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Karla Ramos and Amin Karmali *

Posted Date: 15 April 2026

doi: 10.20944/preprints202602.1369.v2

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 doi:10.20944/preprints202602.1369.v2

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Experimental Planning for Extraction of Secondary Metabolites from *Tithonia diversifolia* (Hmsl.) A. Gray/Asteraceae Leaves: Biological and Chemical Characterization by Synchronous Fluorescence and Phosphorescence Spectroscopy and FTIR

Karla Ramos [1] and Amin Karmali [1,2,*]

[1] Chemical Engineering and Biotechnology Research Center and Chemical Engineering Department of Instituto Superior de Engenharia de Lisboa, Instituto Politécnico de Lisboa Rua Conselheiro Emídio Navarro, Nº. 1, 1959-007 Lisboa, Portugal

[2] CITAB-Centre for the Research and Technology of Agro-Environmental and Biological Sciences, UTAD, 5000-801 Vila Real, Portugal

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

Abstract

S. Tomé and Principe (STP) islands have been studied in recent years for their wide range of medicinal plants which exhibit several biological activities of great medicinal interest for some diseases. Experimental planning for optimization of several parameters was carried out by a full factorial of two levels of three factors for secondary metabolite extraction from *Tithonia diversifolia* leaves by using water and hexane at 25 and 40 ºC and 150 rpm for 0 and 5 days of incubation. The best conditions for highest extraction of phenolic compounds (i.e 72.16 mmoles gallic acid equivalent/g leaves)) was obtained at 40ºC, in H_20 and 5 days of incubation. Several phytochemical assays were performed for characterization of these plant extracts and the highest levels for extraction of reducing power, ABTS and DPPH were obtained at 25 ºC, H_20 and 5 days of incubation whereas highest levels of SOD activity were extracted at 40ºC, H_20 and 5 days of incubation. The present report consists of a novel and intrinsic synchronous fluorescence and phosphorescence characterization of secondary metabolites from this plant extract. Intrinsic and non-destructive synchronous fluorescence was carried out in the range of 250 to 750 nm with a $\Delta\lambda$ range of 5–30 nm which exhibited peaks at 290, 320, 345, 400, 490 and 675 nm in hexane plant extracts whereas aqueous extracts revealed only peaks at 490, 560 and 675 nm. On the other hand, intrinsic and non-destructive synchronous phosphorescence was also performed which exhibited peaks at 325, 400, 490, 550, 675 nm and 500, 560 nm, respectively. 3-D spectra of secondary metabolites confirmed the peaks at 290, 320, 345, 400, 490 and 675 nm in plant extracts. FTIR spectroscopy was selected to investigate the structural properties of secondary metabolites in these plant extracts. Therefore, the present work describes a novel characterization of secondary metabolites by a non-destructive and intrinsic synchronous fluorescence techniques for plant extracts.

Keywords: *Tithonia diversifolia*; plant leaves extracts; experimental planning; phytochemical assay of secondary metabolites; antioxidant activity; intrinsic synchronous fluorescence and phosphorescence spectroscopy; 3 -D. fluorescence spectra; FTIR spectroscopy

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]. Phytochemicals can be divided into six

 doi:10.20944/preprints202602.1369.v2

main groups according to their chemical structure: carbohydrates, lipids, phenolics, terpenoids, alkaloids and nitrogen containing compounds. These plant substances are known as secondary metabolites since there are not essential for plant survival [1]. Quantitative phytochemical assays on plant 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 plant has been widely used in traditional medicine for clinical conditions of diabetes, diarrhoea, menstrual pain, malaria, hematomas, hepatitis, hepatomas, and wound healing among other diseases [5,6]. S. Tomé and Principe (STP) islands in the Gulf of Guinea are very rich in many medicinal plants that are used by the local population with guidance of local traditional healers [7,8]. Several plant extracts of *T. diversifolia from* STP have been prepared in various solvents such as ethanol, water, dichloromethane, petroleum ether, methanol and ethyl acetate for treatment of several diseases [9,10]. The organic extract of this plant leaves has been successfully used for many decades for treatment of malaria in STP [11]. As far as biological and chemical composition of these plant extracts are concerned, they contain phenolic and flavonoids, terpenoids, proteins, carbohydrates and glycosides as well as antioxidant and scavenging superoxide activities [12,13].

There are some published reports about phytochemical assays for phenolics, flavonoids, antioxidant and superoxide scavenging activities from this plant extract as well as some FTIR analysis [10–15]. Although some research work has been carried out on this plant, there is little data on the chemical and biological composition of these plant extracts. Moreover, to the best of our knowledge, we have not found any published report about experimental planning for extraction of secondary metabolites from this plant. This is an important strategy for optimization of secondary metabolite extractions as it provides useful information about significant factors and the interactions between the variables [16]. Optimal extraction yield of secondary metabolites from plant 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 plants [17].

Therefore, the current work is concerned with optimization of secondary metabolites extraction from plant leaves by experimental planning by using water and hexane as extraction solvents at 25 and 40 ºC. These plants extracts were analysed by phytochemical assays for quantification of phenolic compounds, flavonoids, reducing power, antioxidant activity with DPPH, ABTS, superoxide scavenging activity and SOD activity since no previous works have investigated in detail these phytochemical parameters [9,10]. On the other hand, intrinsic synchronous fluorescence and phosphorescence as well as 3-d fluorescence spectroscopy are very useful analytical techniques for identification of secondary metabolites in plant extracts as they are fast, sensitive and non-destructive [18,19]. However, to the best of our knowledge, there are no reports in the literature about fluorescence characterization of this plant extract. Therefore, the present work involved fluorescence characterization of these secondary metabolites by intrinsic synchronous fluorescence, phosphorescence and 3-d fluorescence spectroscopy as well as by FTIR analysis.

2. Materials and Methods

2.1. Chemicals

Phenazine methosulfate, NADH, nitro blue tetrazolium (NBT), Riboflavin, superoxide dismutase (SOD) from bovine erythrocytes (3,000 units/mg protein) deuterated water, gallic acid, catechin, Trolox, ascorbic acid, Folin & Ciocalteu, DPPH and ABTS (2,2'-Azino-di [3-

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ethylbenzthiazoline sulfonate]) were obtained from Sigma-Aldrich (USA). All other reagents were of analytical grade.

2.2. Plant Collection

Plant leaves were collected on the island of S. Tomé, *T. Diversifolia* (Hmsl.) A. Gray. in the S. Tomé botanical garden, in the interior of the island, at 6 am, with very high humidity and average temperature of 24 °C in January 2018 (GPS coordinates: 0°17'16.0"N 6°38'14.0"E) and they were free of pests and diseases from the same tree. These plants were collected under the guidance of the healers, and they were identified at the S. Tomé e Príncipe National Herbarium and STP Agronomical Research Centre (CIAT-STP). A voucher specimen of the plant was deposited in the herbarium of S. Tomé (08-01-2018), Voucher Number 001 / 2019 and the samples were cut into small fragments and evenly packed.

2.3. Methods

2.3.1. Preparation of Plant Extract

The plant leaves were properly washed with distilled water, dried overnight in an oven at 40 º C. The dried plant material was ground through a IKA A10 universal grinder and the plants extracts were prepared with 15 grams of ground dry matter from the leaves of *T. Diversifolia* in either 150 mL of demineralized water or 150 ml of hexane. All plant extracts were transferred to an orbital shaking either at 25ºC or 40 ºC according to experimental planning procedure. The plant extract of day 0 was shaken for 30 min in orbital shaker at 150 rpm and centrifuged at 10.000 rpm for 30 min at room temperature, the supernatant was recovered and stored in Eppendorf tubes at -20º C for further analysis. The remaining plant extracts were incubated in orbital shaker at 150 rpm for 5 days at 25 and 40 ºC with either water or hexane. After 5 days, the same procedure was carried out to recover the supernatant in Eppendorf tubes and stored at -20 ºC in dark containers protected from light.

