

Article

Screening Factorial Design for Extraction of Bioactive Metabolites from *Tithonia diversifolia* (Hmsl.) A. Gray/ Asteraceae Leaf Extract: Phytochemical Assays and Chemical Characterization by Synchronous Fluorescence and Phosphorescence Spectroscopy

Karla Ramos ¹ and Amin Karmali ^{1,2,*}

¹ Chemical Engineering and Biotechnology Research Center, Department of Chemical Engineering, Instituto Superior de Engenharia de Lisboa, Instituto Politécnico de Lisboa, Rua Conselheiro Emídio Navarro, N.º. 1, 1959-007 Lisboa, Portugal; karlajosefatr@gmail.com

² CITAB—Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Universidade de Trás-os-Montes e Alto Douro—UTAD, 5000-801 Vila Real, Portugal

* Correspondence: akarmali@deq.isel.ipl.pt

Abstract

Recent studies highlight the rich medicinal flora of São Tomé and Príncipe (STP) islands due to their therapeutic potential against various diseases. To maximize bioactive compounds' extraction from *Tithonia diversifolia* leaves, we have used a two-level, three-factor (2^3) full factorial design. The screening evaluated water and hexane solvents at 25 °C and 40 °C, across incubation periods of 0 and 5 days, maintaining a constant agitation of 150 rpm. The highest TPC among the tested conditions was 72.16 µmole gallic acid equivalent/g of leaves using water at 40 °C with a 5-day incubation period. The leaf extracts were characterized through several phytochemical assays, including TFC, reducing power, ABTS, DPPH, and SOD activity. This report introduces a novel, non-destructive analytical fingerprinting approach using intrinsic synchronous fluorescence and phosphorescence. Intrinsic, non-destructive synchronous fluorescence spectroscopy was performed across a wavelength range of 250 to 750 nm, utilizing a $\Delta \lambda$ interval of 5 to 30 nm. The hexane leaf extract displayed distinct peaks at 290, 320, 345, 400, 490, and 675 nm. Conversely, the aqueous extracts only showed peaks at 490, 560, and 675 nm. Intrinsic synchronous phosphorescence testing revealed peaks at 325, 400, 490, 550, and 675 nm for the hexane extract, and at 500 and 560 nm for the aqueous extract. Additionally, 3D fluorescence fingerprinting spectra successfully validated the emission peaks at 290, 320, 345, 400, 490, and 675 nm. Therefore, the present work describes novel SFS, SPS and 3D-SFS as fingerprinting/comparative profiling techniques for leaf extracts which require definitive compound identification methods.

Keywords: *Tithonia diversifolia*; screening factorial design; phytochemicals; bioactive compounds; antioxidant activity; synchronous fluorescence and phosphorescence spectroscopy; 3D fluorescence fingerprinting; Leaves extracts



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1. Introduction

Plants synthesize thousands of secondary metabolites which play major roles for their protection from various diseases, exhibiting many biological activities, such as antibacterial, antifungal, cytotoxic, antioxidant, anti-inflammatory, and antimalarial activities [1]. Based

on their chemical structure, phytochemicals are classified into six primary groups: carbohydrates, lipids, phenolics, terpenoids, alkaloids, and nitrogen-containing compounds. These plant substances are known as secondary metabolites since they are not essential for plant survival [1]. Quantitative phytochemical assays on leaf extracts provide data on concentration of secondary metabolites or bioactive compounds present in such extracts. This information reveals the potential therapeutic efficacy and medicinal uses of these plant extracts for human health benefits [2]. Therefore, phenolic compounds have shown antioxidant capacity and radical scavenging activity, whereas flavonoids exhibited anti-inflammatory and antioxidant potential. These assays play an important role for translating traditional folk medicine knowledge into scientific pharmaceutical and nutraceutical compounds for public health applications [3].

Tithonia diversifolia is native to North and Central America, but it has also been found in Africa, Australia, and Asia [4]. This leaf extract has been widely used in traditional medicine for clinical conditions of diabetes, diarrhea, menstrual pain, malaria, hematomas, hepatitis, hepatomas, and wound healing, among other diseases [5,6]. Located in the Gulf of Guinea, São Tomé and Príncipe (STP) possesses a rich diversity of medicinal plants which local communities employ under the supervision of traditional practitioners [7,8]. Various *Tithonia diversifolia* extracts from STP have been produced in solvents such as ethanol, water, dichloromethane, petroleum ether, methanol, and ethyl acetate to treat multiple diseases [9,10]. For decades, leaf extract has been successfully employed to treat malaria in STP [11]. Regarding their chemical and biological profile, these leaves extracts exhibit antioxidant and superoxide scavenging activities, as they have high levels of phenolics, flavonoids, terpenoids, proteins, carbohydrates, and glycosides [12,13].

Some research works have described a few phytochemical assays for phenolics, flavonoids, antioxidant, and superoxide scavenging activities from this leaf extract, as well as some FTIR analysis [10–15]. However, this research work requires a systematic study to investigate phytochemical compounds in leaf extracts, as well as to analyze secondary metabolites by fluorescence techniques. Additionally, to our knowledge, no published studies have utilized a screening factorial design to extract secondary metabolites from these plant leaves. This optimization strategy is essential, as it identifies significant factors and reveals critical interactions between variables [16]. Optimal extraction yield of secondary metabolites from leaf extracts are highly dependent on several factors, such as extraction time, nature of solvent, temperature, pH, solvent concentration, liquid-to-solid ratio, and particle size of leaves [17].

Therefore, the main contribution of this work involved screening factorial design by phytochemical analysis of leaves extracts, as well as analytical fingerprinting of secondary metabolites by fluorescence techniques. The current work is concerned with TPC extraction from leaves by screening factorial design by using H₂O and hexane as extraction solvents. These leaf extracts were analyzed using phytochemical assays to quantify phenolic compounds and flavonoids, and to evaluate reducing power, antioxidant activity (via DPPH and ABTS), superoxide scavenging activity, and SOD activity, as detailed investigations into these parameters are currently lacking. Concurrently, intrinsic synchronous fluorescence spectroscopy (SFS), intrinsic synchronous phosphorescence spectroscopy (SPS), and three-dimensional synchronous fluorescence spectroscopy (3D-SFS) were employed for fingerprinting and comparative profiling. These multi-dimensional spectroscopic techniques [18,19] offer rapid, sensitive, and non-invasive analytical profiling of bioactive compounds in leaf extract, serving as valuable tools prior to definitive compound identification methods.

2. Materials and Methods

2.1. Chemicals

All chemicals used in the present work were of analytical grade, as described previously [20].

2.2. Plant Material Collection

Leaves of *Tithonia diversifolia* (Hemsl.) A. Gray were collected from the São Tomé Botanical Garden (GPS coordinates: 0°17'16.0" N; 6°38'14.0" E). A voucher specimen (No. 001/2019) was kept in the Herbarium of São Tomé, following previously described procedures [20].

2.3. Methods

2.3.1. Preparation of Leaf Extract

The collected leaves were thoroughly washed with distilled water and dried overnight in an oven at 40 °C. The dried material was then ground into a fine powder. Leaf extracts were prepared according to the methodology described previously [20].

2.3.2. Phytochemical Assays

Only one biological replicate was used in the present study. All assays were performed according to screening factorial design mentioned in Section 2.3.4.

Quantification of Total Phenolic Content (TPC)

TPC was assayed by the Folin–Ciocalteu methodology [21–23], with minor changes, to a final volume of 300 µL, as reported previously [20].

Quantification of Total Flavonoids Content (TFC)

TFC was assayed by using the spectrophotometric method [24], with minor changes, as mentioned previously [20].

2.3.3. Antioxidant Activity of Leaf Extract

DPPH Radical Scavenging Activity

This parameter was assayed according to the method reported previously [23], with minor changes to a final volume of 310 µL, as mentioned previously [20].

ABTS Scavenging Activity

This parameter was assayed [24,25] with minor changes, as mentioned previously [20].

Reducing Power

This parameter was assayed [24] with minor changes, as reported previously [20].

Superoxide Radical Scavenging Activity

This assay was carried out [26] with some modifications, as described previously [20].

Assay of Superoxide Dismutase (SOD) Activity

This enzyme assay was performed by the NBT method [27], with minor changes, as mentioned previously [20].

2.3.4. Screening Factorial Design for Extraction of TPC

Only one biological replicate was used in the present study. A 2³ factorial screening design was utilized, consisting of eight experiments performed in duplicate (technical

replicates). This design evaluated the effects of three independent variables: extraction/incubation temperature (25 °C and 40 °C) and extraction/incubation time (0 and 5 days) as numerical factors, and solvent type (H₂O and hexane) as a categorical factor. The low (−) and high (+) levels were selected based on data from prior pilot tests. After constructing the experimental matrix, the results were fitted to the following factorial model equation:

$$y = \beta_0 + \beta_A x_A + \beta_B x_B + \beta_C x_C + \beta_{AB} x_A x_B + \beta_{AC} x_A x_C + \beta_{BC} x_B x_C + \beta_{ABC} x_A x_B x_C$$

where y is the concentration of phenolic compounds, β_0 is the mean of all responses, β is the regression coefficient, and x is the prediction variable. The statistical analysis of the model was evaluated according to ANOVA methodology by using Design-Expert software version 10.

2.3.5. SFS of Leaf Extract

SFS was carried out on a spectrofluorometer (JASCO JP-8300, Jasco International Co., Ltd., Hachioji, Tokyo, Japan), as described before [28].

2.3.6. SPS of Leaf Extract

SPS of secondary metabolites was performed as reported before [28].

2.3.7. 3D-SFS Analysis of Leaf Extract

These measurements of secondary metabolites were carried out as reported previously [28].

2.3.8. Replication Structure and Statistical Analysis

Only one biological replicate was used in the present study.

The independent experimental unit in this study was the extraction experiment (i.e., one extraction performed under a specific combination of extraction factors). The factorial design consisted of the eight treatment combinations performed in duplicate, resulting in 16 experimental runs, which formed the dataset used for the factorial ANOVA. The corresponding 15 degrees of freedom therefore correctly reflect these 16 observations. The factorial analysis represents a screening study and that the statistical inference should be interpreted within the limitations of this replication structure.

