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Experimental Design for Extraction of Secondary Metabolites from *Rauvolfia caffra* Sond. Leaves: Biological and Chemical Characterization by Synchronous Fluorescence, Phosphorescence and FTIR Spectroscopy

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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 design for optimization of several parameters was carried out by a full-factorial test of two levels of three factors for secondary metabolite extraction from *Rauvolfia caffra* leaves. The best conditions for highest extraction of phenolic compounds (i.e., 89.90 µmoles gallic acid equivalent/g leaves) were obtained at 25 °C in H_2O and at 5 days of incubation. Several phytochemical assays were performed for characterization of these plant extracts, and the highest levels of TFC, DPPH and reducing power were obtained with aqueous plant extraction at 25 °C and for 5 days of incubation, whereas leaf extraction with water at 40 °C for 5 days of incubation revealed the highest levels of ABTS scavenging activity. The levels of SOD and superoxide radical scavenging activities were highest in plant extraction, with hexane at 25 and 40 °C for 5 days of incubation, respectively. 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 several fluorescence peaks in hexane and aqueous plant extracts. On the other hand, intrinsic and non-destructive synchronous phosphorescence was also performed which also exhibited several peaks in aqueous and hexane extracts. 3D spectra of secondary metabolites confirmed the fluorescence peaks observed in SFS 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 technique for plant extracts.

Keywords: *Rauvolfia caffra*; plant leaves extracts; experimental design; characterization of secondary metabolites; antioxidant activity; intrinsic synchronous fluorescence and phosphorescence spectroscopy; 3D fluorescence spectra; FTIR spectroscopy

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1. Introduction

Rauvolfia caffra Sond is a plant species belonging to the family Apocynaceae which has been found in several African countries [1]. This medicinal plant has been widely used in various African countries for treatment of various clinical conditions such as sexually transmitted diseases, swollen legs, severe abdominal pains, abdominal disorders, wounds fever, swellings, abscesses, hepatitis, pneumonia, measles, skin lesions or itching rashes, cough, malaria and toothaches [2,3]. 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 traditional healers [2,3]. Several plant extracts of *Rauvolfia caffra* have been prepared in various solvents such as ethanol, methanol and water to investigate their chemical and biological properties [3–6]. The methanolic extract of *Rauvolfia caffra* was found to exhibit antimalarial activity in vitro [1], and it has been widely used for malaria treatment. As far as biological and chemical composition of these plant extracts are concerned, they contain phenol and flavonoids, terpenoids, proteins, carbohydrates, alkaloids, flavonoids, tannins, saponins, and glycosides as well as antioxidant and reducing power activities [3–8].

There are few published reports in the literature about phytochemical assays for only reducing power and antioxidant activity from this plant extract [1,3–8]. Therefore, it is of great interest to investigate in detail the phytochemical parameters of this plant extract in order to understand its biological activity in several clinical conditions [1,3–8]. Moreover, to our knowledge, we have not found any published reports about experimental design for extraction of secondary metabolites from leaves of *Rauvolfia caffra*. This is an important strategy for optimization of secondary metabolite extractions as it provides useful information about significant factors and interactions between the variables [9]. Intrinsic synchronous fluorescence and phosphorescence as well as 3D fluorescence spectroscopy are very useful analytical techniques for identification of secondary metabolites in plant extracts as they are fast, sensitive and non-destructive [10,11]. The current report involved the investigation of endogenous and intrinsic fluorescence properties of these secondary metabolites without the use of exogenous fluorescence probes. Moreover, these analytical techniques are very sensitive to conformational changes in these secondary metabolites as well as their micro-environment, and huge samples can be processed by the use of a fluorescence microtiter plate reader in a spectrofluorometer [10,11]. Additional information can be generated by using chemometric techniques with these fluorescence data, which involves the use of mathematical and statistical methods to analyze complex, high-dimensional spectral data to identify bioactive compounds. However, to the authors' knowledge, there are no reports in the literature about fluorescence characterization of this plant extract. Therefore, the present work involved experimental design for optimization of secondary metabolite extraction from leaves of *Rauvolfia caffra* [12]. The current report was carried out by using two solvents of opposite polarities to allow for extraction of as many secondary metabolites as possible from these plant extracts. According to previous reports on phytochemical assays of secondary metabolites in plant extracts [12–14], several parameters were analyzed in the current report for quantification of phenolic compounds, flavonoids, reducing power, antioxidant activity with DPPH, ABTS, superoxide scavenging activity and SOD activity. Subsequently, these secondary metabolites were investigated by intrinsic synchronous fluorescence, phosphorescence and 3D fluorescence spectroscopy, as well as by FTIR analysis.