Phytochemical assays

All phytochemical assays were carried out by using 8 samples from experimental planning design described below

2.3.2. Determination of Total Phenolic Content (TPC)

Phenolic compounds from plant extracts were determined by the Folin-Ciocalteau procedure [20–22] with some modifications to a final volume of 300 µL. In a 96-well microplate, 30 µL of plant extract was added to 150 µL of aqueous Folin-Ciocalteau reagent solution (diluted 1:10, V/V), and finally 120 µL of sodium carbonate (0. 25 mg / mL) was added to a final volume of 300 µL. After shaking, the microplate was incubated at 40 ° C for 30 min protected from light. Absorbances were read at 765 nm in a microtiter plate reader in triplicates (FLUOstar OPTIMA-BMG Labtec, Ortenberg, Germany). A calibration curve of gallic acid (0.25mg / mL), (R^2 = 0.99) was carried out with the following volumes: 0, 5, 10, 15, 20, 25 µL, adjusted to final volume of 30 µL with water. The results of the total phenolic compounds were expressed as µmole gallic acid equivalent per g of leaves.

2.3.3. Determination of Total Flavonoids Content (TFC)

Flavonoids quantification was carried out by using the colorimetric method described previously [23], with some modifications. A calibration curve was carried out with the catechin standard solution (0.3 mg / mL) by using concentrations in the range of $1.5x10^{-2}$ - 1.0mM, which revealed a R^2 = 0.9958 and the results were expressed in µmole catechin equivalent / g of leaves. Therefore, a 25 µL aliquot of extracts were diluted with 110 µl demineralized water and 7.5 µL sodium nitrite (5%, w/v). 7.5 µL aluminium chloride (10%, w/v) were added to the microplate and incubated for 6 minutes at room temperature and protected from light. Subsequently, 100 µL of a sodium hydroxide solution (4%) was added to a final volume of 250 µL of the reaction mixture which

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was mixed manually and incubated for 15 minutes. The absorbance was measured at 510 nm in a microtiter plate reader (FLUOstar OPTIMA – BMG Labtec and its accompanying software Optima 2.10 R3) and compared with that of a blank reaction mixture which contained deionized water. All assays were carried out in triplicate.

Antioxidant Activity of plant extracts

2.3.4. DPPH Radical Scavenging Activity

Quantification of DPPH free radical scavenging activity was based on the method described previously [22], with some modifications to a final volume of 310 µl. An aliquot of plant extract was pipetted (0, 4, 8, 12, 16, 20 µL) to 290 µL of the 0.3 mM DPPH solution which was prepared by using methanol: water mixture (80:20, v/v). The microplate was incubated at room temperature in the dark for 1h and read at 550 nm on a microplate reader (FLUOstar OPTIMA-BMG Labtec). The DPPH scavenging effect was determined as follows:

$$\text{DPPH Scavenging Effect (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

where $A_{control}$ - the absorbance of the negative control and A_{sample} - the absorbance at 550 nm of the tested samples or standard. The test was performed in triplicate and Trolox was used as standard in the range of 0 to 6 µg/well. The IC_{50} values for each plant extract were calculated from the graph of DPPH scavenging effect against the concentration of extracts (mg/mL).

2.3.5. ABTS Scavenging Activity

This assay was carried out as described previously [23,24] with some modifications. The reaction of ABTS (8 mM) and $K_2S_2O_8$, (2.45 mM) in demineralized water (H_2O) was allowed to react for 12 h protected from light. The working solution involved the use of 0.5 mL stock solution (described above) along with (14.5 mL) 50 mM phosphate buffer pH 7.4. In a 96-dark well microplate, 16 µL of plant extract and 280 µL of $ABTS^{\bullet+}$ radical solution was added to a final volume of 296 µL which was mixed manually and the microplate was incubated for 30 minutes, protected from light. The absorbance was read at 655 nm, all assays were carried out in triplicates and Trolox (0.3 mg/ mL) was used as standard in the range of 0 to 6 µg/well. The following expression was used:

$$\text{ABTS scavenging effect (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

where $A_{control}$ - the absorbance of the negative control and A_{sample} - the absorbance at 655 nm of the tested samples or standard. The IC_{50} values for each plant extract were calculated from the graph of ABTS scavenging effect against the concentration of plant extracts (mg/mL).

2.3.6. Reducing Power

This assay was carried out as described previously [23] with some modifications. Different concentrations of 25 µL of plant extracts were added to 25 µL of 0.2 M sodium phosphate buffer pH 6.6 and 25 µL of 1% (w/v) K_3FeCN_6. The mixture was incubated at 50 ° C for 20 minutes. After incubation, 80 µL trichloroacetic acid (TCA,10%, w/v) was added to the reaction mixture as well as 100 µL of demineralized water (H_2O) and 20 µL $FeCl_3$ (0.1%,) to a final volume of 275 µL and the absorbance was read at 655 nm. Trolox (0.3mg / mL) was used as the standard in the range of 0 to 7.5 µg/well, the results were expressed as µmole Trolox equivalents per g of leaves and the assays were carried out in triplicates.

2.3.7. Superoxide Radical Scavenging Activity

The superoxide radical scavenging activity was carried out as described previously [25] with minor changes. Each sample of 25 L was mixed with 25 µL of 80 µM phenazine methosulfate (PMS), 25 µL of 625 µM NADH, 25 µL of 200 µM nitro blue tetrazolium (NBT) and 100 L of 100 mM sodium phosphate buffer pH 7.4 to a final volume of 200 µL. After 5 min at room temperature the absorbance was measured at 550 nm (microplate reader Bio-Rad 680). All absorbance measurements were carried

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out in triplicate and ascorbic acid (0.3mg / mL) was used as standard in the range of 0 to 7.5 µg/well. The following expression was used:

$$\text{Superoxide Radical Scavenging Activity (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

where $A_{control}$ - the absorbance of the negative control and A_{sample} - the absorbance at 550 nm of the tested samples or standard. The IC_{50} values for each plant extract were calculated from the graph of Superoxide Radical Scavenging Activity against the concentration of plant extracts (mg/mL).

2.3.8. Superoxide Dismutase (SOD) Activity Assay

SOD activity was carried out by NBT method described previously [26] with some modifications. The assay mixture consisted of 70 µl Tris buffer (20 mM, pH 7.8) containing 1 mM EDTA, 10 µl plant extract, 10 µl NBT (2 mM), and 10 µl riboflavin (2mM) to a final volume of 90 µL. The absorbance at time 0 of the reaction mixture was measured at 550 nm (microtiter plate reader Bio-Rad 680) and the reaction mixture was exposed to a 25 W light for 10 min and the absorbance was read again immediately at 550 nm. A negative control (water instead of the sample) and a positive control (commercial SOD, 1.5 units) were evaluated at the same time per microtiter plate. One unit of SOD was defined is as the amount of enzyme that provides a 50% inhibition of the riboflavin-mediated initial rate of reduction of NBT, at pH 7.8 and room temperature. All assays were carried out in triplicate.

2.3.9. Experimental Design to Optimize Extraction of Secondary Metabolites

The experimental design was conducted using three factors and two levels with duplicates: extraction temperatures (25 and 40ºC), extraction time (0 and 5 days) and solvents (H_20 and hexane). The upper (+) and lower (−) levels were defined based on the preliminary results based on different solvents, temperatures and extraction/incubation time of bioactive compounds. The experimental design matrix was obtained with Design Expert version 10 software, and the results were fitted to the following factorial model equation:

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

where y represents the concentration of phenolic compounds, β0 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 the ANOVA methodology

2.3.10. Intrinsic Synchronous Fluorescence Spectroscopy (SFS) of Plant Extracts

The samples containing secondary metabolites were investigated on a spectrofluorometer (JASCO JP-8300, JASCO INTERNATIONAL CO., LTD., Hachioji, Tokyo, Japan) as reported previously [27].

2.3.11. Intrinsic Synchronous Phosphorescence Spectroscopy (SPS) of Plant Extracts

Intrinsic synchronous phosphorescence spectroscopy (SPS) of secondary metabolites was performed in a spectrofluorometer (JASCO JP-8300, JASCO International Co. Ltd. 11-10, Myojin-cho 1-chome. Hachioji, Tokyo 192-0046, Japan) in quartz cuvettes with a 1 cm optical path length. Spectra Manager software ver. 2.5 was obtained for spectral acquisition and processing (Spectra analysis). Synchronous phosphorescence spectra were obtained by using the following parameters: range of measurement λ of 210–750 nm; data intervals of 2 nm; data points of 271; excitation bandwidth of 20 nm; emission bandwidth of 20 nm; very low sensitivity; chopping period of 100 msec; delay time of 10 msec; integration time of 65 msec, variation in delta wavelength (Δλ) of 5, 10, 20 and 30 nm; response of 0.2 s; light source of Xe lamp and scan speed of 10,000 nm/min.

