay.

Determination of lipase inhibitory activity

The inhibitory potential of OpTh on the lipase enzyme, which plays a role in lipid breakdown metabolic processes, was evaluated using adapted in vitro tests for a microplate reader (Moldovan *et al*. 2023). Each well contained 40 µL of the tested sample and 40 µL of type II lipase (L3126, Sigma-Aldrich, Saint Louis, MO, USA) from porcine pancreas (2.5 mg/mL prepared in Tris-Buffer (100 mM Tris–HCl and 5 mM CaCl2, pH 7.0)). After preincubation for 15 min at 37 ºC, 20 µL of 10 mM pNPB (p-nitrophenyl butyrate) solution was added to each well, and samples were incubated for another 10 min at 37 ºC. Finally, the absorbance of the samples was measured at 405 nm.

Determination of acetylcholinesterase inhibitory activity

The inhibitory potential of OpTh substance on the acetylcholinesterase enzyme, which plays a role in various neurological, metabolic, and cutaneous conditions, was evaluated in a laboratory context. For this purpose, in vitro tests were performed using a modified microplate reader. The procedure involved combining 25 µL of various sample solutions with varying concentrations, 50 µL of Tris-HCl buffer (50 mM, pH = 8), 125 µL of DTNB (0.9 mM), and 25 µL of acetylcholinesterase enzyme solution (0.078 U/mL). The resulting mixture was pre-incubated for 15 min at 37 °C. Afterward, 25 µL of ATCI (4.5 mM) was added to the reaction mixture, and the test plate was further incubated for 10 min at 37 °C. Finally, the absorbance of light at a wavelength of 405 nm was measured, and the results were expressed as IC_{50} values (the concentration at which the effect is reduced by 50%) in µg/mL. For comparison, galantamine was used as a positive reference point, tested at different concentrations (Babotă *et al*. 2021).

Determination of tyrosine inhibitory activity

The inhibitory potential of OpTh on the tyrosinase enzyme, which is involved in various metabolic processes and biochemical reactions, was examined using in vitro tests adapted for a microplate reader (Tanase *et al*. 2023). The metabolic processes in which tyrosinase is involved include protein synthesis, hormone synthesis, neurotransmitter synthesis, pigment synthesis, and melatonin production. Therefore, 40 µL of sample solution at different concentrations were mixed with 80 µL of potassium phosphate buffer (50 mM, pH = 6.5) and 40 µL of tyrosinase enzyme solution (125 U/mL) in a 96-well plate. The resulting mixture was pre-incubated for 5 min at 37 °C. Subsequently, 40 µL of L-DOPA (10 mM) was added, and the new mixture was incubated for an additional 15 min. The final absorbance of the reaction mixture was read at 492 nm, and the results were expressed as percentages of inhibition. Kojic acid was used as a positive control.

The percentage of inhibition for all the enzymes studied with OpTh was calculated according to Equation I, and the final results were expressed as IC_{50} values (µg/mL).

$$Inhibition(\%) = \frac{(A_b - A_c) - (A_d - A_e)}{(A_b - A_c)} x100 \quad (1)$$

where A_b is the absorbance of the control blank, A_c is the absorbance of the control, A_d is the absorbance of the sample blank, and A_e is the absorbance of the sample.

Statistical Analysis

The data were presented as mean ± standard deviation. The statistical analysis was performed using the GraphPad Prism 9.4.1 software (GraphPad Software, San Diego, CA, USA). The normality of the sample data was assessed by conducting the Shapiro-Wilk test and visually examining quantile-quantile (Q-Q) plots. Spearman's correlation coefficients were used to assess the relationships between total phenolic content (TPC), antioxidant capacity, and enzymatic inhibition. The statistical analysis associated with the experimental design was conducted directly in the *MODDE* software through the examination of the replicated graph and by performing regression analysis (Eriksson *et al*. 2000). For the chord diagram, the ORIGIN 9 software (OriginLab Corporation, Northampton, MA, USA) was used. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Experimental Design and Fitting of Experimental Models