Analytical triplicates were performed only for the quantification of each extract to improve measurement precision and were averaged before statistical analysis; they were not treated as independent observations. Likewise, the standard deviations reported in Table 1 reflect the variability between the duplicate extraction experiments rather than analytical replicates. Table 1 was revised to implement a standard Compact Letter Display (CLD) derived from Tukey's HSD post hoc procedure. In this new presentation, means within the same column sharing the same superscript letter are not significantly different ($p > 0.05$), while different letters indicate statistically significant differences ($p \leq 0.05$). Additionally, the table footnote was expanded to clearly state the sample sizes ($n = 2$ technical replicates per group; total observations, $n = 16$ per assay), the specific independent factor tested ("Extraction Condition"), and the multiplicity-adjusted post hoc procedure (Tukey's HSD, $\alpha = 0.05$). Correlation and regression analyses of phytochemical parameters were carried out as reported previously [28].

Table 1. Phytochemical parameters of leaf extracts.

Leaf Extracts	TPC (μ moles Gallic Acid Equivalent/g Leaves)	TFC (μ moles Catechin Equivalent/g Leaves)	Reducing Power (μ moles TE Equivalent/g Leaves)	ABTS Inhibition IC ₅₀ (mg Extract/mL)	DPPH Inhibition IC ₅₀ (mg Extract/mL)	Superoxide Radical Scavenging Activity C ₅₀ (mg Extract/mL)	SOD Activity (Units/g Leaves)
H ₂ O, 25 °C, 0 days	14.24 $\pm$ 0.52 bc	3.41 $\pm$ 0.24 d	21.63 $\pm$ 2.17 ^b	0.90 $\pm$ 0.01 e	2.20 $\pm$ 0.02 de	1.35 $\pm$ 0.01 d	1101.95 $\pm$ 34.14 ^a
H ₂ O, 25 °C, 5 days	15.32 $\pm$ 0.01 bc	5.61 $\pm$ 0.93 abc	26.63 $\pm$ 0.36 ^a	0.09 $\pm$ 0.01 f	1.35 $\pm$ 0.02 e	0.90 $\pm$ 0.02 d	1107.72 $\pm$ 91.15 ^a
Hexane, 25 °C, 0 days	12.04 $\pm$ 1.31 c	6.56 $\pm$ 0.82 ab	4.82 $\pm$ 0.15 cd	6.63 $\pm$ 0.05 ^b	6.48 $\pm$ 0.03 bc	1.06 $\pm$ 0.01 d	576.47 $\pm$ 40.71 ^b
Hexane, 25 °C, 5 days	13.46 $\pm$ 0.88 bc	3.23 $\pm$ 0.15 d	0.39 $\pm$ 0.03 e	3.19 $\pm$ 0.01 d	2.65 $\pm$ 0.09 de	0.65 $\pm$ 0.01 d	388.26 $\pm$ 25.42 ^{bc}
H ₂ O, 40 °C, 0 days	14.87 $\pm$ 0.12 bc	4.63 $\pm$ 0.38 bcd	0.87 $\pm$ 0.20 de	0.52 $\pm$ 0.01 ef	2.23 $\pm$ 0.07 de	1.06 $\pm$ 0.01 d	145.62 $\pm$ 23.07 ^c
H ₂ O, 40 °C, 5 days	71.95 $\pm$ 0.30 ^a	6.20 $\pm$ 0.31 ab	12.13 $\pm$ 0.20 ^{bc}	0.32 $\pm$ 0.004 f	1.51 $\pm$ 0.07 ab	0.92 $\pm$ 0.03 d	1249.53 $\pm$ 160.04 ^a
Hexane, 40 °C, 0 days	1.53 $\pm$ 0.02 d	4.36 $\pm$ 0.34 cd	2.08 $\pm$ 0.15 de	10.87 $\pm$ 0.07 ^a	8.78 $\pm$ 0.14 ^a	9.09 $\pm$ 0.02 ^a	549.05 $\pm$ 260.88 ^{bc}
Hexane, 40 °C, 5 days	7.62 $\pm$ 0.33 cd	5.22 $\pm$ 0.38 abcd	2.63 $\pm$ 0.25 de	4.07 $\pm$ 0.04 ^c	7.61 $\pm$ 0.36 ^{ab}	8.23 $\pm$ 0.02 ^b	237.00 $\pm$ 18.37 ^c

Values are expressed as mean $\pm$ SD ($n = 2$ technical replicates). For each column, means followed by the same lowercase letter are not significantly different according to Tukey's honestly significant difference (HSD) test, following one-way ANOVA ($p < 0.05$).

3. Results and Discussion

3.1. Testing the Chemical Properties of Leaf Extract

Phytochemical screening methods are very useful in the identification and quantitative analysis of bioactive compounds present in plants. These methods provide valuable information about the presence of various phytochemicals in plants such as alkaloids, flavonoids, tannins, saponins, terpenoids, and phenolics, which play vital roles in the therapeutic properties of medicinal plants [17,29]. This understanding of phytochemical composition of leaf extract is of great interest for novel pharmaceutical discovery, quality control and safety assessment, standardization of traditional herbal medicines, and leaf chemistry for ethnobotanical investigations. However, these assays also present challenges and limitations, such as interference from complex plant matrices, the need to use a confirmatory advanced analytical technique, variation in levels of phytochemical substances due to environmental factors, and the need to standardize methods for increased reproducibility [17,29]. Table 1 presents the analysis of various phytochemical assays for these leaf extracts which were obtained by using one biological replicate in the present study. The highest levels of total phenolic content (TPC) and superoxide dismutase (SOD) were achieved using an aqueous extraction at 40 °C with a 5-day incubation period. Conversely, H₂O extraction of leaves at 25 °C for 5 days yielded the highest reducing power, ABTS, and DPPH levels. Regarding total flavonoid content (TFC) and superoxide radical scavenging activity, the maximum values were obtained using hexane at 25 °C with incubation periods of 0 and 5 days, respectively. In comparison, the literature results for *T. diversifolia* leaf extracts showed TPC and TFC values of 52 and 59 μ moles/L in H₂O extracts, respectively [10]. The levels of TPC and TFC for flowers were reported to be 57.81 mg tannic acid equivalent and 109.39 mg quercetin equivalent/g dry weight [12]. On the other hand, the levels of superoxide scavenging activity and DPPH were 5.5 μ g/mL and 4.3 mg/mL of leaf extract, respectively [10,14]. The data on TPC and TFC in the current work were 72.16 μ moles gallic acid equivalent/g leaves and 6.56 μ moles catechin equivalent/g leaves, respectively (Table 1). The leaf extract exhibited superoxide scavenging and DPPH activities of 0.65 mg/mL and 1.35 mg/mL, respectively (Table 1). Direct comparison with the literature is challenging due to variations in plant parts, methodologies, and reporting units [10,12,14]. According to the literature survey of *T. diversifolia* leaf extract, FTIR analysis, and only some phytochemical assays (i.e., TPC, TFC, DPPH, and superoxide radical scavenging activity) were performed [10,12,14]. Besides all of these phytochemical parameters and FTIR analysis, the present work has performed additional phytochemical analysis (i.e., reducing power, and ABTS and SOD levels), screening factorial design and analytical fingerprinting of leaf extracts by SFS, SPS, and 3D spectra.

Phenolic compounds are found in plants which have revealed scavenging activity due to their hydroxyl groups. Moreover, phenolic substances such as tannins, flavonoids, and phenolic acid have exhibited several biological activities, such as anti-carcinogenic, antibacterial, and anti-inflammatory activities [17,29]. Several research works have suggested that medicinal plants contain high levels of polyphenols, which have exhibited high antioxidant capacity in the defensive process against ROS by provoking molecular injuries to pests, microorganism, herbivores, and insects. In plants, flavonoids are very useful to combat oxidative stress, and some possible mechanism of their action may involve the inhibition of various enzymes such as lipoxygenase and xanthine oxidase. Alternatively, DPPH serves as a valuable free radical for assessing the non-enzymatic antioxidant activity of natural products. Although superoxide anions are weak oxidants, they generate highly destructive hydroxyl radicals and singlet oxygen, which induce oxidative stress and suppress defense enzymes like catalase and glutathione peroxidase [30–32]. Bioactive compounds such as phenolics, flavonoids, tannins, and other polar fractions can eliminate these anions. Their electron-donating capacity and ability to stabilize free radicals enable them to effectively scavenge various reactive oxygen species [30–32].

3.2. Screening Factorial Design for Extraction of TPC from Leaf Extracts

The main contribution of this work involved screening factorial design by phytochemical analysis of leaf extracts, which were obtained by using one biological replicate in the present study. The model is based on duplicate experimental runs, thus limiting the strength of the statistical inference. In order to extract bioactive compounds from leaves, a systematic work was carried out to investigate various parameters that influence the extraction of these bioactive substances. In the present work, two solvents (water and hexane) with a different polarity index (9 and 0, respectively) were used for the extraction of bioactive compounds [30]. Since this was a preliminary study for extraction of bioactive compounds from leaf extract, we decided to investigate only these two solvents with different polarity index values. Water belongs to the group of green solvents which are nontoxic, biodegradable, recyclable, and renewable, and it has a boiling point. It is a non-selective solvent, and it can extract polar compounds such as saponins, phenolics, polysaccharides, glycosides, alkaloids, terpenoids, and carbohydrates. Alternatively, hexane belongs to the group of organic solvents with a relative polarity of 0.009, which can be used for extraction of lipophilic compounds such as oils, flavonoids, alkaloids, and anthocyanins. There are other parameters that affect the extraction efficiency of secondary metabolites such as extraction time, solvent-to-solute ratio, temperature, and extraction method [30].