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2.3.12. Intrinsic 3D Fluorescence Spectra Measurements of Plant Extracts

Three-dimensional intrinsic fluorescence spectra of of secondary metabolites were performed across a 3D space (excitation λ, emission λ and fluorescence intensity). The samples containing of secondary metabolites were analysed on a spectrofluorometer (JASCO JP-8300) in quartz cuvettes with a 1 cm optical path length. Spectra Manager software was purchased for spectral acquisition and processing (Interval data analysis). Intrinsic 3D fluorescence spectra were obtained by using the following parameters: scan speed of 10,000 nm/min and light source of Xe lamp; measurement range of 260–750 nm; data interval of 0.5 nm; excitation λ of 250.0 nm; emission bandwidth of 5 nm; response of 10 msec; high sensitivity; start at 260 nm and end at 750 nm; data interval of 0.5 nm; data points of 981; interval measurement of λ (nm) points of 98; start at 250 nm and end at 735 nm; interval of 5 nm; mode of emission and excitation bandwidth of 5 nm.

2.3.13. FTIR Analysis of Plant Extracts

The structural information of secondary metabolites was investigated by FTIR analysis as described previously [27].

2.3.14. Statistical Analysis

Correlation and regression analyses were carried out with the Excel software 2024 package (Academic License, Microsoft of Portugal). Sigma Plot 16.0 (2011–2012 Systat Software Inc., Hounslow, Middlesex, UK) was purchased to draw graphs in this manuscript. Experimental results are means of three parallel measurements, and the results are presented as mean values ± standard deviation (SD). Statistical analysis was carried out by using one-way analysis of variance (ANOVA). The significance of the p-value is represented with letters (a,b,c,d,e,f) which indicate significance of the p-value less than 0.1,0.05, 0.01, 0.005 and 0.001 respectively.

3. Results and Discussion

3.1. Phytochemical Assays of Plant Extracts

Phytochemical screening methods play an important role 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 therapeutic properties of medicinal plants [17,28]. This understanding of phytochemical composition of plant extracts is of great interest for novel pharmaceutical discovery, quality control and safety assessment, standardization of traditional herbal medicines and plant chemistry for ethnobotanical investigations. However, these assays also present challenges and limitations such as interference from complex plant matrices, 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 [17,28]. Several phytochemical parameters were analysed in these plant extracts as shown in Table 1. The highest levels of TPC and SOD were obtained with aqueous plant extraction at 40ºC and for 5 days of incubation whereas leaves extraction with water at 25º C for 5 days of incubation revealed highest levels of reducing power, ABTS and DPPH. The levels of TFC and superoxide radical scavenging activity were highest with plant extraction with hexane, 25ºC and 0 days of incubation and with hexane, 25ºC and 5 days of incubation, respectively. The data on phytochemical assays from *T. diversifolia* plant extracts published in the literature revealed 52 and 59 mmoles/L of aqueous plant extracts for TPC and TFC, respectively [10]. The levels of TPC and TFC for flowers was 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 mg/mL and 4.3 mg/mL of plant extract. respectively [10,14]. The data on TPC and TFC in the current work were 72.16 mmoles gallic acid equivalent/g leaves and 6.56 mmoles catechin equivalent/g leaves, respectively (Table 1).

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The levels of superoxide scavenging activity and DPPH were 0.65 mg/mL and 1.35 mg/mL of plant extract. respectively (Table 1). These data are difficult to compare with published data as the units and part of plant are different as well as the methodology used [10,12,14].

Phenolic compounds are found in plants which have revealed scavenging activity due to their hydroxyl groups. Moreover, phenolic compounds such as tannins, flavonoids, and phenolic acid have exhibited several biological activities such as anti-carcinogenic, anti-bacterial, and anti-inflammatory activities [17,28]. Several studies have suggested that medicinal plants contain high levels of polyphenol which have exhibited high antioxidant capacity in the defensive process against ROS by causing molecular damage to pests, microorganism, herbivores, and insects. In plants, flavonoids play an important role to combat oxidative stress and some possible mechanism of their action may involve the inhibition of various enzymes such as lipoxygenase and xanthine oxidase. On the other hand, DPPH is widely used as free radical for evaluating the antioxidant activity of natural antioxidants by a non-enzymatic reaction. Superoxide anions are weak oxidants that generate extremely potent and dangerous hydroxyl radicals and singlet oxygen, which are the oxidative stress-causing agents. They can decrease the activity of various antioxidant defence enzymes, such as catalase and glutathione peroxidase [29–31]. The bioactive compounds responsible for eliminating superoxide anions may include various antioxidants such as phenolic compounds, flavonoids, tannins, and other polar compounds. These compounds are known for their ability to scavenge reactive oxygen species, including superoxide anions, due to their electron-donating properties and ability to stabilize free radicals [29–31].

Table 1. Some phytochemical assays of plant extracts.

Plant extracts	TPC	TFC	Reducing power	ABTS inhibition	DPPH inhibition	Superoxide radical	SOD activity
	(mmoles gallic acid	(mmoles catechin	(mmoles TE equivalent /g	IC_{50}	IC_{50}	scavenging activity	(Units/g leaves)
	equivalent/g leaves)	equivalent /g leaves)	leaves)	(mg extract/mL	(mg extract/mL)	IC50 (mg extract/mL)	
H_20,25ºC, 0 days	14.24±0.52[a]	3.41±0.24[a]	21.63±2.17[b]	0.90±0.01[a]	2.20±0.02[a]	1.35±0.01[a]	1101.95±34.14[a]
H_20,25ºC, 5 days	15.32±0.01[a]	5.61±0.93[b]	26.63±0.36[a]	0.09±0.01[c]	1.35±0.02[b]	0.90±0.02[c]	1107.72±91.15[c]
Hexane,25ºC,0 days	**12.04±1.31[a]**	6.56±0.82[a]	4.82±0.15[a]	6.63±0.05[b]	6.48±0.03[b]	1.06±0.01[b]	576.47±40.71[c]
Hexane,25ºC,5 days	**13.46±0.88[a]**	3.23±0.15[a]	0.39±0.03[b]	3.19±0.01[c]	2.65±0.09[d]	0.65±0.01[d]	388.26±25.42[c]
H_20,40ºC, 0 days	14.87±0.12[b]	4.63±0.38[c]	0.87±0.20[a]	0.52±0.01[a]	2.23±0.07[c]	1.06±0.01[b]	145.62±23.07[d]

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H2O,40ºC, 5 days	71.95±0.30[c]	6.20±0.31[d]	12.13±0.20[d]	0.32±0.004[d]	1.51±0.07[d]	0.92±0.03[b]	1249.53±160.04[c]
Hexane, 40ºC,0 days	**1.53±0.02[b]**	4.36±0.34[b]	2.08±0.15[e]	10.87±0.07[e]	8.78±0.14[c]	9.09±0.02[d]	549.05±260.88[f]
Hexane, 40ºC,5 days	**7.62±0.33[c]**	5.22±0.38[d]	2.63±0.25[e]	4.07±0.04[f]	7.61±0.36[b]	8.23±0.02[d]	237.00±18.37[e]

Statistical analysis via one-way ANOVA: [a] $p < 0.1$, [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.005$, [e] $p < 0.001$, [f] $p < 0.0001$.

3.2. Optimization of Extraction of Secondary Metabolites from Plant Extracts

In order to optimize the extraction of secondary metabolites from plant leaves, a systematic study was developed to analyze several factors that affect the extraction of these compounds. In the present work, two solvents (water and hexane) with different polarity index (9 and 0, respectively) were used for extraction of secondary metabolites [29]. Water belongs to the group of green solvents which are nontoxic, biodegradable, recyclable, renewable and it has a boiling point. It is a non-selective solvent, and it can extract hydrophilic compounds such as saponins, phenolics, polysaccharides, glycosides, alkaloids, terpenoids and carbohydrates. On the other hand, 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 extraction efficiency of secondary metabolites such as extraction time, solvent to solute ratio, temperature and extraction method [29].

The experimental design was conducted using a 2^3-way full factorial design, with a total of eight duplicate experiments. After the experimental runs, statistical significance, the effect of each variable, and multivariate interactions on extraction of secondary metabolites were evaluated (Table 2).