Multiple criteria can be considered when assessing the choice of the experimental designs. In this study, "the number of conditions" has been mainly used as statistical parameter (Mandenius and Brundin 2008). In the Design of Experiments (DoE), the "number of conditions" measures the stability of the design matrix, with the ideal value being <1. A high number indicates instability in the analysis, while a low one is preferable (Carlson 2001; Eriksson *et al*. 2000). The experimental design that has been used consists in a central composite face-centered model (CCF), with a total of 14 experimental runs. CCF models are able to maintain low and high factor levels, with axial point centers on the faces of the experimental cube. At the same time, the design contains a central point corresponding to three replicate parameters, which allowed the assessment of the reproducibility of the model. This concept reduces the number of trials in the development of production methods, making it valuable in the pharmaceutical industry for pre-formulation optimization, reducing early experiments. Optimizations decrease overall costs by more efficiently allocating resources and revealing individual influences and interactions of process factors, thus enhancing process efficiency (Babotă *et al*. 2022) (Colobatiu *et al*. 2019).

The Design of Experiments (DoE) registered a condition number of 5.84. The studied response was TPC, which was tested for each individual sample (Table 1).

Table 1. Variation of the Total Polyphenol Content (TPC) in Relation to the Selected Extraction Parameter Values for the Optimization Process

Sample Code	Run Order	EtOH (%)	Extraction Time (min)	Amplitude (%)	TPC (mg GAE/mL)
N1	1	30	5	30	22.45
N12	2	70	5	30	23.29
N2	3	30	15	30	24.65
N13	4	70	15	30	25.21
N3	5	30	5	50	22.08
N14	6	70	5	50	20.80
N4	7	30	15	50	25.05
N15	8	70	10	40	27.18
N5	9	50	5	40	27.21
N6	10	50	15	40	28.98
N7	12	50	10	30	25.44
N8	13	50	10	50	23.18
N9	14	50	10	40	27.68
N10	15	50	10	40	29.00
N11	16	50	10	40	27.25

The resulting data were incorporated into a matrix developed for Design of Experiments (DoE), and the adaptation of the experimental data was achieved by applying multiple linear regression (MLR). Model fit assessment involved using standard statistical measures, among which the most robust are: R^2 (coefficient of determination), Q^2 (prediction coefficient), ANOVA examination, and model reproducibility assessment. These results were calculated and presented according to the number of replicates specified in the design matrix. The values describing each parameter are presented in Table 2. Good agreement is evident through high values of model evaluation indicators, as close as

possible to 1. Additionally, for a model to be considered valid, the difference between R^2 and Q^2 values should not exceed 0.2 to 0.3, as larger differences suggest an improper model selection. Regarding reproducibility, it should be significantly above 0.5.

Table 2. Parameter Values Employed for Assessing the Fit of an Experimental Model

	R^2	R^2Adj.	Q^2	SDY	RSD	N	Model Validity	Reproducibility
TPC	0.97	0.93	0.73	5.84	0.69	15	0.92	0.84

The summary of the fit assessment and statistical indicators were derived from the data collected in the experimental design. The conclusions supported the statistical robustness of the developed model, indicating a significant influence of the factors on the outcomes and the absence of discrepancies in the model fit. Similar statistical conclusions have been observed in other research where Design of Experiments (DoE) was implemented for optimization purposes (Moldovan *et al*. 2021).

Influence of Process Variables on Extracted Total Phenolic Content (TPC)

The regression coefficient values were automatically determined for the analyzed variables using the Design of Experiments (DoE) model. To clarify the influence of the chosen factors, statistical significance of the interactions between them was analyzed, as represented in Fig. 1. This analysis highlighted the major importance of amplitude-amplitude interactions and solvent-amplitude interactions in increasing TPC values. This reveals the leveling effect of the solvent on extraction yield, where an increase in alcohol concentration can partially compensate for a decrease in amplitude or extraction times.