2. Materials and Methods

2.1. Chemicals

Phenazine methosulfate, NADH, nitro blue tetrazolium (NBT), Riboflavin, deuterated water, gallic acid, catechin, Trolox, ascorbic acid, Folin and Ciocalteu, DPPH and ABTS (2,2′-Azino-di [3-ethylbenzthiazoline sulfonate]) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Plant Collection

Plant leaves were collected on the island of S. Tomé, *Rauvolfia caffra Sond.* in the S. Tomé botanical garden, in the interior of the island, at 6 am, with very high humidity and an average temperature of 24 °C in January 2018 (GPS coordinates: 0°21′31.3″ N 6°42′03.9″ E), and they were free of pests and diseases from the same tree. Additional plant leaves were also collected from different plants to confirm that the selected sample is representative of the species. 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 Center (CIAT-STP). A voucher specimen of the plant was deposited in the herbarium of S. Tomé (08-01-2018), Voucher Number 002/2019, 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 and dried overnight in an oven at 40 °C. The dried plant material was ground through an IKA A10 (IKA-Werke GmbH & Co. KG, Staufen, Germany) universal grinder, and the plants extracts were prepared with 15 g of ground dry matter from the leaves of *Rauvolfia caffra* in either 150 mL of demineralized water or 150 mL of hexane. All plant extracts were transferred to an orbital shaker either at 25 °C or 40 °C according to the experimental design procedure. The plant extract of day 0 was shaken for 30 min in the orbital shaker and centrifuged at 10,000 rpm for 30 min at room temperature; the supernatant was recovered and stored in Eppendorf tubes at −20 °C for further analysis representing the sample at 0 days. The remaining plant extracts were incubated in the orbital shaker at 150 rpm for 5 days at 25 and 40 °C 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 °C in dark containers protected from light, which represents the sample at 5 days.

2.3.2. Phytochemical Assays

All phytochemical assays were carried out by using eight samples from the experimental design described below. All phytochemical parameters were analyzed by using plant extract concentration in the range of 0 to 5 mg/mL, and blank corrections were carried out by using either H_2O or hexane.

Determination of Total Phenolic Content (TPC)

Phenolic compounds from plant extracts were determined by the Folin–Ciocalteau procedure [14–16] with some modifications. 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. After shaking, the microplate was incubated at 40 °C for 30 min and 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 (0.25 mg/mL), (R^2 = 0.99) was carried out with the following volumes: 0, 5, 10, 15, 20, and 25 µL, adjusted to a final

volume of 30 µL with water. The results of the total phenolic compounds were expressed as micromoles gallic acid equivalent per g of leaves.

Determination of Total Flavonoids Content (TFC)

Flavonoids quantification was carried out by using the colorimetric method described previously [13], 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 1.5×10^{-2}–1.0 mM, 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 was diluted with 110 µL demineralized water and 7.5 µL sodium nitrite (5%). An amount of 7.5 µL aluminum chloride (10%) were added to the microplate and incubated for 6 min at room temperature and protected from light. Subsequently, 100 µL of a sodium hydroxide solution (4%) was added, and the reaction mixture was mixed manually and incubated for 15 min. 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.

2.3.3. Antioxidant Activity of Plant Extracts

All antioxidant activity parameters were analyzed by using plant extract concentration in the range of 0 to 5 mg/mL, and blank corrections were carried out by using either H_2O or hexane.

DPPH Radical Scavenging Activity

Quantification of DPPH free radical scavenging activity was based on the method described previously [16], with some modifications. 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 80:20 methanol:water. The microplate was incubated at room temperature in the dark for 1 h 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 $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance at 550 nm of the tested samples or standard. The test was performed in triplicate, and Trolox was used as standard. The IC_{50} values for each plant extract were determined from the graph of DPPH scavenging effect against the concentration of plant extracts (mg/mL).