Table 2. 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
ABC	659.32	1	659.32	1751.98	< 0.0001

 doi:10.20944/preprints202602.1369.v2

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%.

The data in Table 2 summarized the statistical analysis of variance (ANOVA). The F-value of 2558.67 indicated that the model is significant and that there was only a 0.01% probability of such an F-value being due to noise. The coefficient of determination R^2 = 0.9996 indicated that there was a statistical correlation between the response and the variables considered and that only 0.01% of the total variation was not explained by the model. The predicted R^2 of 0.9982 is in reasonable agreement with the adjusted R^2 of 0.9992 whereby the difference is less than 0.2. Adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. A ratio of 162.323 presented in this work indicates an adequate signal. The statistical analysis shows that the significant factors for phenolic compounds extraction are all considered: the temperature (variable A), the time (variable B), the solvent (variable C), and the interactions between the variables AB, AC, BC, and ABC (Table 3). However, the time, the solvent and the interaction between the temperature and the solvent exhibited the greatest effect. The following empirical equation was obtained to estimate the extraction of phenolic compounds from plant extracts (Table 3):

$$y = 18,88+5,12xA+8,21xB-10,21xC+7,58xAB-9,20xAC-6,33xBC-6,42xABC$$

where: y is the concentration of phenolic compounds, A is the temperature, B is the time and C is the solvent. This equation was used to facilitate plotting the response surfaces which are represented in Figure 1A and 1B.

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

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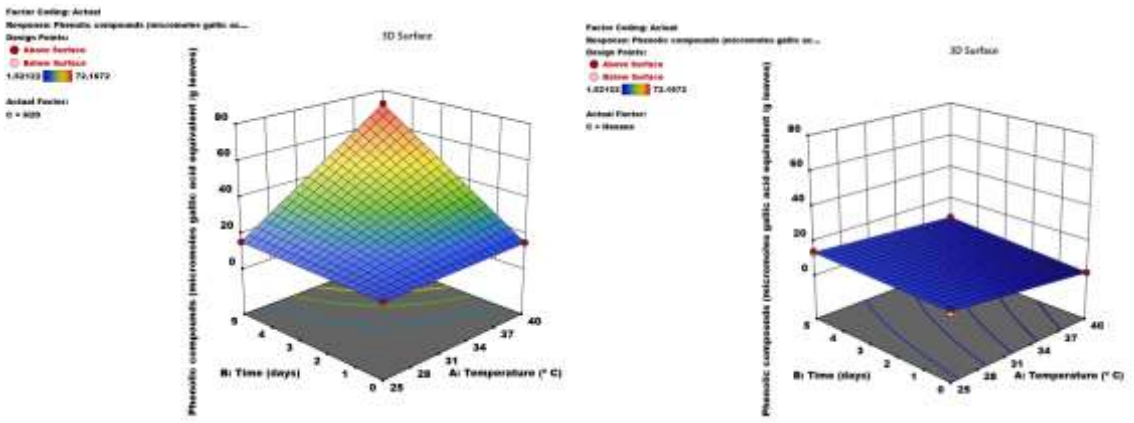

A B

Figure 1. Three-dimensional representation of the interaction of extraction/incubation time and temperature of incubation on the extraction of phenolic compounds. A- Water as solvent and B- Hexane as solvent.

The data in Figure 1A and 1B exhibited the effect of optimized factors on phenolic compounds extraction from plant extracts. The highest extraction of phenolic compounds (i.e 72.16 mmoles gallic acid equivalent/g leaves)) was obtained at 40ºC, in H_20 and 5 days of incubation (Fig, 1A). Regarding the experimental planning of secondary metabolite extraction from these plant leaves, there is only one published report in the literature about optimization of extraction by using a 2^2 factorial design matrix for this plant extract [12]. Therefore, the present data of experimental planning is difficult to compare with published data for TPC and TFC due to different parts of plant and factorial design matrixes as well as different units presented [12].

3.3. Synchronous Fluorescence Spectroscopy (SFS)

SFS involved simultaneous scans of both the excitation and emission wavelengths of a sample at a constant wavelength difference ($\Delta\lambda$) to produce a simple spectrum. It exhibits sharper and narrower spectra, and it has several advantages over conventional fluorescence spectroscopy such as eliminating light scattering interference, amplifying the small spectral features, enhancing selectivity and improving spectral resolution. The $\Delta\lambda$ in SFS is an important parameter to obtain the best resolution, sensitivity and spectral shape for a specific analyte. To the author's knowledge, there ae no reports in the literature on intrinsic fluorescence spectroscopy of secondary metabolites from *T. diversifolia* leaves. Moreover, there are very few published reports on fluorescence properties of secondary metabolites in plant leaves extracts in general [18.19]. Therefore, synchronous fluorescence spectroscopy (SFS) of secondary metabolites from this plant extract was investigated in a spectrofluorometer with different $\Delta\lambda$ at high and medium sensitivity for leaves extracts with hexane at 25 and 40 ºC, respectively (Figure 2 A and B). In both cases, there are several fluorescence peaks at 290, 320, 345, 400, 490 and 675 nm which exhibited an increase in fluorescence as a function of $\Delta\lambda$. The emission peaks in the region of 280–320 nm may be due to the presence of a protein moiety containing aromatic amino acids such as tyrosine and tryptophan residues. As far as the spectral region of 325-450 nm is concerned, these emission peaks may be due to phenolic compounds, hydroxycinnamic acids and stilbenes whereas in the region 500-550 nm, flavanols, flavonoids and alkaloids are apparently responsible for these peaks. Finally, the emission peak at 675 nm is due to the presence of chlorophyll in chloroplasts [32,33]

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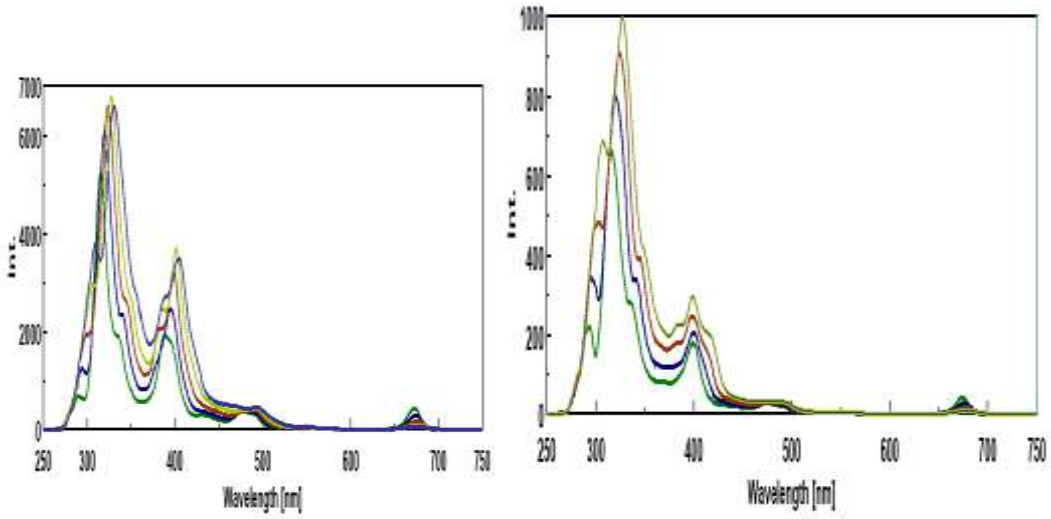
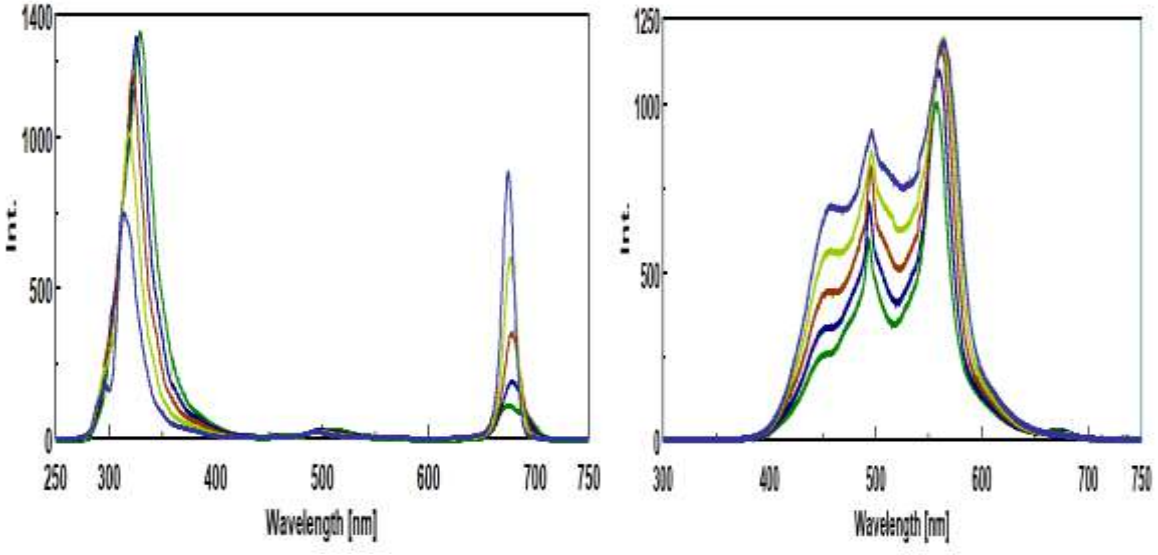