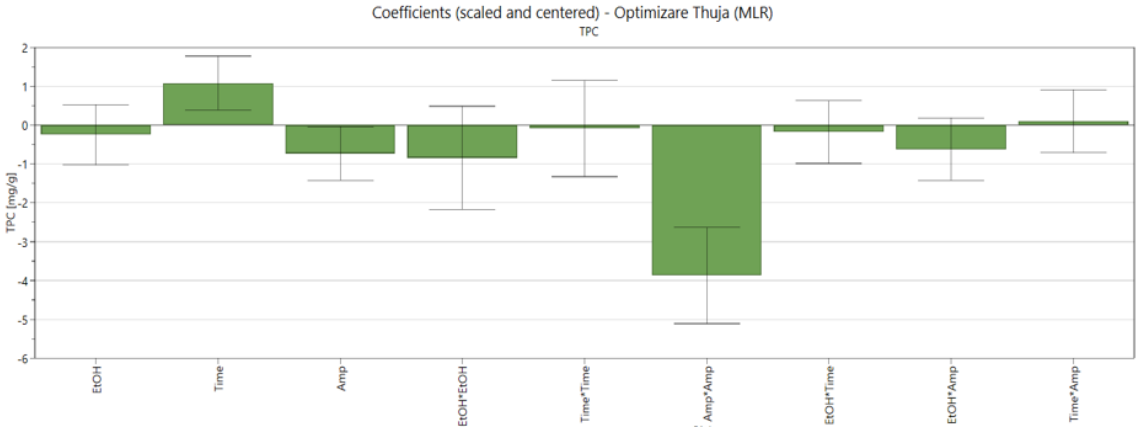

Fig. 1. The representation of the interaction between extraction parameters and the statistical correlation of these interactions with TPC values. The coefficients have been represented as scaled and centered values.

The TPC shows a proportional increase with the intensification of ultrasound wave amplitude, time period, and the solvent-to-feed ratio (Imtiaz *et al*. 2023). The effects of ultrasound waves generate significant torsional forces capable of disrupting cell membranes, allowing the solvent to penetrate plant cells and release compounds from within (Belwal *et al*. 2018). Increasing ultrasound amplitude leads to the intensification of cavity formation, damage to the plant's cellular structure, and increased tissue permeability,

facilitating the release of a significant quantity of compounds into the solvent. This phenomenon results in an increase in the TPC yield (Rodrigues *et al*. 2008). Conversely, decreasing ultrasound amplitude leads to reduced sensitivity and the detection of effects (Abbas *et al*. 2021). If there is an interaction between solvent and another solvent, it suggests that the measurements are stable and replicable. If there is an interaction between solvent and amplitude, this may suggest that these two variables have a strong impact on the response. This can help identify important factors for the experiment (Dahmoune *et al*. 2014).

Design Space and Process Optimization

After validating the experimental model, the ideal extraction conditions were determined. Based on these conditions, a theoretical target value for the TPC was established. According to Table 3, the optimal predictions for the extraction parameters were consistent with the experimentally determined TPC values. Optimization resulted in a reduction in the alcohol content of the extraction solvent, even though the optimal extraction duration and amplitude corresponded to the initial upper limits of these parameters in the experimental plan. The prediction of optimal conditions considered possible experimental errors that could have occurred pre- or post-validation, with the *MODDE* software determining a 0.54% chance of model failure. This highlights the benefits of combining ultrasound-assisted extraction and the optimization process to obtain the optimized extract (OpTh) compared to traditional extraction approaches.