A 2³-way full factorial design was used for the screening process, consisting of eight duplicate experiments as technical replicates. Table 2 summarizes the analysis of the model variables, showing both the average responses and the predicted results. Design-Expert software was utilized to determine the effects of individual variables, multi-variable interactions, and predetermined process objectives. The model is based on duplicate experimental runs and limit the strength of the statistical inference.

Table 2. Analysis of variance (ANOVA) of the factorial design model proposed.

	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-Value
Model	6740.36	7	962.91	2558.67	<0.0001
A-Temperature	418.77	1	418.77	1112.76	<0.0001
B-Time	1077.58	1	1077.58	2863.39	<0.0001
C-Solvent	1668.91	1	1668.91	4434.70	<0.0001
AB	920.44	1	920.44	2445.83	<0.0001
AC	1354.75	1	1354.75	3599.90	<0.0001
BC	640.57	1	640.57	1702.16	<0.0001

Table 2. Cont.

	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-Value
ABC	659.32	1	659.32	1751.98	<0.0001
Pure Error	3.01	8	0.3763		
Cor Total	6743.37	15			

Std. Dev. = 0.6135; $R^2 = 0.9996$; Mean = 18.88; Adjusted $R^2 = 0.9992$; CV = 3.25%; adequate precision = 162.32; contribution of A = 6.21%; contribution of B = 15.98%; and contribution of C = 24.75%.

An F-value of 2558.67 confirms that the model is highly significant, indicating a negligible probability that such a high value occurred due to noise. The coefficient of determination ($R^2 = 0.9996$) demonstrates a strong statistical correlation between the response and the studied variables, with only 0.01% of the total variation left unexplained by the model. Furthermore, the predicted R^2 of 0.9982 is in close agreement with the adjusted R^2 of 0.9992, as the difference is well below the 0.2 threshold. Adequate precision, which measures the signal-to-noise ratio, ideally should exceed 4. The ratio of 162.323 obtained in this study indicates an excellent signal-to-noise ratio. Statistical analysis confirms that all primary factors influencing phenolic compound extraction are significant: temperature (A), time (B), solvent (C), and their interactions (AB, AC, BC, and ABC) (Table 3). Among these, time, solvent, and the temperature–solvent interaction (AC) exerted the most substantial effects. The following empirical equation was derived to estimate phenolic compound extraction from leaf extract (Table 3):

$$y = 18.88 + 5.12 \times A + 8.21 \times B - 10.21 \times C + 7.58 \times AB - 9.20 \times AC - 6.33 \times BC - 6.42 \times ABC$$

where y is the concentration of phenolic compounds, A is the temperature, B is the time, and C is the solvent. This equation was subsequently used to generate the response surfaces illustrated in Figure 1A,B.

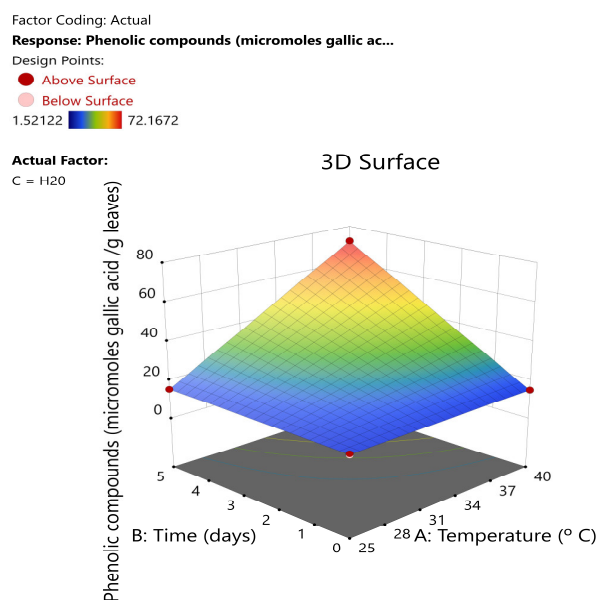
Table 3. Coefficients in terms of coded factors (sum contrasts).

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	18.88	1	0.1534	18.53	19.24	
A—Temperature	5.12	1	0.1534	4.76	5.47	1.0000
B—Time	8.21	1	0.1534	7.85	8.56	1.0000
C—Solvent	−10.21	1	0.1534	−10.57	−9.86	1.0000
AB	7.58	1	0.1534	7.23	7.94	1.0000
AC	−9.20	1	0.1534	−9.56	−8.85	1.0000
BC	−6.33	1	0.1534	−6.68	−5.97	1.0000
ABC	−6.42	1	0.1534	−6.77	−6.07	1.0000

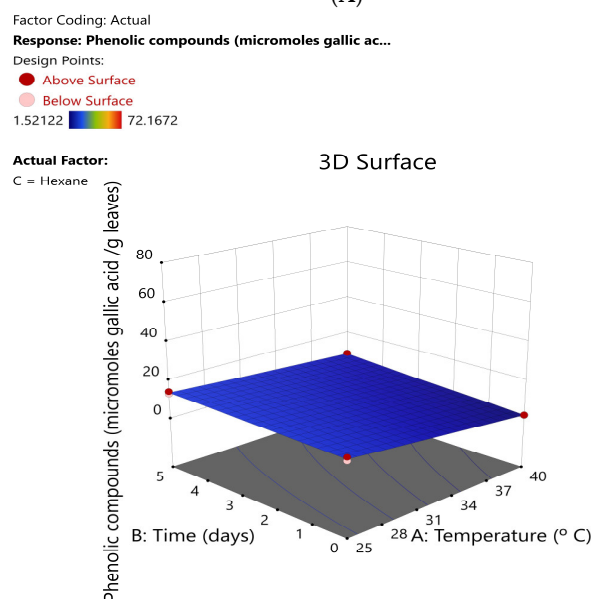
The use of TPC as sole response in this screening factorial design was justified since the main goal was simply to establish extraction factors (time, temperature, and solvent ratio) maximizing the total yield of TPC. Moreover, there is a high positive correlation in many plants of TPC with antioxidant capacity. TPC response involves simplicity and cost-effectiveness, as it is fast, simple, and cheap to carry out for processing many thousands of samples. In the present work, the highest levels of TPC were exhibited in H₂O extracts at 40 °C and 5 days (Table 1).

The data in Figure 1A,B exhibited the screening factorial design for extraction of phenolic compounds from leaf extract. The highest extraction of phenolic substances (i.e., 72.16 µmoles gallic acid equivalent/g leaves) was exhibited at 40 °C in H₂O and 5 days of extraction (Figure 1A). Preliminary experiments were carried out about extraction times of secondary metabolites from leaf extracts which revealed that extraction times of 0 and

5 days were adequate for extraction of key secondary metabolites according to phytochemical assays. Regarding the screening factorial design for secondary metabolite extraction from this leaf in the literature, there is only one published report about extraction by using a 2^2 factorial design matrix for plant flowers extract [12]. Therefore, the present data of screening factorial design are difficult to compare with published data for TPC and TFC due to the different parts of plants and factorial design matrices used, as well as different units presented [12].



(A)



(B)

Figure 1. Screening factorial design for extraction of TPC. Three-dimensional representation for interaction of two key parameters: Extraction/incubation time and temperature of incubation on the extraction of bioactive metabolites. (A) H₂O and (B) hexane.

3.3. SFS

The main contribution of this work involved spectroscopic fingerprints or tentative assignments of secondary metabolites by fluorescence techniques. The $\Delta\lambda$ value in SFS represents a crucial factor for achieving peak resolution, sensitivity, and spectral perfor-

mance. To the authors' knowledge, there are no published reports on intrinsic fluorescence spectroscopy of bioactive compounds from *T. diversifolia* leaves. Moreover, there are very few research works in the literature on fluorescence properties of bioactive compounds in leaf extract in general [18,19]. Therefore, SFS of bioactive compounds from this leaf extract was analyzed with different $\Delta\lambda$ at high and medium sensitivity for leaf extracts with hexane at 25 and 40 °C, respectively (Figure 2A,B). In both cases, there are various fluorescence peaks at 290, 320, 345, 400, 490, and 675 nm which exhibited an increase in fluorescence as a function of $\Delta\lambda$. Published data suggest that the emission peaks observed at 280–320 nm stem from a protein containing aromatic amino acids like Tyr and Trp [33,34]. Regarding the 325–450 nm spectral range, previous studies [33,34] suggest that these emission peaks stem from phenolic substances, hydroxycinnamic acids, and stilbenes. Conversely, the 500–550 nm region likely reflects the presence of flavanols, flavonoids, and alkaloids. Lastly, the emission peak at 675 nm is provisionally attributed to chloroplast chlorophyll [35,36].