A B

Figure 2. -Intrinsic synchronous fluorescence spectra with different Δλ of secondary metabolites from plant extracts. A- Plant extract with hexane at 25ºC and 0 days at high sensitivity as follows: ___ 10 nm; ___ 15nm; ___ 20 nm; ___ 25 nm and ___ 30 nm of Δλ ; B- Plant extract with hexane at 40ºC and 0 days at medium sensitivity as follows: ___ 10 nm; ___ 15nm; ___ 20 nm; and ___ 25 nm of Δλ.

The data in Figure 3 A and B has revealed increase in fluorescence intensity in all emission peaks as a function of Δλ except the chlorophyll peak that has exhibited a decrease in fluorescence. It is important to point out that aqueous extract exhibited a different emission pattern with fluorescence peaks at 490, 560, 675 nm (Figure 3 B).



A B

Figure 3. - Intrinsic synchronous fluorescence spectra with different Δλ of secondary metabolites from plant extracts. A Plant extract with hexane at 40ºC and 5 days at medium sensitivity as follows: ___ 30 nm; ___ 25nm; ___ 20 nm; ___ 15 nm and ___ 5 nm of Δλ; B- Plant extract with H_20 at 25ºC and 0 days at high sensitivity as follows: ___ 10 nm; ___ 15nm; ___ 20 nm; ___ 25 nm and ___ 30 nm of Δλ.

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These secondary metabolites were analysed by SFS which were extracted with aqueous and hexane solutions at different temperatures and incubation times as shown in Fig, 4. The data in Figure 4 A and B exhibited different fluorescence emission profiles of aqueous extracts compared with hexane extracts (Figure 4 C) as emission peaks were observed at 490 and 560 nm for Figure 4 A and 490, 540 and 565 nm for Figure 4 B.

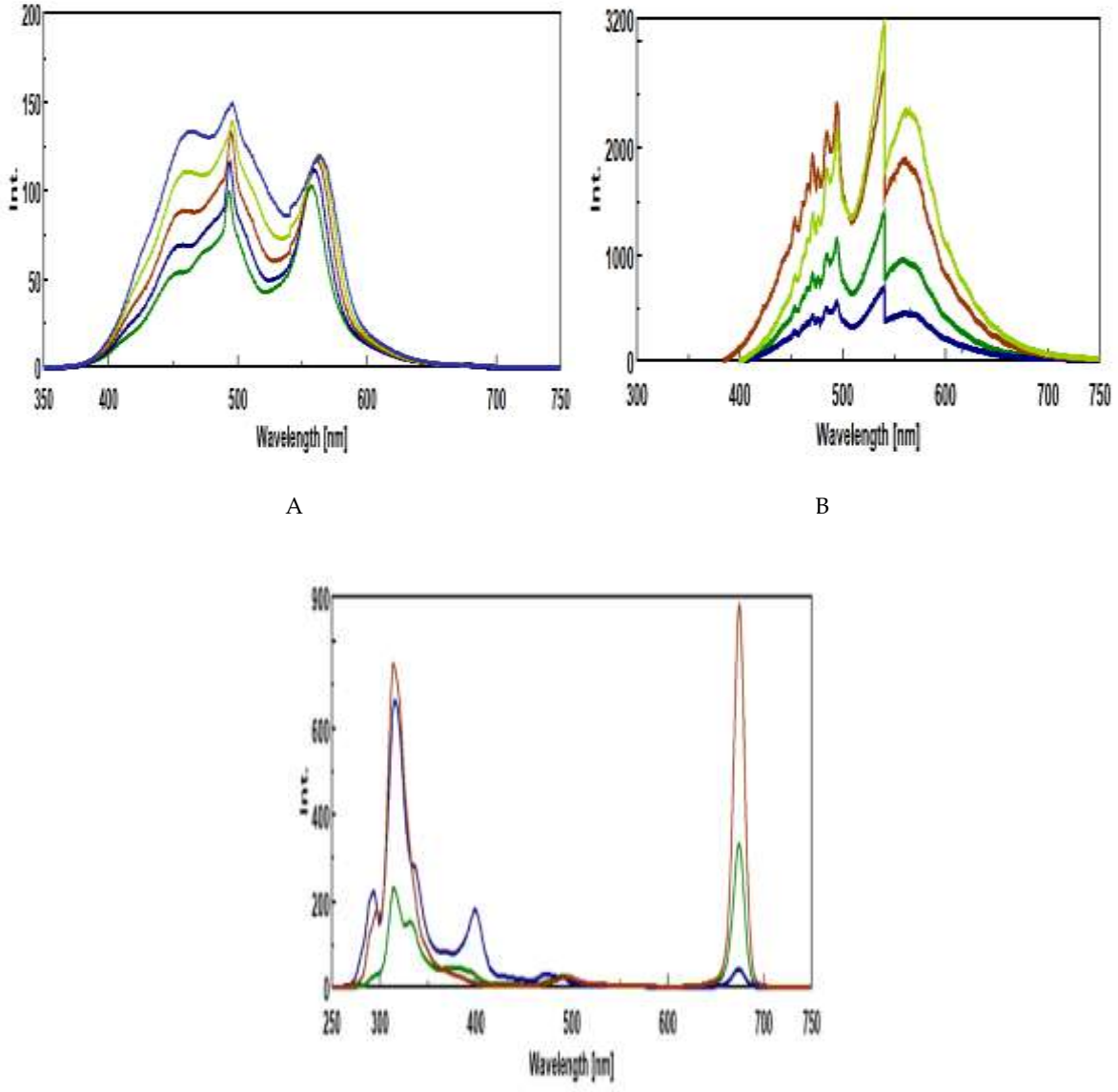

Figure 4. - Intrinsic synchronous fluorescence spectra with different Δλ of secondary metabolites from plant extracts. A Plant extract with H_20 at 40ºC and 0 days at low sensitivity as follows: ___ 10 nm; ___ 15nm; ___ 20 nm; ___ 25 nm and ___ 30 nm ; B- Plant extract with H_20 at medium sensitivity and 5 nm of Δλ as follows: ___ 25 ºC and 0 days; ___ 40 ºC and 0 days ; ___ and 40 ºC and 5 days ___ and C- plant extracts with hexane at 10 nm of Δλ and medium sensitivity as follows: ___ 25 ºC and 5 days; ___ 40 ºC and 0 days ; ___ 40 ºC and 5 days.

Although there are few reports in the literature about SFS of plant materials, this analytical technique is very useful as a diagnostic tool for detection of physiological conditions of plants, nutrients, phytochemicals, environmental pressures and diseases due to its remarkable high sensitivity and specificity [32,33].