Table 3. Optimal Extraction Parameters and the Theoretical and Experimentally Determined TPC Values for the Optimized Extract

TPC (mg GAE/mL extract)					Recovery (%) Predicted *vs.* Measured value
Objective			DoE predicted	Experimentally measured	
Minimum	Target	Maximum			
25	29	33	20.69	29.164	140.96
Extraction Parameter					Value
Exposure time (min)					15
Ultrasounds amplitude (%)					40
Ethanolic concentration (%)					44

All these premises were subsequently confirmed in the modeling and validation stage of the experimental extraction design, where the TPC values of the extracts were correlated with variations in the extraction parameters. The graphical representation of these correlations is known as the "design space".

Surface response graphs allow for a preliminary assessment of interactions between factors and the identification of "confluence points," where extraction should achieve maximum efficiency. Graphically, these aspects are highlighted by the appearance of red zones on the "response surface" of the experiment, as can be seen in Fig. 2. The surface response graphs revealed the importance of amplitude and exposure time as primary factors influencing the extraction of total polyphenols from the plant product. An important aspect that can be observed is related to the extraction time, and the higher times produced an increased TPC. However, since the aim of the experimental design was to obtain an efficient extraction process, higher time values were not taken into consideration.

Moreover, the maximal efficiency of this model was clearly obtained in the case of ultrasound amplitude, and approximatively 40% amplitude induced the maximal TPC values for all the used concentrations of EtOH.

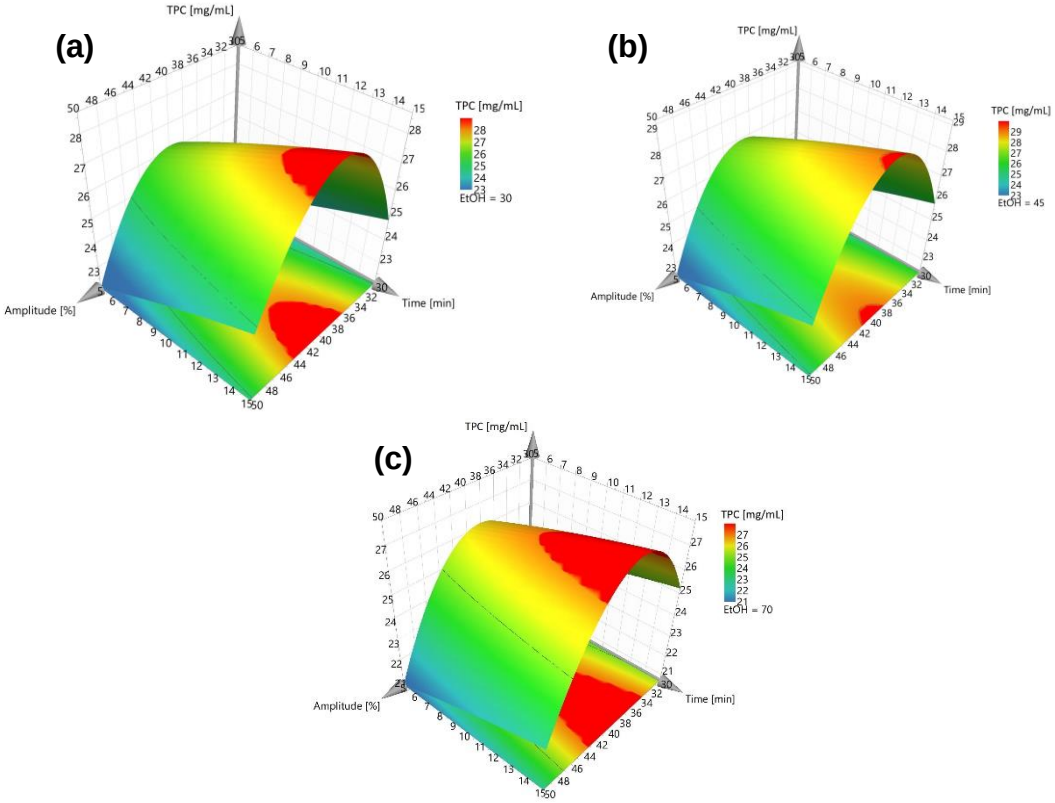

Fig. 2. Design space for EtOH 30% extract (a), EtOH 45% extract (b), EtOH 70% extract (c)