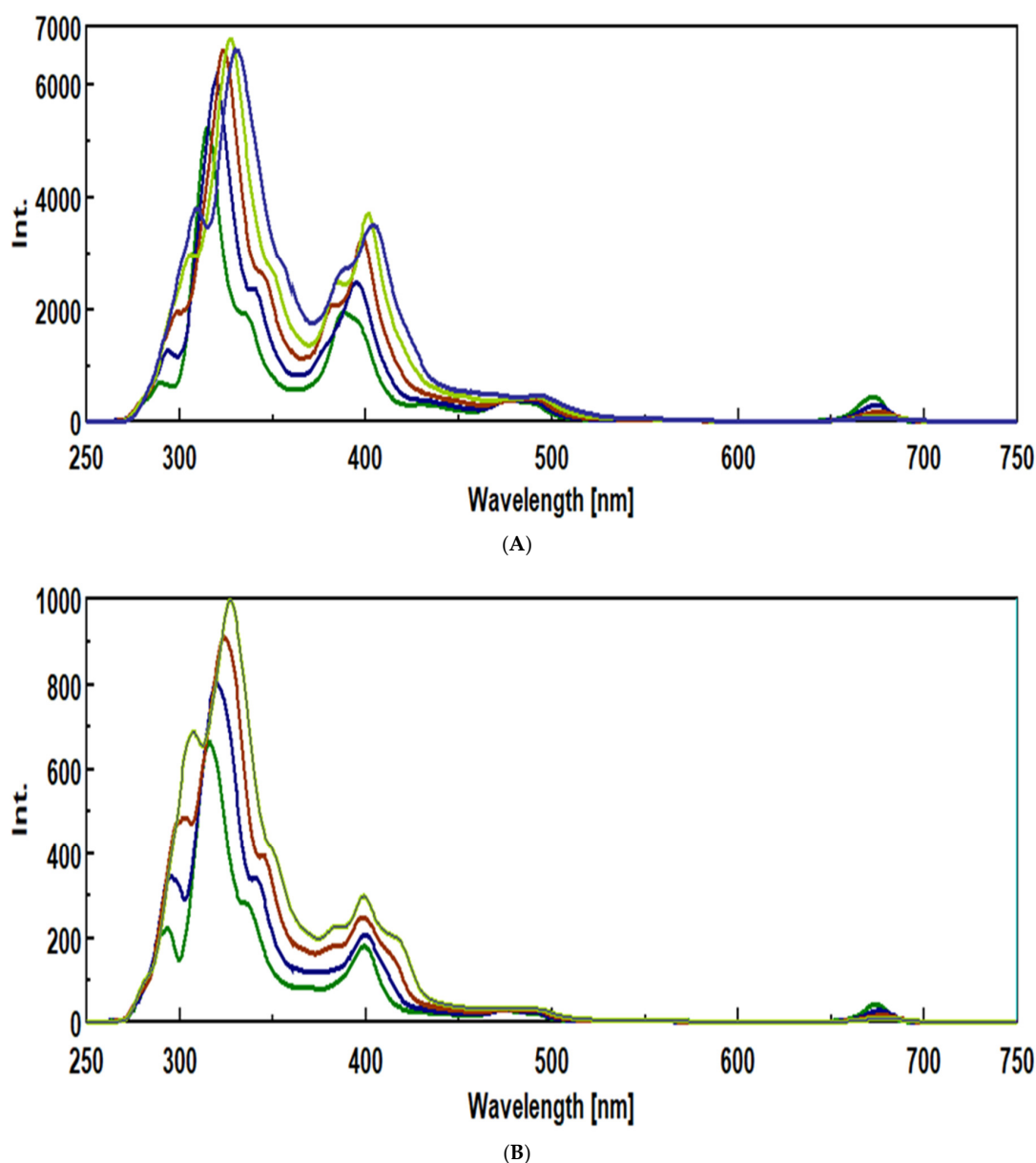


Figure 2. SFS with different $\Delta\lambda$ of bioactive compounds from leaf extract. (A) Leaf extract with hexane at 25 °C and 0 days at high sensitivity: — 10 nm; — 15nm; — 20 nm; — 25 nm; and — 30 nm of $\Delta\lambda$. (B) Leaf extract with hexane at 40 °C and 0 days at medium sensitivity: — 10 nm; — 15nm; — 20 nm; and — 25 nm of $\Delta\lambda$.

As shown in Figure 3A,B, the fluorescence intensity across all emission peaks rose alongside $\Delta\lambda$, with the sole exception of the chlorophyll peak, which demonstrated a decline. Notably, the H₂O extract displayed a distinct emission profile, characterized by fluorescence peaks at 490, 560, and 675 nm (Figure 3B).

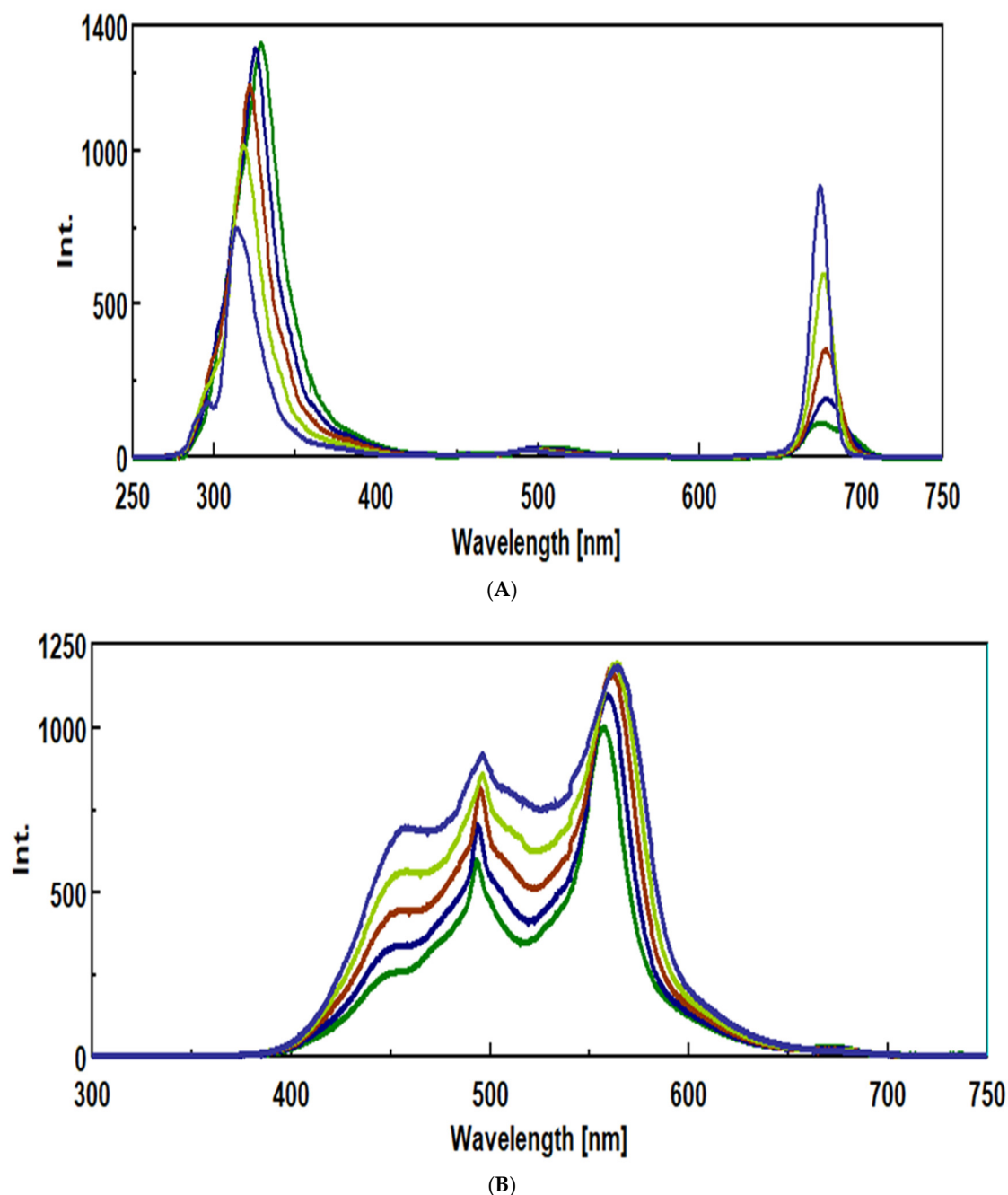


Figure 3. SFS with different $\Delta\lambda$ of bioactive compounds from leaf extract. (A) Leaf extract with hexane at 40 °C and 5 days at medium sensitivity: — 30 nm; — 25 nm; — 20 nm; — 15 nm; and — 5 nm of $\Delta\lambda$. (B) Leaf extract with H₂O at 25 °C and 0 days at high sensitivity: — 10 nm; — 15 nm; — 20 nm; — 25 nm; and — 30 nm of $\Delta\lambda$.

These secondary metabolites were analyzed by SFS and were extracted with aqueous and hexane solutions at different temperatures and incubation times, as shown in Figure 4. The results in Figure 4A,B exhibit different fluorescence emission patterns of H₂O extracts compared with hexane extracts (Figure 4C), as emission peaks were revealed at 490 and 560 nm for Figure 4A; and 490, 540, and 565 nm for Figure 4B.

The present report involved SFS as fingerprinting/comparative profiling technique for leaf extract which requires definitive compound identification methods.

According to the literature survey on intrinsic fluorescence studies (i.e., SFS) of leaf extracts from *T. diversifolia*, there are no such reports; however, some published works have been described on intact parts of leaves, bark, and flowers of other plants by fluorescence spectroscopy [33,34]. Nevertheless, this methodology is essential for evaluating leaf health, nutrient levels, bioactive components, and infections [35,36].