ABTS Scavenging Activity

This assay was carried out as described previously [16,17], 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-well microplate, plant extract (16 µL) and $ABTS^{\bullet+}$ radical solution (280 µL) were added; after manual homogenization, the microplate was incubated for 30 min, protected from light. Absorbance was read at 655 nm, and all assays were carried out in triplicates and Trolox (0.3 mg/mL) was used as standard. The following expression was used:

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

where $A_{control}$ is the absorbance of the negative control and A_{sample} is 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).

Reducing Power

This assay was carried out as described previously [16] with some modifications. Different concentrations of plant extracts (25 µL) were added to sodium phosphate buffer (25 µL, 0.2 M pH 6.6) and 1% K_3FeCN_6 (25 µL). The mixture was incubated at 50 °C for 20 min. After incubation, 80 µL of trichloroacetic acid (10% TCA) was added to the reaction mixture, and finally 100 µL, of demineralized water (H_2O) and 20 µL of $FeCl_3$ (0.1%) were added; the absorbance was read at 655 nm. Trolox (0.3 mg/mL) was used as the standard; the results were expressed as µmole Trolox equivalents per g of leaves and the assays were carried out in triplicates.

Superoxide Radical Scavenging Activity

The superoxide radical scavenging activity was performed as described previously [18] with minor changes. Each sample (25 µL) was mixed with 80 µM phenazine methosulfate (PMS, 25 µL), 625 µM NADH (25 µL), 200 µM nitro blue tetrazolium (NBT, 25 µL) and 100 µL of 100 mM sodium phosphate buffer pH 7.4. After 5 min at room temperature, the absorbance was read at 550 nm (microplate reader Bio-Rad 680, Bio Rad, Hercules, CA, USA). All absorbance readings were performed in triplicate, and ascorbic acid (0.3 mg/mL) was used as a standard. The following expression was used:

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

where $A_{control}$ is the absorbance of the negative control and A_{sample} is 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 superoxide radical scavenging activity against the concentration of plant extracts (mg/mL).

Superoxide Dismutase (SOD) Activity Assay

SOD activity was carried out by NBT method described previously [19], 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 (2 mM). The absorbance at time 0 of the reaction mixture was measured at 550 nm (microtiter plate reader Bio-Rad 680); 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) were evaluated at the same time per microtiter plate. One unit of SOD was defined 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.

2.3.4. Experimental Design to Optimize Extraction of Secondary Metabolites

The experimental design was performed by using three factors and two levels with duplicates: extraction temperatures (25 and 40 °C), extraction time (0 and 5 days) and solvents (H_2O and hexane). Preliminary experiments were carried out on these parameters, and the incubation time of 0 and 5 days provided the best results. The upper (+) and lower (−) levels were defined based on the preliminary results about 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, 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 the ANOVA methodology.

2.3.5. 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., Tokyo, Japan) as reported previously [20]. All fluorescence spectra were blank corrected by using either H_2O or hexane.

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

SPS of plant extracts was carried out as described previously [20]. All fluorescence spectra were blank corrected by using either H_2O or hexane.

2.3.7. Intrinsic 3D Fluorescence Spectra Measurements of Plant Extracts

3D fluorescence spectrum of plant extracts was performed as reported previously [20]. All fluorescence spectra were blank corrected by using either H_2O or hexane.

2.3.8. FTIR Analysis of Plant Extracts

The structural information of secondary metabolites was analyzed by FTIR analysis as reported previously [20].