Optimization of Extraction Parameters and Total Polyphenol Quantity

The TPC of the ethanolic extract was determined after the lyophilization process and reconstitution, considering the absorption of the samples following the Folin-Ciocâlteu reaction and the calibration curve of gallic acid standards: $y = 11.767x + 0.2737$, $R^2 = 0.9984$. The results were expressed as mg GAE (Gallic Acid Equivalent) per gram of dry weight and are presented in Table 4.

Table 4. Total Polyphenolic Content (TPC) and Antioxidant Activity (DPPH and ABTS)

Sample	TPC (mg GAE/g d.w)	IC_{50} DPPH (µg/mL)	IC_{50} ABTS (µg/mL)
OpTh	161.84 ± 23.48	10.13 ± 1.21	0.93 ± 0.17

In the methanolic extract of *Thuja occidentalis* leaves, the total quantity of polyphenols is 0.125 mg/g GAE (Tekaday *et al*. 2020). The total polyphenol content (TPC) for the methanolic extract obtained from *Thuja occidentalis* leaves has been measured at 135.3 mg/g GAE (Nazir *et al*. 2016). Furthermore, the values of total polyphenol concentration in *T. occidentalis* extracts exhibit variations based on different geographical locations. As altitude increases, the quantitative level of TPC shows a corresponding

increase (Thakur *et al*. 2023). The inhibition percentage (at 50 and 100 µg/mL) of extract 1 (ethyl acetate: chloroform: ethanol (40: 30: 30)) is similar to the inhibition percentage of ascorbic acid. On the other hand, the inhibition percentage of extract E2 (70% methanolic extract) from *Thuja occidentalis* is higher (70.4% and 85.2%) than that of the control (ascorbic acid) (Nakuleshwar *et al*. 2013).

Assay of the Antioxidant Activity

In Fig. 3, the results of antioxidant activity assessed through two methods, DPPH and ABTS, are presented. The inhibition of the ABTS radical was more pronounced compared to that of the DPPH radical. Additionally, a distinct pattern of inhibition was evident for each radical separately. In the case of ABTS, inhibition occurred rapidly and reached saturation, with a consistent maintenance of activity even at relatively low extract concentrations (this is justified by the plateau shape in the inhibition curve). In contrast, for the DPPH radical, the inhibition curve indicated a concentration-dependent inhibition relationship over a much wider range. This can be explained by both the different chemical characteristics of the two radicals (DPPH typically carries a negative charge, while ABTS carries a positive charge) and the varying capacity of the extract components to interact with them (Huang *et al*. 2005).

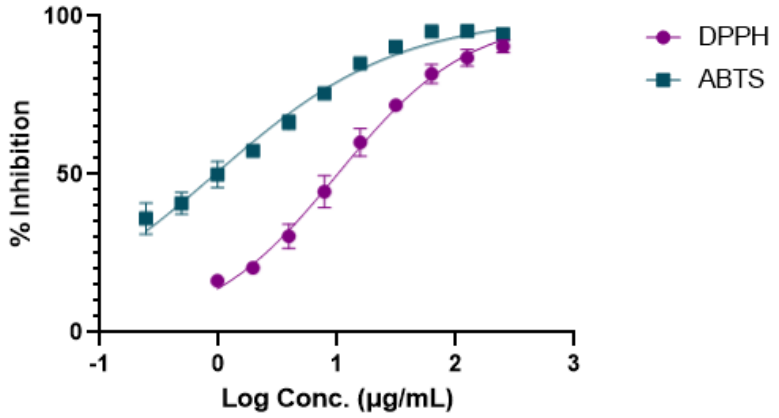

Fig. 3. Percentage inhibition graphical representation for the DPPH and ABTS free radical scavenging capabilities of the optimized OpTh extract