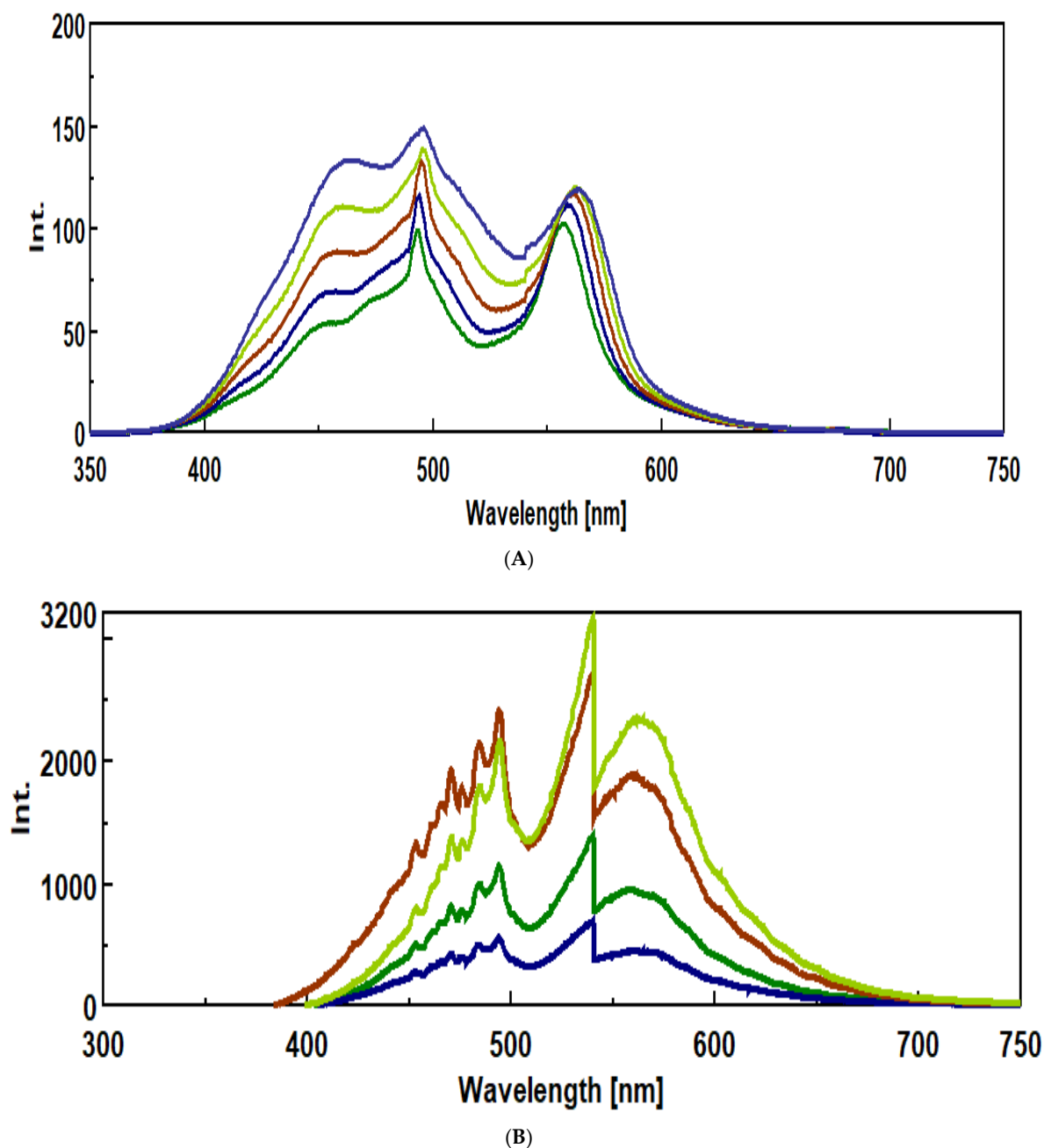


Figure 4. Cont.

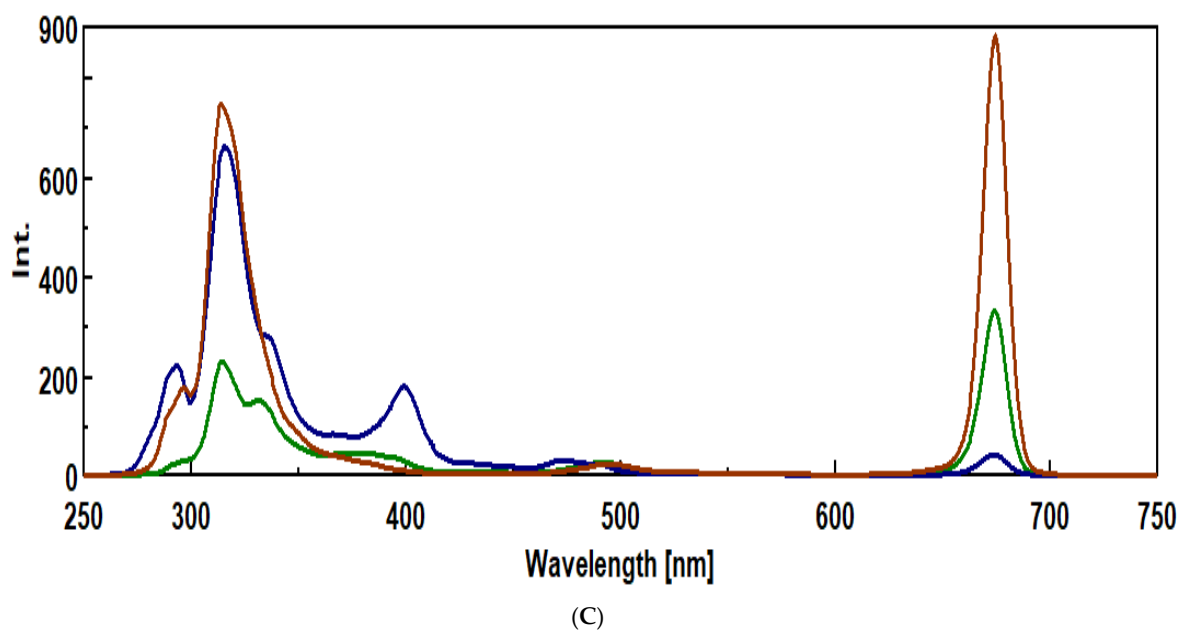


Figure 4. SFS with different $\Delta\lambda$ of bioactive compounds from leaf extract. (A) H₂O leaf extract at 40 °C and 0 days at low sensitivity: — 10 nm; — 15nm; — 20 nm; — 25 nm; and — 30 nm. (B) H₂O leaf extract at medium sensitivity and 5 nm of $\Delta\lambda$: — 25 °C and 0 days; — 40 °C and 0 days; — 40 °C and 5 days; — 25 °C and 5 days. (C) leaf extract with hexane at 10 nm of $\Delta\lambda$ and medium sensitivity: — 25 °C and 5 days; — 40 °C and 0 days; and — 40 °C and 5 days.

3.4. SPS

The $\Delta\lambda$ value in SPS represents a crucial factor for achieving peak resolution, sensitivity, and spectral performance of a specific bioactive compound. Hence, Figure 5 have revealed several fluorescence peaks of SPS at 325, 390, 490, 550, and 675 nm by using hexane extracts. The results in Figure 5A revealed various SPF spectra at 10 nm of $\Delta\lambda$ for several leaf extracts which revealed the highest fluorescence peaks with hexane extract at 40 °C for 5 days of extraction. On the other hand, the data presented in Figure 5B revealed an increase in fluorescence at low $\Delta\lambda$ both at 400 and 675 nm.

The results in Figure 6 exhibited several SPS spectra for various aqueous and organic leaf extracts. It should be emphasized that H₂O extracts revealed only two fluorescence peaks at 500 and 560 nm (Figure 6B) compared with several peaks for hexane extract (Figure 6A,C). The present work involved SPS as a fingerprinting/comparative profiling technique for leaf extracts which requires definitive compound identification methods.

Consistent with SFS data, prior studies [33,34] attribute the emission peaks between 280 and 320 nm to proteins rich in aromatic amino acids, particularly tryptophan and tyrosine residues. In the 325–450 nm wavelength range, the observed emission peaks are associated with phenolic substances, stilbenes, and hydroxycinnamic acids [33–36]. Furthermore, the literature suggests that peaks appearing in the 500–550 nm region stem from flavonoids, flavanols, and alkaloids [33–36]. Lastly, the emission peak recorded at 675 nm is linked to chlorophyll located within chloroplasts [36,37]. SPS is a valuable important tool to obtain specific information in plants about stress detection and damage in photosynthetic pigments [35,36].

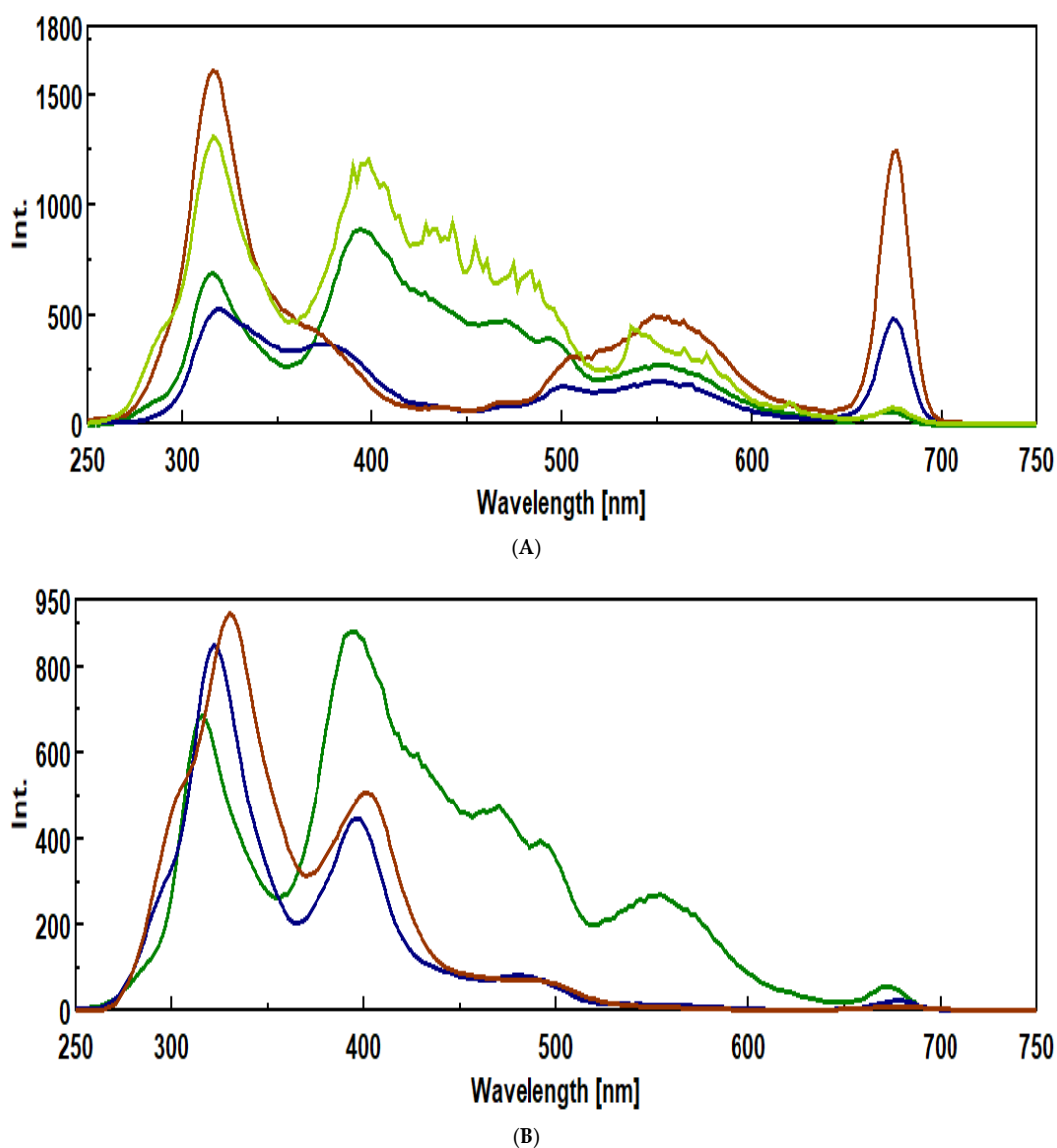


Figure 5. SPS of bioactive compounds from leaf extract. (A) Leaf extract with hexane at low sensitivity and 10 nm of $\Delta\lambda$: — 25 °C and 0 days; — 25 °C and 5 days; — 40 °C and 5 days; and — 40 °C and 0 days. (B) Leaf extract with hexane at 25 °C and 0 days with different $\Delta\lambda$: — 10 nm; — 20 nm; and — 30 nm.