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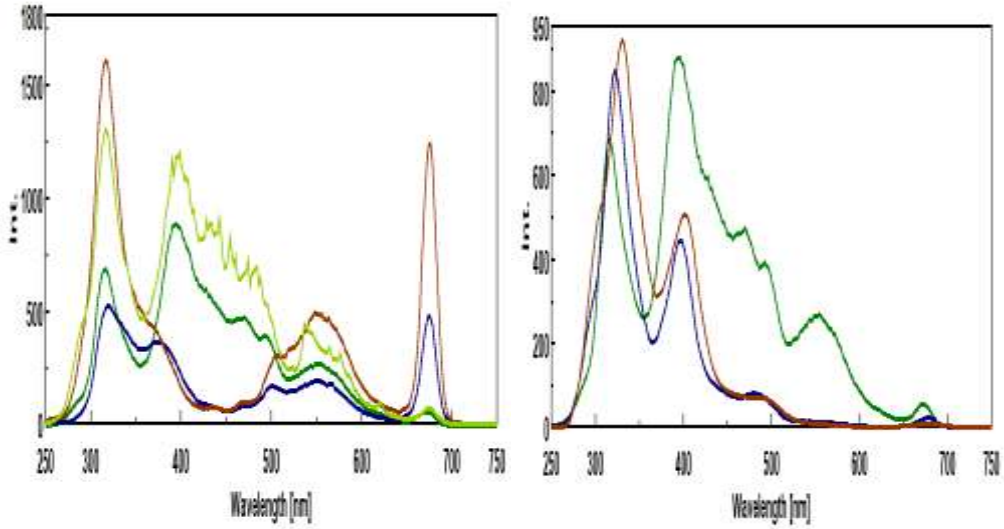
3.4. Intrinsic Synchronous Phosphorescence Spectroscopy (SPS)

Regarding SPS, it involves the delayed and often long-lasting emission of light from a phosphorescent material that takes place after it has been excited by a light source. The main difference between fluorescence and phosphorescence is because the fact that the former is a fast, active measurement technique, whereas synchronous phosphorescence describes a property of slow-decaying light emission.

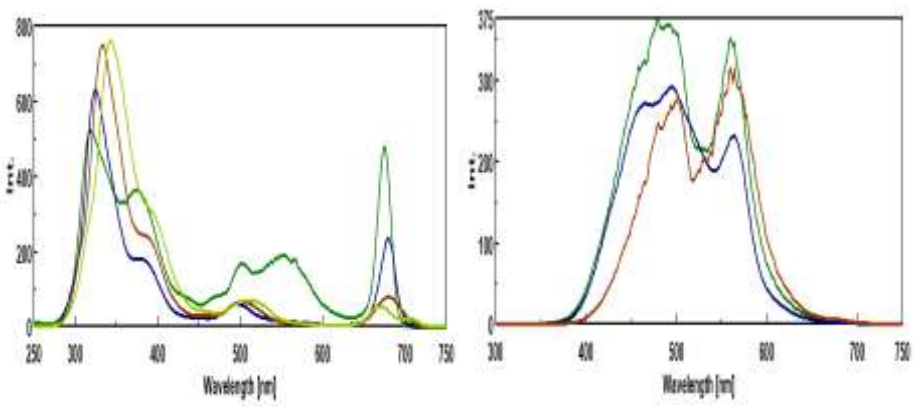
The $\Delta\lambda$ in SPS is an important parameter to obtain the best resolution, sensitivity and spectral shape for a specific analyte. Therefore, Fig, 5 have revealed several fluorescence peaks of SPS at 325, 390, 490, 550 and 675 nm by using hexane extracts. The data in Figure 5 A exhibited several SPF spectra at 10 nm of $\Delta\lambda$ for several plant leaves extracts which revealed the highest fluorescence peaks with hexane extract at 40 ºC for 5 days of incubation. On the other hand, the data presented in Figure 5 B revealed an increase in fluorescence at low $\Delta\lambda$ both at 400 and 675 nm



A B

Figure 5. - Intrinsic synchronous phosphorescence spectra of secondary metabolites from plant extracts. A- Plant extracts with hexane at low sensitivity and 10 nm of $\Delta\lambda$ as follows: ___ 25 ºC and 0 days; __ ;25 ºC and 5 days ___ 40 ºC and 5 days and __ 40 ºC and 0 days; B- Plant extracts with hexane at 25 ºC and 0 days with different $\Delta\lambda$ as follows: ___ 10 nm ; ___ 20 nm and ___ 30 nm.

The data in Figure 6 exhibited several SPS spectra for various aqueous and organic plant extracts. It is important to point out that the aqueous extracts revealed only two fluorescence peaks at 500 and 560 nm (Figure 6 B) compared with several peaks for hexane extract (Figure 6 A and C).



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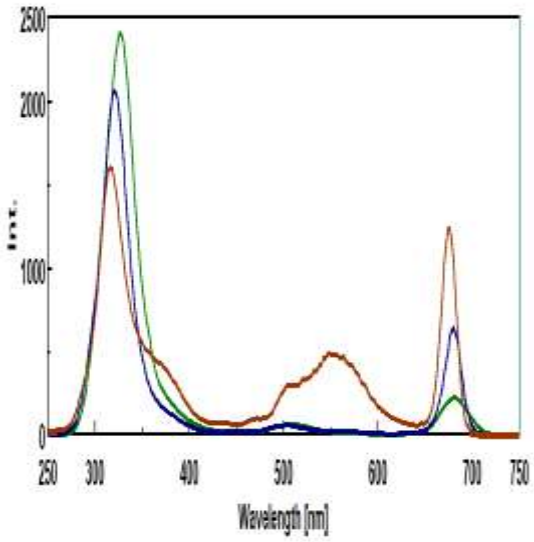

C

Figure 6. - Intrinsic synchronous phosphorescence spectra of secondary metabolites from plant extracts. A- Plant extracts with hexane at 25 ºC and 5 days at low sensitivity with different Δλ as follows: ___ 10 nm; ___ ;20 nm ___ 30 nm ___ 40 nm; B- Plant extracts with H_20 at 40 ºC with different Δλ as follows: ___ 0 days and 20 nm; ___ 0 days and 30 nm and ___ 5 days and 20 nm. C- Plant extracts with hexane at 40 ºC and 5 days at low sensitivity with different Δλ as follows: ___ 30 nm ; ___ ;20 nm; ___ 10 nm.

In a similar manner to SFS, the emission peaks in the region of 280–320 nm may be due to the presence of a protein moiety containing aromatic amino acids such as tyrosine and tryptophan residues. As far as the spectral region of 325-450 nm is concerned, these emission peaks may be due to phenolic compounds, hydroxycinnamic acids and stilbenes whereas in the region 500-550 nm, flavanols, flavonoids and alkaloids are responsible for these peaks. Finally, the emission peak at 675 nm may be due to chlorophyll in chloroplasts [34,35]. SPS can provide very useful information in plants namely stress detection by identification of photodynamic stress and damage in photosynthetic pigments. Moreover, SPS can be used to obtain useful structural information on the organization of pigment-protein complexes as well as to track changes in chlorophyll biosynthesis [32,33].

3.5. Intrinsic 3D Fluorescence Spectroscopy

Three-dimensional fluorescence spectra are also emission–excitation matrices (EEM); therefore, by using excitation and emission monochromators successively, it is possible to obtain emission spectra for different excitations λ. Hence, a range of emission spectra at different excitations λ is obtained in this constant step, and EEM exhibited two dimensions: excitation λ and emission λ. Therefore, fluorescence matrices revealed a fluorescence map of all fluorophores present in a sample for their characterization. The data in Figure 7A–F have revealed 3-D spectra in different formats for secondary metabolites from plant extract with hexane at 40 ºC and 5 days of extraction as well as a synchronous 2D spectrum which exhibited fluorescence peaks at 290 and 675 nm as shown in Figure 7 F. The data in Figure 7 related to 3- D spectra exhibited several fluorescence peaks (i.e 290, 320, 345, 550 and 675 nm) which are in agreement with SFS data. However, the chlorophyll peak at 675 nm has been subdivided into 5 peaks with different excitation λ (Figure 7 A, B, D, E) which are due to complex, overlapping contributions from different pigment-protein complexes, photosynthetic photosystems as well as their degradation products [36]. Therefore, the 3D spectra of plant extracts behaved as fingerprint region of the photosynthetic system's state, subdividing the main emission