In a study conducted by Nazir *et al*. (2016), it was found that the methanolic extract of *Thuja occidentalis* exhibited significant antioxidant potential. This potential was indicated by high results in terms of scavenging DPPH radicals, ABTS tests, NO, and lipid peroxidation. In a study focused on essential oil extracts obtained from *Thuja occidentalis* leaves, an augmented antioxidant activity (200 µg/mL Trolox) was observed compared to essential oil extracts derived from cones (150 µg/mL Trolox) (Bellili *et al*. 2018).

In vitro Enzyme Inhibitory Properties of OpTh Extract

The inhibitory capacity of the OpTh extract (Table 5) was tested against α-glucosidase, α-amylase, lipase, tyrosinase, and acetylcholinesterase. These enzymes are important targets in the therapy of several non-transmissible chronic diseases such as diabetes mellitus, dermatological conditions characterized by excessive melanin production, hypercholesterolemia, Alzheimer's disease, or other neurological disorders.

Table 5. Overview of *in vitro* Enzymatic Inhibition Potential of OpTh

Enzymatic Assay		IC_{50} µg/mL
α-Glucosidase	OpTh	344.48 ± 41.49
	Acarbose	21.28 ± 1.42
α-Amilase	OpTh	2084.32 ± 242.60
	Acarbose	21.28 ± 1.42
Lipase	OpTh	4476.71 ± 366.87
	Orlistat	68.47 ± 2.98
Acetylcholinesterase	OpTh	869.48 ± 75.87
	Galantamine	1.42 ± 0.25
Tyrosinase	OpTh	1459.10 ± 264.56
	Kojic acid	50.20 ± 0.15

From the perspective of its potential antidiabetic effects, it was observed that the extract exerted the most significant inhibitory action on α-glucosidase, while α-amylase was poorly inhibited. Considering that the literature associates *in vitro* antidiabetic potential with an optimal inhibition ratio of 1:3 between the activities of these two enzymes, as well the IC_{50} values below 100 µg/mL for the positive control (acarbose), it can be concluded that the obtained extract could be considered for further evaluations in order to support its potential use as adjuvant agent in antidiabetic therapy (Tadera *et al*. 2006; Tanase *et al*. 2023).

The inhibition of lipase is of interest in breaking down fats into smaller molecules for absorption into the body. In the context of managing body weight, lipase can be used to help break down fats in food, thereby contributing to the reduction of calorie intake and fat absorption (Guerrand 2017). Among the enzymes studied in this study, lipase was found to exert the weakest inhibitory action, considering that the normal value is less than 61 U/L (Patel *et al*. 2021).

Acetylcholinesterase (AChE) is an enzyme that breaks down the neurotransmitter acetylcholine in the brain. In Alzheimer's disease and other neurological disorders, low levels of acetylcholine are associated with cognitive impairment. Adjuvant treatments that inhibit acetylcholinesterase can increase acetylcholine levels and temporarily improve cognitive function (Ellman *et al*. 1961). *Thuja occidentalis* exhibits inhibitory activity (IC_{50}) against the AChE enzyme at 869.5 µg/mL.

Inhibiting tyrosinase is of interest in the treatment of skin hyperpigmentation caused by overproduction of melanin, with relevance in both pharmaceutical and cosmetic fields. Numerous studies have demonstrated the effectiveness of extracts obtained from the barks of various trees as anti-tyrosinase agents (Tanase *et al*. 2019b, 2023). The analyzed extract was found to exert weak inhibitory action on this enzyme, given that the literature cites IC_{50} values of 4.44 µg/mL for the positive control used in this test (kojic acid).