3.5. Intrinsic 3D Fluorescence Spectroscopy (3D-SFS)

By utilizing 3D-SFS, a comprehensive fluorescence map of all fluorophores within the sample was generated to enable detailed characterization. The results displayed in Figure 7A–F present 3D spectra in various formats for bioactive molecules extracted from the leaves using hexane at 40 °C over a 5-day incubation period. Additionally, Figure 7F includes a synchronous 2D spectrum highlighting distinct fluorescence peaks at 290 nm and 675 nm. The 3D spectral results in Figure 7 revealed multiple fluorescence peaks at 290, 320, 345, 550, and 675 nm, matching the SFS results. Notably, the chlorophyll emission peak at 675 nm was resolved into five separate peaks across different excitation wavelengths (Figure 7A,B,D,E). This splitting stems from the complex, overlapping signals of diverse pigment–protein complexes, photosynthetic systems, and their respective degradation products [37,38]. Consequently, the 3D spectra of these leaf extracts serve as a diagnostic fingerprint for the state of the photosynthetic apparatus, fragmenting the primary 675 nm

emission peak into distinct components that correspond to specific functional and structural elements of the chloroplast [19].

Consistent with the SFS results, the 3D spectra in Figure 8 display multiple fluorescence peaks at 290, 320, 345, 550, and 675 nm, mirroring the trends observed in Figure 7. However, the profiles in Figure 8 correspond to the hexane extract obtained at 40 °C with 0 days of extraction. This lack of incubation explains the lower chlorophyll fluorescence peak at 675 nm compared to the 5-day hexane extract shown in Figure 7. The present report involved 3D-SFS as a fingerprinting/comparative profiling technique for leaf extract which requires definitive compound identification methods.

Even though the results on the 3D fingerprinting spectra for these leaf extracts have not been reported, there are published works in the literature about intact leaves of other plants by this analytical 3D technique [19]. However, 3D fluorescence spectroscopy is an important tool in several research fields, namely precision farming, location of leaf harvest, purity testing, and phyto-forensics of leaf-based products [19,39–41].

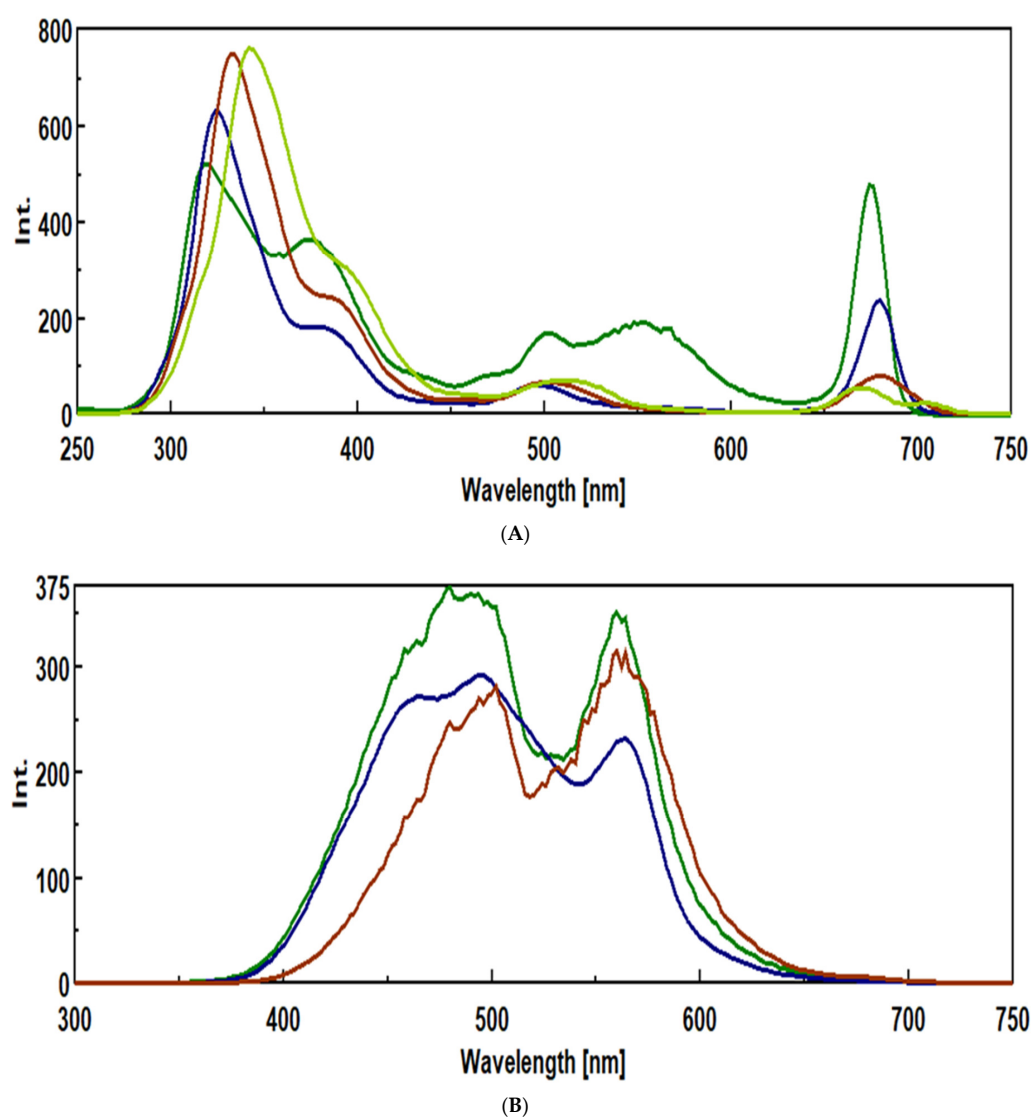


Figure 6. *Cont.*

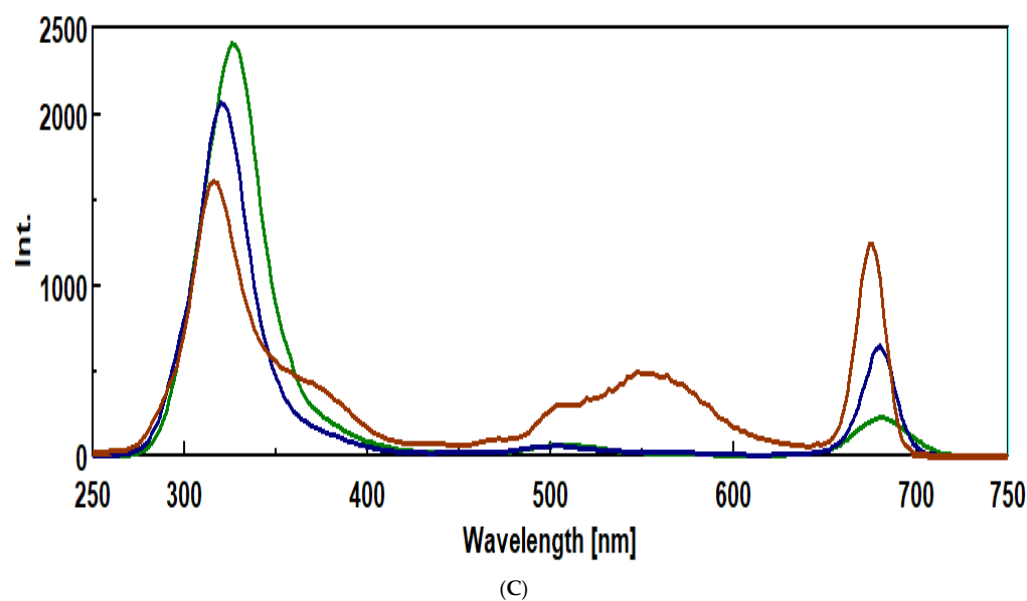


Figure 6. SPS of bioactive compounds from leaf extract. (A) Leaf extract with hexane at 25 °C and 5 days at low sensitivity with different $\Delta\lambda$: — 10 nm; — 20 nm; — 30 nm; and — 40 nm. (B) H₂O leaf extract at 40 °C with different $\Delta\lambda$: — 0 days and 20 nm; and — 0 days and 30 nm and — 5 days and 20 nm. (C) Leaf extract with hexane at 40 °C and 5 days at low sensitivity with different $\Delta\lambda$: — 30 nm; — 20 nm; and — 10 nm.