2.3.9. Statistical Analysis

Correlation and regression analyses were performed with the Excel software 2024 package (Academic License, Microsoft of Portugal). Experimental results are means of three parallel measurements, and the results are presented as mean values ± standard deviation (SD). Statistical analysis was performed 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

Several phytochemical parameters were analyzed in these plant extracts as shown in Table 1. The highest levels of TPC, TFC, DPPH and reducing power were obtained with aqueous plant extraction at 25 °C and for 5 days of incubation whereas aqueous leaves extraction at 40 °C for 5 days of incubation revealed highest levels of ABTS scavenging activity. The levels of SOD and superoxide radical scavenging activities were highest with plant extraction with hexane at 25 and 40 °C for 5 days of incubation, respectively. The data of phytochemical assays from *Rauvolfia caffra* plant extracts published in the literature revealed some data on DPPH antioxidant activity as well as reducing power in stem bark extracts in methanol [12]. The IC_{50} for DPPH inhibition was found to be 0.213 mg/mL which compares with 0.26 mg/mL for plant extract with H_2O, 25 °C and 5 days in the present report [12]. The data on reducing power of stem bark extract is difficult to compare because it is presented in IC_{50} (1.22 mg/mL) whereas in the current report the data are presented in µmoles TE equivalent/g leaves [12]. Similar data on DPPH inhibition and reducing power have been reported in the literature by using % inhibition for these methanol plant extracts of stem bark and roots [5,6,8]. These data are difficult to

compare with the data of the present work as the units, methodology, solvents and part of plant are different for these phytochemical assays.

Table 1. Some phytochemical assays of plant extracts.

Plant Extracts	TPC	TFC	Reducing Power	ABTS Inhibition	DPPH Inhibition	Superoxide Radical Scavenging Activity	SOD Activity
	(µmoles Gallic Acid Equivalent/g Leaves)	(µmoles Catechin Equivalent/g Leaves)	(µmoles TE Equivalent/g Leaves)	IC_{50} (mg Extract/mL)	IC_{50} (mg Extract/mL)	IC_{50} (mg Extract/mL)	(Units/g Leaves)
H_2O, 25 °C, 0 days	15.78 ± 0.30^{a}	2.03 ± 0.31^{a}	23.49 ± 2.11^{b}	0.97 ± 0.02^{a}	3.48 ± 0.03^{a}	1.01 ± 0.003^{a}	596.14 ± 137.88^{b}
H_2O, 25 °C, 5 days	80.60 ± 13.15^{a}	48.05 ± 2.70^{a}	51.67 ± 2.37^{b}	0.95 ± 0.01^{c}	0.26 ± 0.001^{b}	2.07 ± 0.02^{a}	1083.92 ± 21.40^{a}
Hexane, 25 °C, 0 days	9.90 ± 0.58^{a}	1.06 ± 0.16^{b}	2.09 ± 1.82^{b}	4.03 ± 0.04^{c}	19.09 ± 0.11^{c}	1.19 ± 0.05^{b}	621.33 ± 102.26^{b}
Hexane, 25 °C, 5 days	27.50 ± 3.79^{a}	1.75 ± 1.19^{c}	2.31 ± 0.15^{c}	1.23 ± 0.03^{b}	84.41 ± 0.94^{c}	1.86 ± 0.02^{b}	1091.41 ± 17.77^{a}
H_2O, 40 °C, 0 days	11.17 ± 1.92^{a}	0.89 ± 0.08^{b}	9.90 ± 0.87^{c}	0.69 ± 0.01^{d}	2.21 ± 0.08^{b}	1.85 ± 0.01^{b}	325.62 ± 5.72^{c}
H_2O, 40 °C, 5 days	19.43 ± 0.88^{a}	2.03 ± 0.37^{b}	8.11 ± 0.10^{b}	0.66 ± 0.006^{b}	12.97 ± 0.93^{c}	1.01 ± 0.007^{d}	657.28 ± 182.13^{d}
Hexane, 40 °C, 0 days	8.01 ± 0.03^{a}	0.57 ± 0.13^{b}	2.17 ± 0.23^{d}	4.07 ± 0.08^{e}	12.55 ± 0.16^{f}	2.36 ± 0.01^{e}	282.25 ± 147.96^{b}
Hexane, 40 °C, 5 days	9.55 ± 0.79^{a}	0.80 ± 0.08^{c}	2.06 ± 0.07^{d}	1.80 ± 0.01^{f}	2.70 ± 0.02^{e}	0.69 ± 0.02^{d}	982.80 ± 31.35^{f}

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 investigate various factors that affect the extraction of these substances. The experimental design was performed by 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.