Spearman's Rank Correlation Coefficient

The Spearman correlation test (Fig. 4a) was applied to examine the relationships between antioxidant activity (DPPH and ABTS) and relevant biochemical factors (α-glucosidase, α-amylase, lipase, acetylcholinesterase, and tyrosinase). There was a very strong but statistically nonsignificant correlation between TPC - α-Amylase and between α-Glucosidase - Lipase, indicating a possible connection between these two variables. This can also be observed in Fig. 4b through the analysis of line intensity, where the thicker or more intensely colored the line, the stronger the correlation.

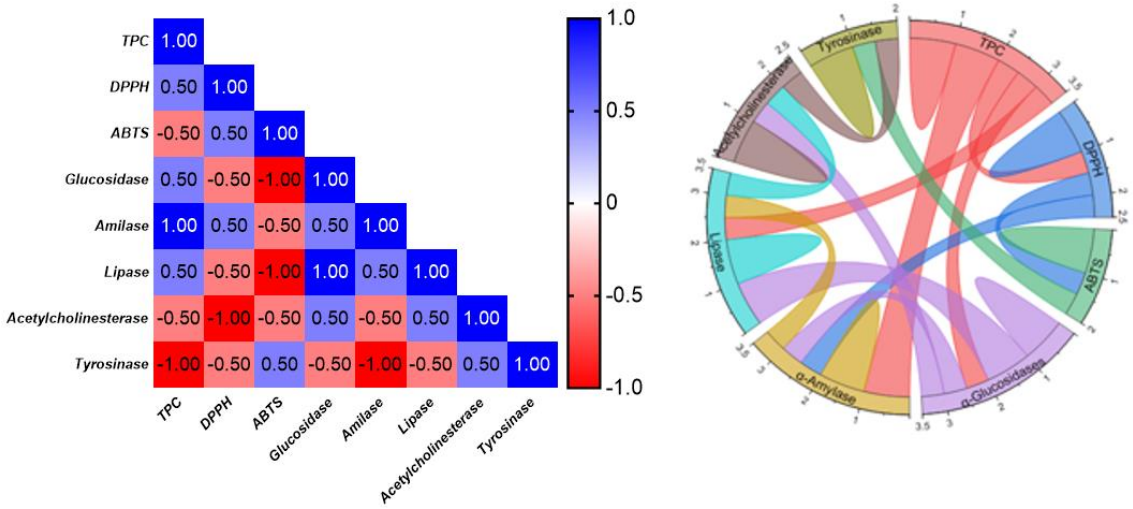

Fig. 4. (a) Spearman's correlation coefficient (ρ) and (b) chord diagram presenting the correlation between TPC, DPPH, ABTS, α-glucosidases, α-amylase, lipase, acetylcholinesterase, tyrosinase among tested extracts

Furthermore, the results revealed ~~very~~ strong negative correlations between TPC - Tyrosinase, DPPH - Acetylcholinesterase, ABTS - α-Glucosidase, ABTS - Lipase, as well as between α-Amylase - Tyrosinase, signaling potential complex interactions among these factors. In addition to these findings, other weak correlations were observed, either positive or negative, that did not reach statistical significance, indicating the need for further investigations to determine whether there is a significant relationship between these biochemical variables or not.

CONCLUSIONS

1. The experimental model used allowed for the determination of optimal extraction parameters (hydroethanolic solvent 44% *v:v*, exposure time of 15 min, and ultrasound amplitude of 40%). These parameters were validated and confirmed by achieving the target value of predicted TPC through mathematical modeling using the *MODDE* software. The lyophilized optimized extract exhibited a high polyphenolic content (161.84 ± 23.48 mg GAE/g dry extract) and demonstrated good antioxidant properties. The potential antidiabetic effect was correlated with significant inhibitory activity against α-glucosidase and α-amylase, while the inhibition of lipase, tyrosinase, and acetylcholinesterase was weak.
2. The obtained results serve as a starting point for further assessments to confirm the qualitative and quantitative presence of individual polyphenolic metabolites in the analyzed extract and to establish the mechanisms through which they impart bioactive properties to the extract.

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