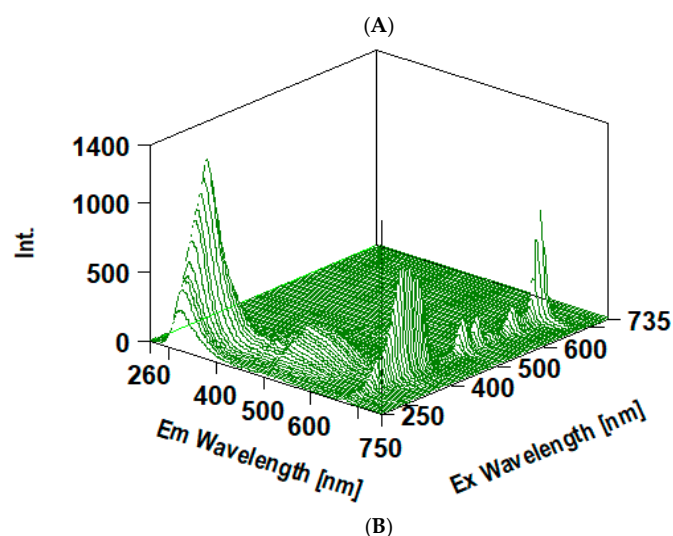
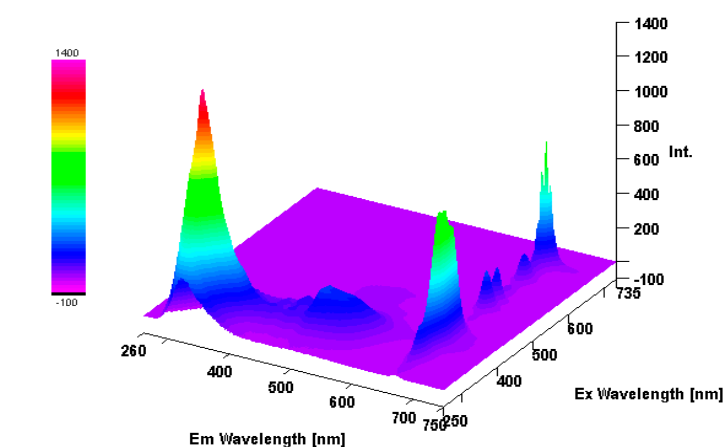
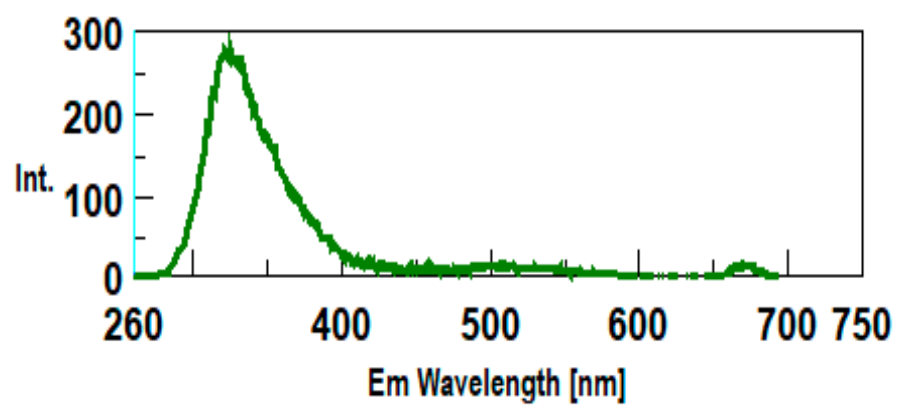
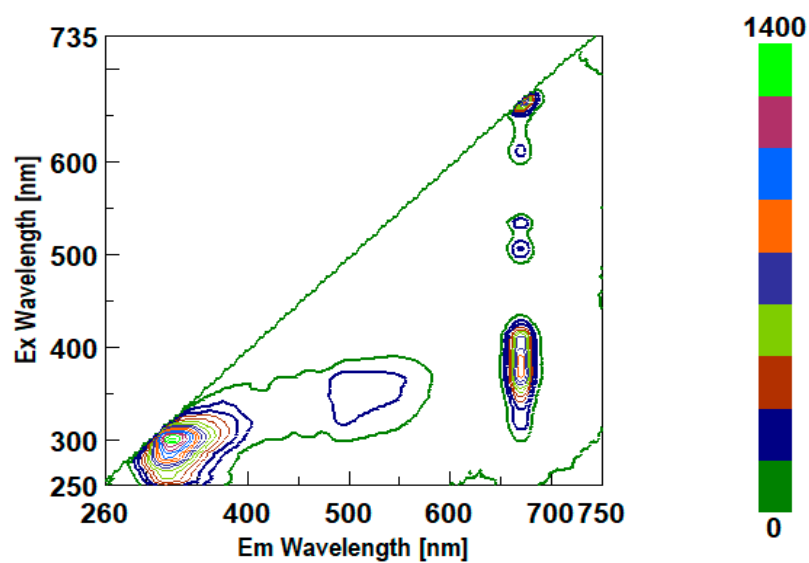


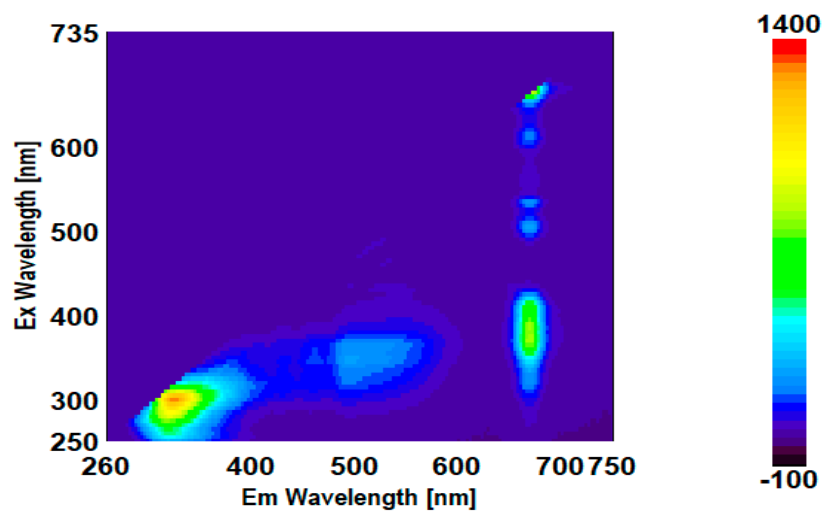
Figure 7. Cont.



(C)



(D)



(E)

Figure 7. Cont.

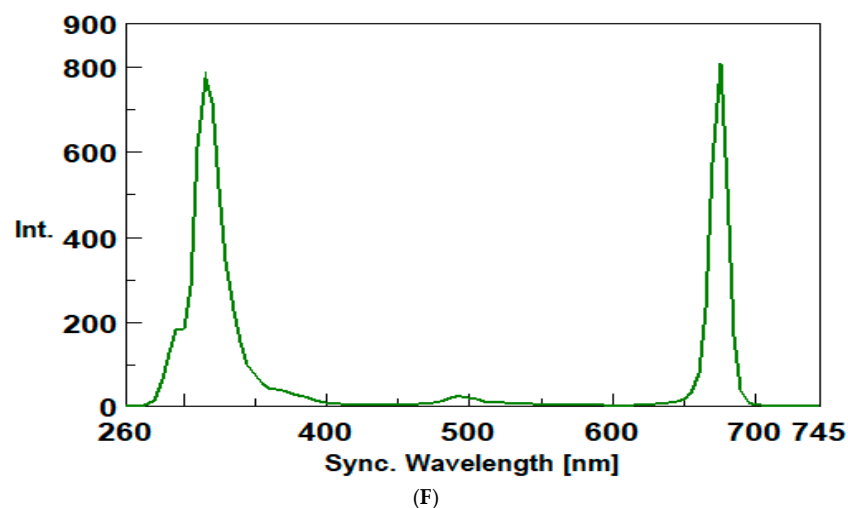


Figure 7. Bioactive compounds of leaf extract with hexane at 40 °C and 5 days with medium sensitivity and 10 nm of $\Delta\lambda$. (A) Color 3D view, (B) three-dimensional spectrum view, (C) 2D spectrum view, (D) contour view, (E) color view, and (F) synchronous two-dimensional spectrum.