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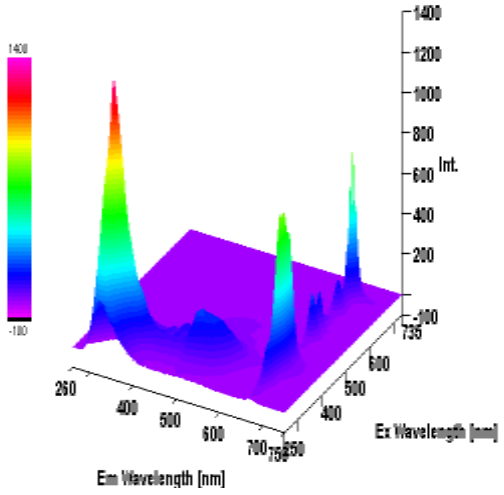
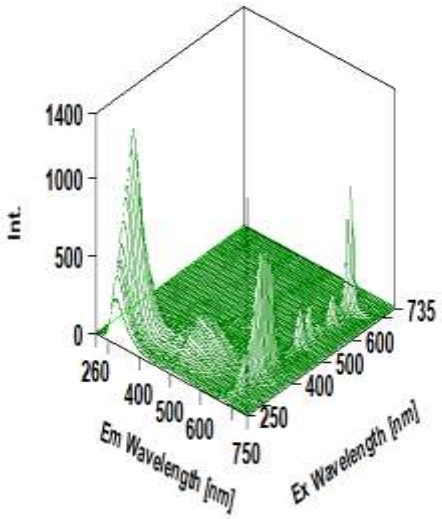
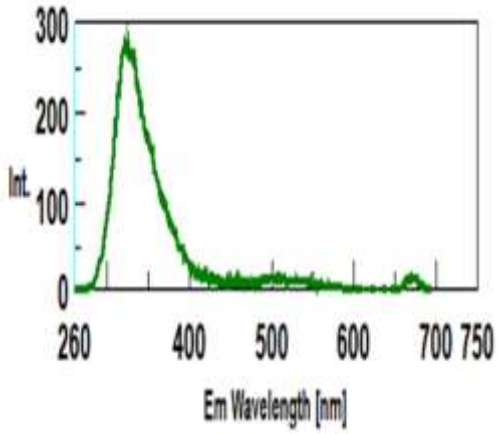
peak at 675 nm into various components which represent different functional and structural parts of the chloroplast [34].



A



B



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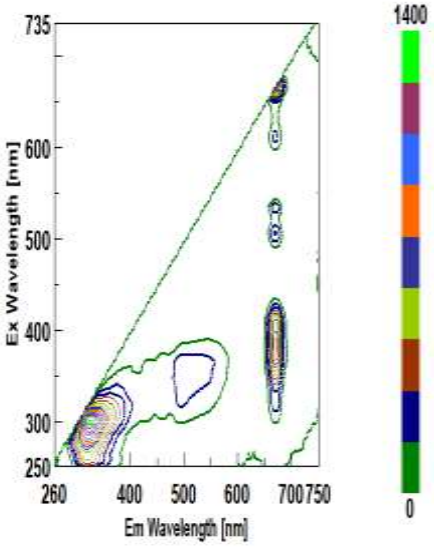

D

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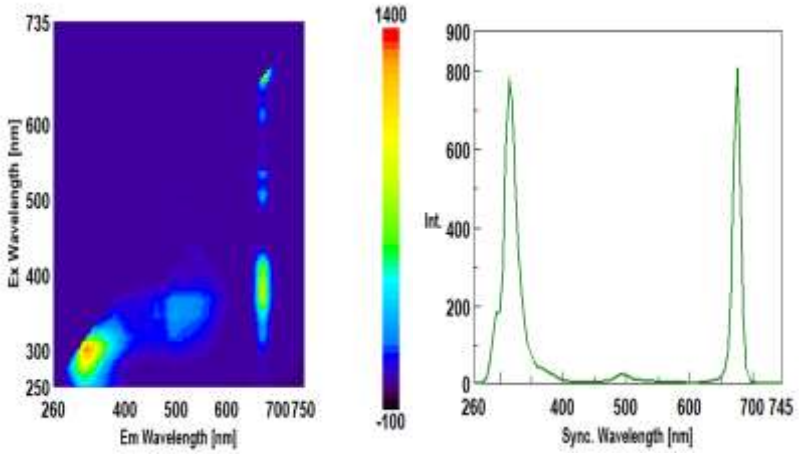
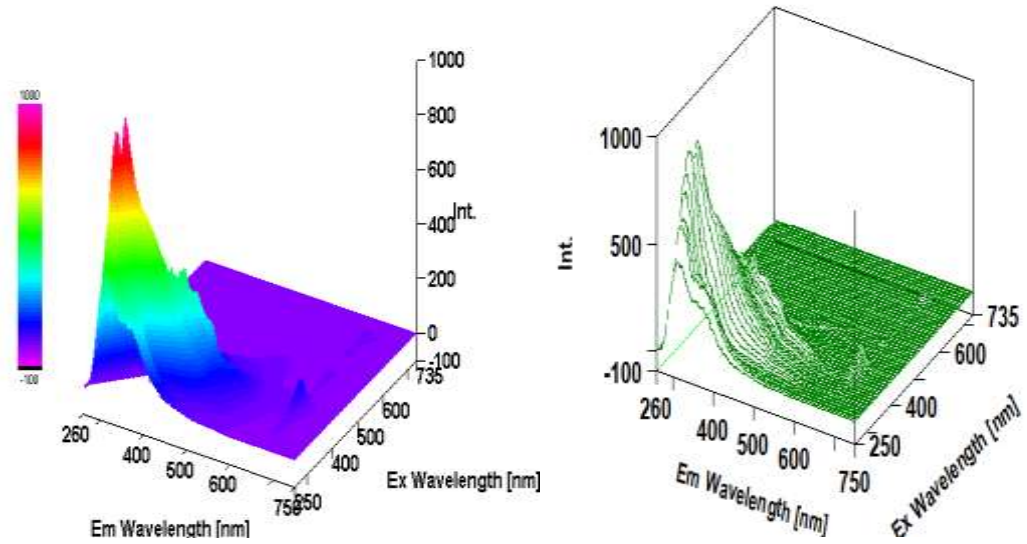


E F

Figure 7. Secondary metabolites of plant extract with hexane at 40ºC and 5 days with medium sensitivity and 10 nm of Δλ. (A)- Colour 3D view; (B) Three-dimensional spectrum view; (C) 2-D spectrum view; (D) Contour view; E- Colour view; F- Synchronous 2D spectrum.

In a similar manner to Figure 7, the data in Figure 8 related to 3- D spectra exhibited several fluorescence peaks (i.e 290, 320, 345, 550 and 675 nm) which are in agreement with SFS data. However, these data in Figure 8 are due to hexane extract at 40ºC for 0 days of incubation/extraction which explains a low fluorescence peak of the chlorophyll at 675nm compared to hexane extract at 40ºC for 5 days (Fig, 7).