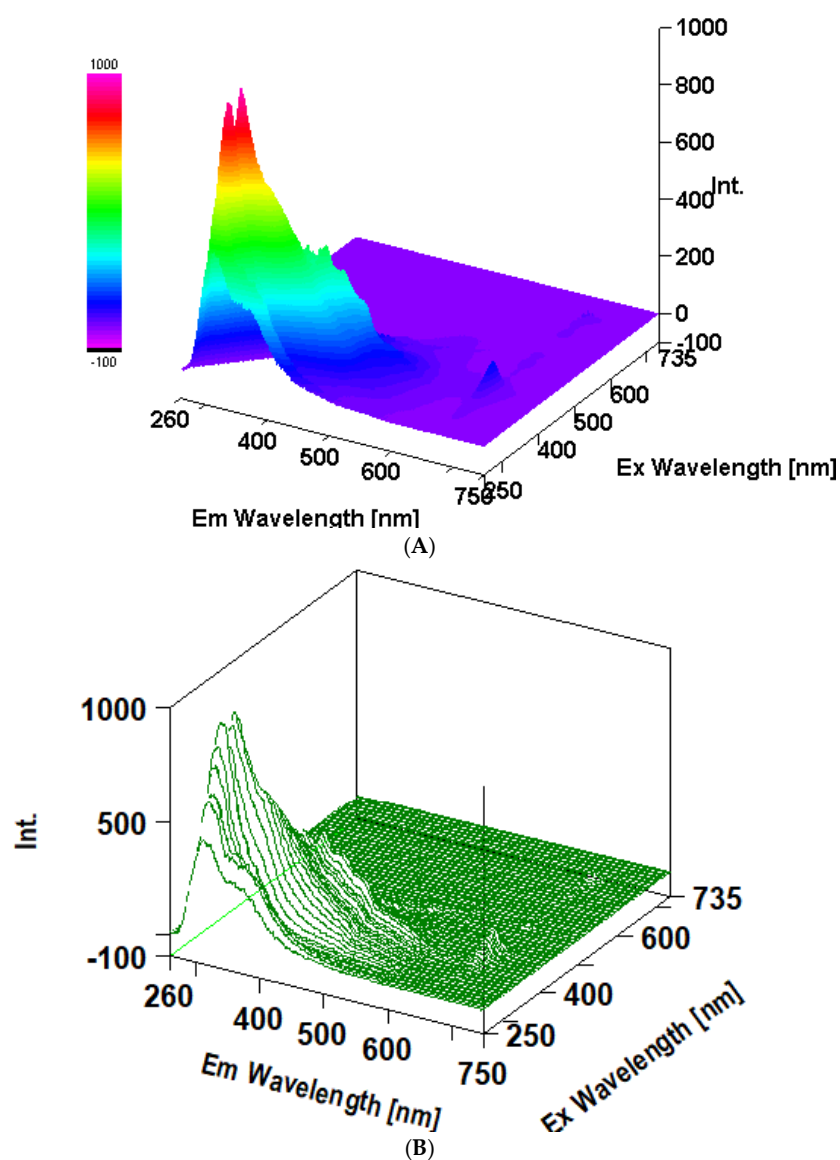
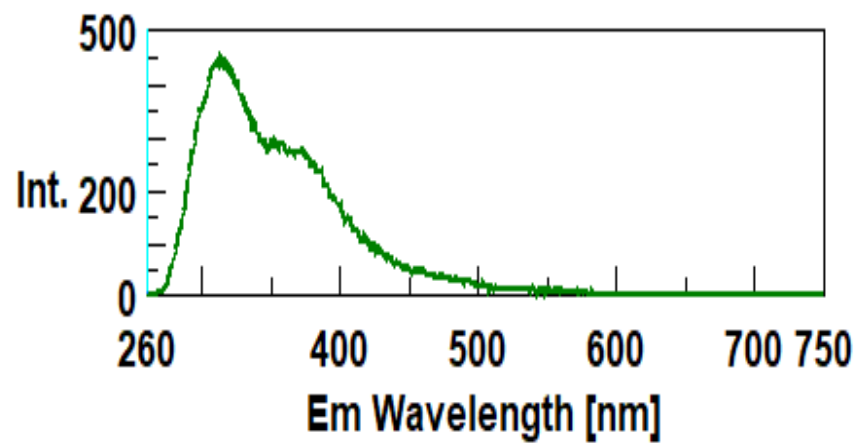
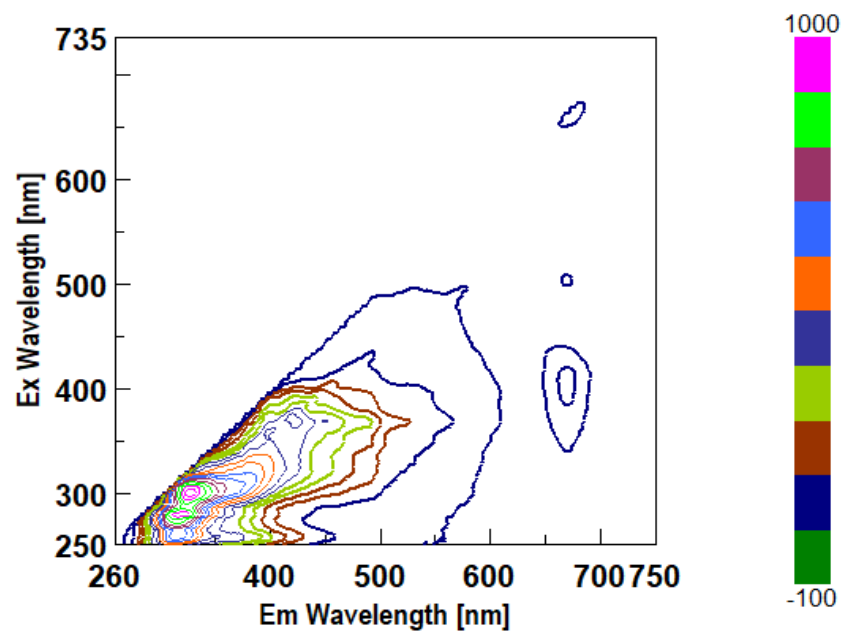


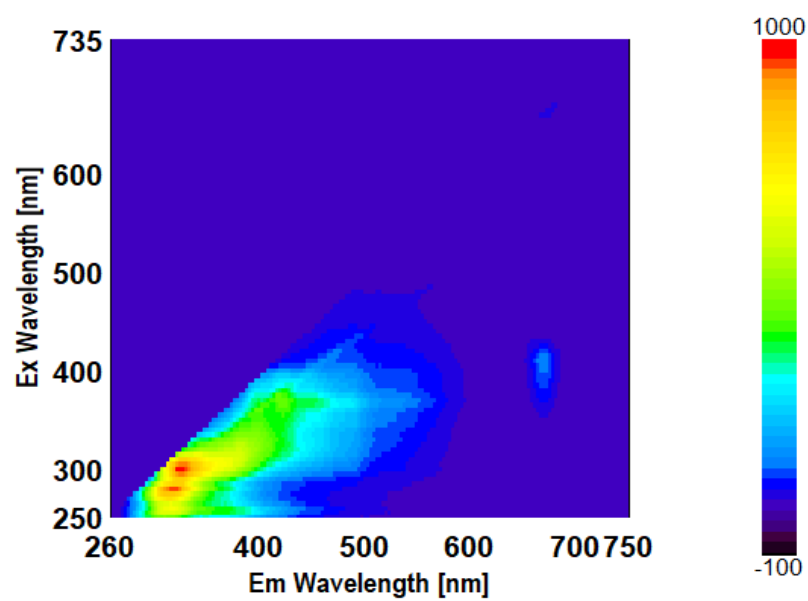
Figure 8. *Cont.*



(C)



(D)



(E)

Figure 8. Cont.

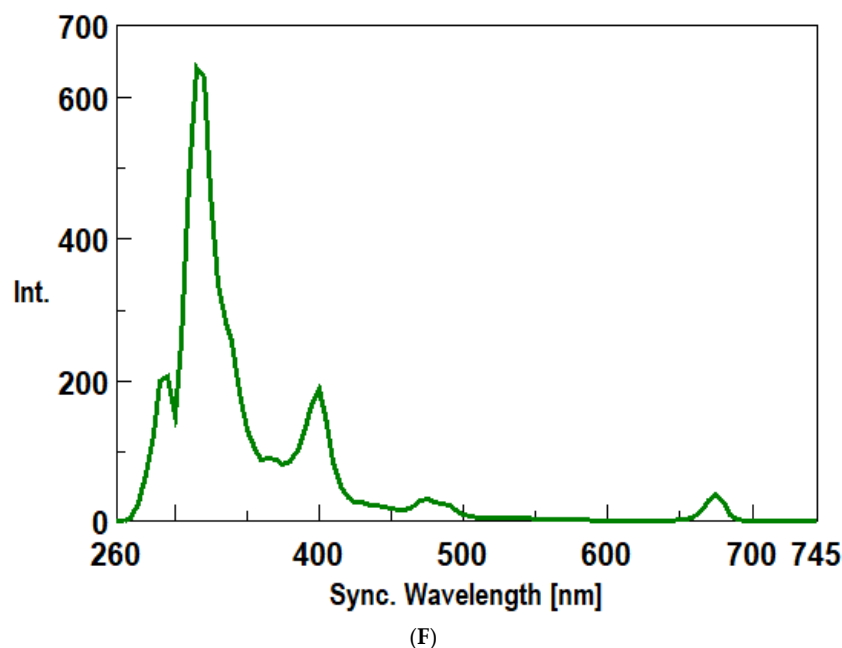


Figure 8. Bioactive compounds of leaf extract with hexane at 40 °C and 0 days with medium sensitivity and 10 nm of $\Delta\lambda$. (A) Color 3D view, (B) three-dimensional spectrum view, (C) 2D spectrum view, (D) contour view, (E) color view, and (F) synchronous two-dimensional spectrum.

4. Conclusions

The main contribution of this work involved screening factorial design for phytochemical analysis of leaf extracts, as well as spectroscopic fingerprints or tentative assignments of secondary metabolites by fluorescence techniques. To the best of our knowledge, this study represents the first comprehensive evaluation combining multiple phytochemical assays (ABTS, DPPH, superoxide scavenging activity, TPC, TFC, reducing power, and SOD levels) with advanced analytical fluorescence fingerprinting techniques (specifically SFS, SPS, and 3D fluorescence spectra) to analyze bioactive metabolites in leaf extracts. Additionally, a screening factorial design was successfully applied to optimize the extraction of TPC. Our findings strongly demonstrate that the extracts contain high concentrations of phytochemicals, validating their robust in vitro antioxidant properties. A comparative analysis revealed that SPS yields higher fluorescence intensity for bioactive metabolites than SFS in these leaf matrices. Furthermore, when dealing with complex leaf matrices, SPS and 3D-SFS effectively overcome the traditional limitations of standard SFS. They simplify excitation-emission contours, resolve overlapping fluorophores, and enhance detection sensitivity, thereby providing a clearer analytical fingerprint for structurally similar compounds. The novelty of this work lies in the detailed integration of SFS, SPS, and 3D-SFS to establish distinct spectroscopic fingerprints and tentative structural assignments for secondary metabolites based on the existing literature. Ultimately, these analytical techniques offer a cost-effective, rapid, non-invasive, and intrinsic approach. Because they do not require exogenous fluorophores, need minimal sample volumes, and provide high sensitivity and selectivity, they are ideal for high-throughput screening in purity testing and phyto-forensic applications. This limitation underscores a classic analytical bottleneck in metabolomics and phytochemistry: the requirement of analytical standards for the definitive identification of these bioactive compounds via NMR, GC-MS, GC-FID, HPLC, ELISA, and chemometrics.

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A.K.; writing—original draft, A.K.; writing—review and editing, A.K.; supervision, A.K.; project administration, A.K. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author due to legal reasons.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
3D-SFS	3D synchronous fluorescence spectroscopy
NBT	Nitro blue tetrazolium
SFS	Synchronous fluorescence spectroscopy
SOD	Superoxide dismutase
SPS	Synchronous phosphorescence spectroscopy
TPC	Total phenolic content
TFC	Total flavonoids content

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