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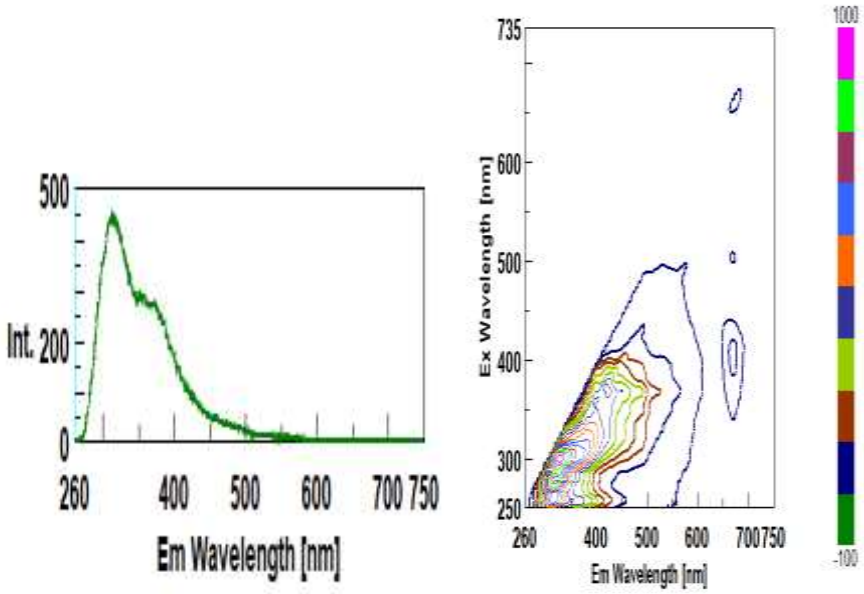
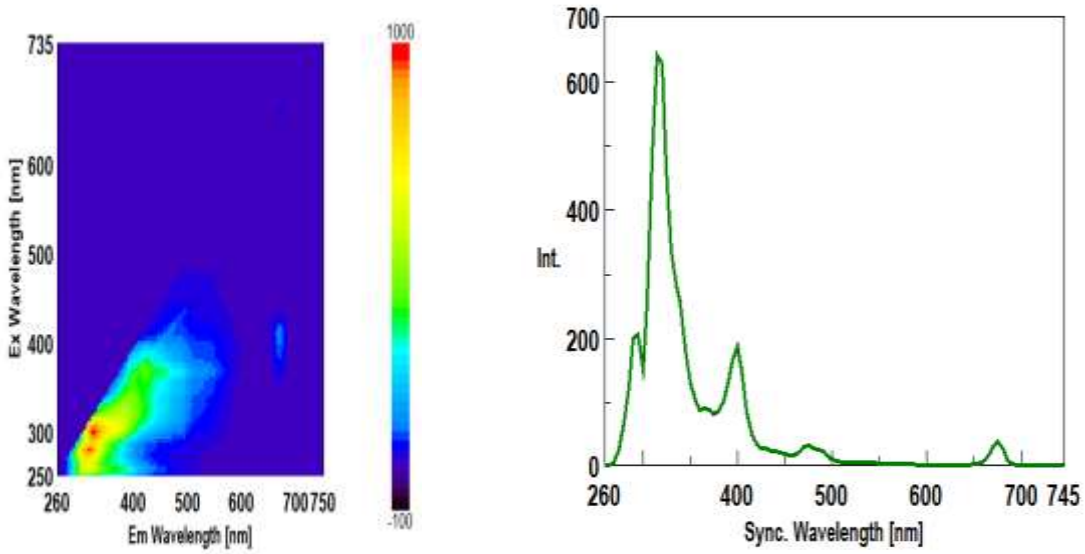


C D



E F

Figure 8. - Secondary metabolites of plant extract with hexane at 40ºC and 0 days with medium sensitivity and 10 nm of Δλ. (A)- Colour 3D view; (B) Three-dimensional spectrum view; (C) 2-D spectrum view; (D) Contour view; (E) - Colour view; (F)- Synchronous 2D spectrum.

Although the data on the 3D spectra measurement for secondary metabolites from this plant extract have not been reported in the literature, this analytical technique of 3D spectra has been widely used in research areas such as smart agriculture, identification of key bioactive substances, geographical origin of plants , evaluation of anti-oxidant capacity of plant extracts and quality control and authentication of plant-based products in terms of adulteration [36–39].

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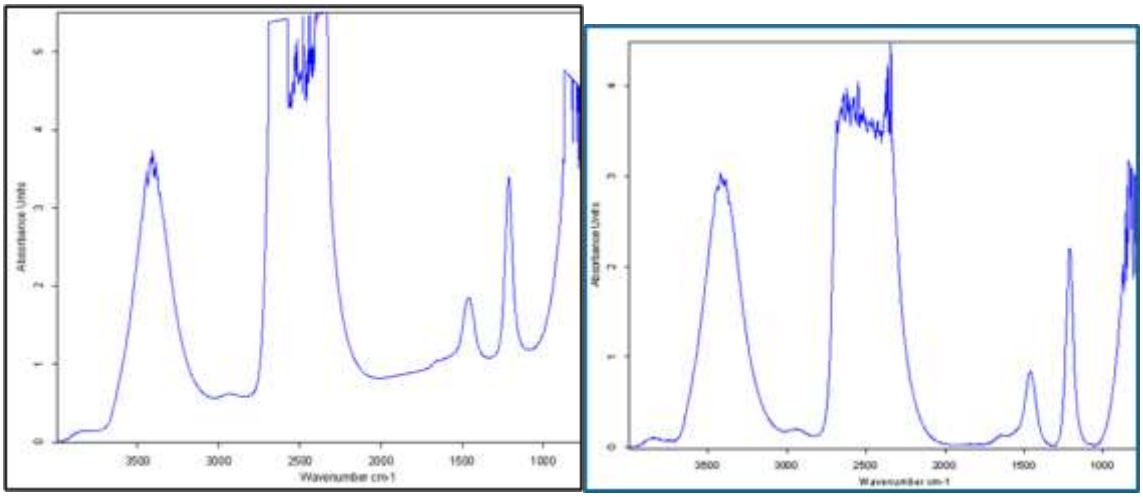
3.6. FTIR Analysis

FTIR spectra of some aqueous plant extracts at 40 ºC and incubated at 0 and 5 days were analysed by FTIR, which revealed typical absorption bands of secondary metabolites (Figure 9). The absorption band at 3350 cm^{-1} is due to the stretching vibration of hydroxyl (O-H) group of alcohols and phenols present in the extract. A weak absorption band at about 2900 – 2950 cm^{-1} corresponds to C-H stretching vibrations of alkanes and alkyl groups. On the other hand, FTIR spectra exhibited a broad absorption band in the region 2300-2750 cm^{-1} which can be attributed to N-H stretching in amine hydrochlorides or amino acids. A weak absorption band was observed at 1630- 1680 cm^{-1} which represents either C=C unsaturated bonds or C=O stretching in carbonyl groups (either amides or ketones). A sharp absorption band at about 1350 – 1450 cm^{-1} corresponds to C-H bending (alkanes), O-H bending and CH_3 stretching of aldehydes and ketones.



A B

Figure 9. - FTIR spectra of selected plant extracts. A- 25 ºC in H_20 for 0 days and B- 40 ºC in H_20 for 0 days.

The absorption band at about 1250 cm^{-1} exhibited O-C stretching of carboxylic acids and derivatives and a broad band at about 700-900 cm^{-1} revealed C-H out-of-plane bending (deformation) vibrations of aromatic rings and C-O-C vibrations. These data on FTIR spectra of the present work are in agreement with the data reported in the literature for *T. diversifolia* plant extracts from FTIR spectra [40]

4. Conclusions

To the authors' s knowledge, this is the first report about phytochemical assays (i.e ABTS, DPPH, superoxide scavenging activity, TPC, TFC, reducing power and SOD levels) and fluorescence properties of plant leaves extracts, which is based on SFS and SPS as well as the characterization of 3D spectra of secondary metabolites. Moreover, experimental planning was carried out to optimize the extraction of secondary metabolites from plant leaves. The data presented in this work revealed that these plant extracts exhibited high levels of phytochemicals which confirmed their biological activities in clinical conditions. The comparative analysis of SFS and SPS strongly suggests that SPS exhibited higher fluorescence intensity for secondary metabolites levels than SFS for these plant leaves extracts. For complex sample matrices of secondary metabolites, SPS and 3D-SFS would provide very useful information compared to SFS in terms of fluorophore identification, simplified spectra, enhanced sensitivity, quantitative analysis, microenvironment and interactions, structural information, conformational changes and sample fingerprinting.

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The data presented in this work is novel since a detailed SFS, SPS and 3D-SFS study was carried out to obtain useful structural, qualitative and quantitative information of secondary metabolites from these plant extracts. These analytical techniques are cheap, fast, non-destructive, intrinsic and do not require exogenous fluorophores, they also exhibit high sensitivity and selectivity, very low sample volumes required and fast analysis by high-throughput screening for quality control and authentication. The limitation of this study lies in the need for full identification of these secondary metabolites by NMR, GC-MS, GC-FID, HPLC, ELISA and chemometric approach by running analytical standards.

Author Contributions: Conceptualization, Karla Ramos and Amin Karmali; Methodology, Karla Ramos; Software, Karla Ramos; Validation, Karla Ramos; Formal analysis, Karla Ramos and Amin Karmali; Investigation, Karla Ramos and Amin Karmali; Resources, Amin Karmali; Data curation, Amin Karmali; Writing – original draft, Amin Karmali; Writing – review & editing, Amin Karmali; Supervision, Amin Karmali; Project administration, Amin Karmali. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by FCT—Portuguese Foundation for Science and Technology, grant number Project UID/AGR/04033/2019.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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

Abbreviations

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

PMS- Phenazine methosulfate

SFS- Synchronous Fluorescence Spectroscopy

SOD – Superoxide dismutase

SPS- Synchronous Phosphorescence Spectroscopy

TCA- Trichloroacetic acid

TPC- Total phenolic content

TFC- Total flavonoids content

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