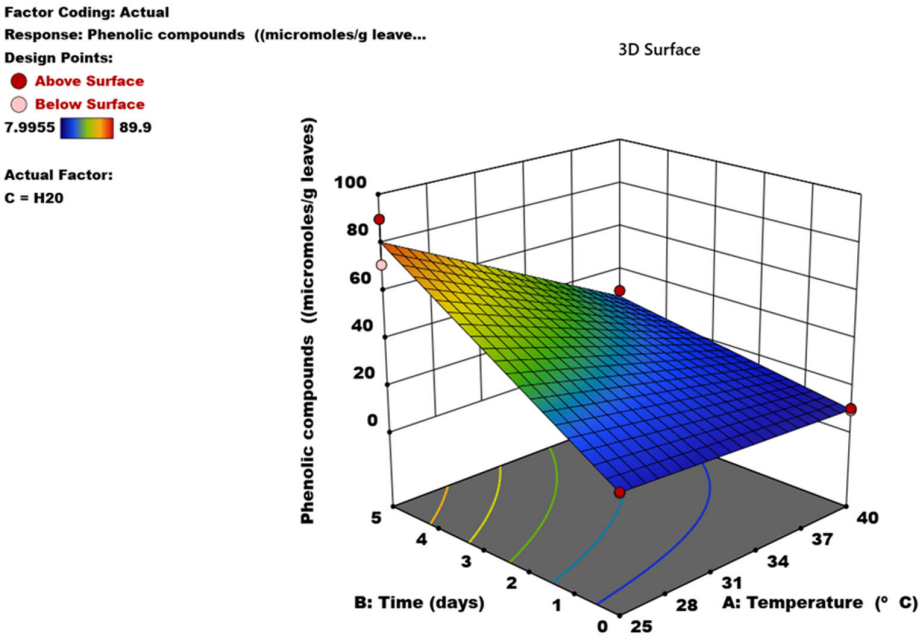
Source	Sum of Squares	Df	Mean Square	F-Value	*p*-Value
Model	8238.17	7	1176.88	48.81	<0.0001 significant
A-Temperature	1296.61	1	1296.61	53.77	<0.0001
B-Time	2126.23	1	2126.23	88.18	<0.0001
C-Solvent	1832.32	1	1832.32	75.99	<0.0001
AB	727.40	1	727.40	30.17	0.0006
AC	527.34	1	527.34	21.87	0.0016
BC	1318.52	1	1318.52	54.68	<0.0001
ABC	409.76	1	409.76	16.99	0.0033
Pure Error	192.90	8	24.11		
Corrected Total	8431.07	15			

$R^2 = 0.9771$; CV = 3.25%; Adequate Precision = 20.9040; Contribution of A = 15.73%; Contribution of B = 25.80%; and Contribution of C = 22.24%.

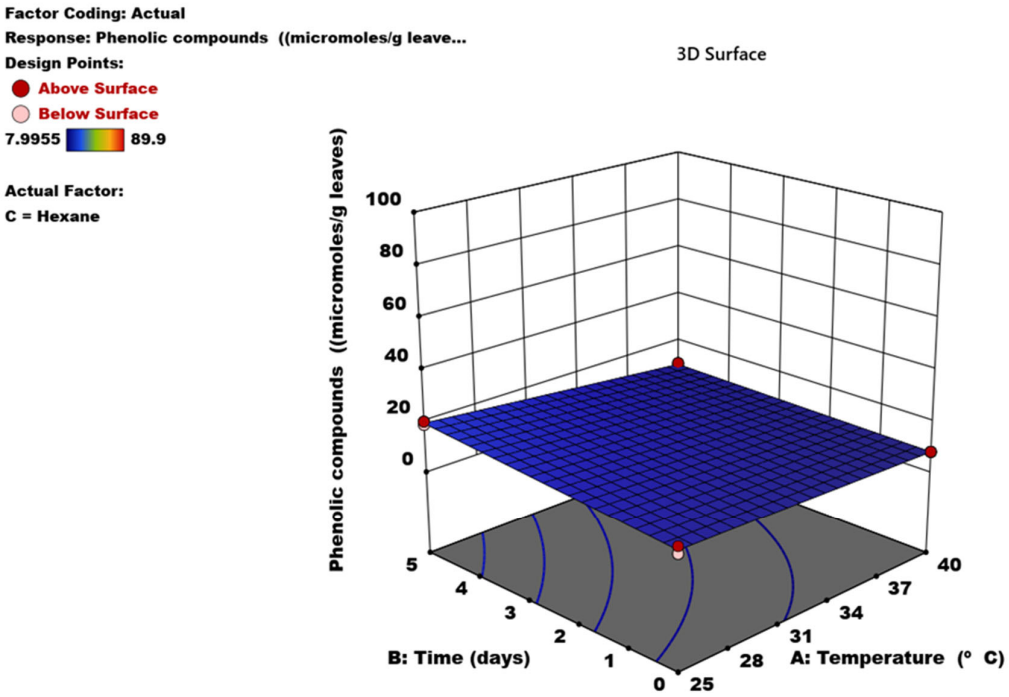
The data in Table 2 summarize the statistical analysis of variance (ANOVA). The F-value of 48.81 suggested that the model is significant and that there was only a 0.01% probability of such an F-value being due to noise. The coefficient of determination $R^2 = 0.9771$ suggested that there was a statistical correlation between the response and the variables considered and that only 0.01% of the total variation was not explained by the model. The statistical analysis revealed that the significant factors for phenolic compounds extraction were 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. However, the time, the solvent and the interaction between the time and the solvent revealed the greatest effect. The empirical equation was obtained to calculate the extraction of phenolic compounds from plant extracts as shown below:

$$Y = 20.06 - 11.05X_A + 6.55X_B - 5.52X_C - 4.05X_{AB} - 0.77X_{AC} - 5.55X_{BC} + 3.91X_{ABC}$$

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



(**A**)



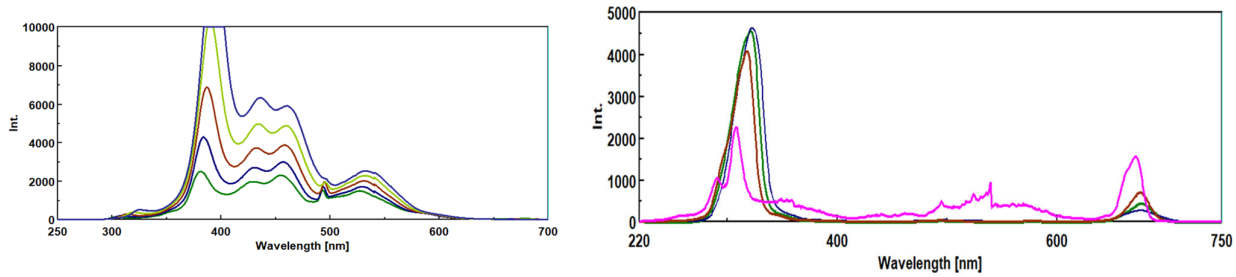
(**B**)

Figure 1. (**A**) 3D representation of the interaction of extraction/incubation time and temperature of incubation on the extraction of phenolic compounds by using water as solvent. (**B**) 3D representation of the interaction of extraction/incubation time and temperature of incubation on the extraction of phenolic compounds by using hexane as solvent.

The data in Figure 1A,B exhibits the effect of optimized factors on phenolic compounds extraction from plant extracts. The highest extraction of phenolic compounds (i.e., 89.9 µmoles gallic acid equivalent/g leaves) was obtained in aqueous extracts at 25 °C and 5 days of incubation (Figure 1A). To the authors' knowledge, there are no published reports about experimental design for extraction of secondary metabolites either from the leaves, stem, bark or roots of this plant.

3.3. Synchronous Fluorescence Spectroscopy (SFS)

SFS consists of simultaneous scans of both the excitation and emission λ of a sample at a constant wavelength difference (Δλ) to produce a simple spectrum. The Δλ in SFS is a very useful parameter to obtain spectra of an analyte with the best resolution and sensitivity. To the authors' knowledge, there are no reports in the literature on intrinsic fluorescence spectroscopy of secondary metabolites from *Rauvolfia caffra* leaves. Moreover, there are very few published reports on fluorescence properties of secondary metabolites in either plant leaves or other parts of plant extracts in general [9,10]. Therefore, synchronous fluorescence spectroscopy (SFS) of secondary metabolites from this plant extract was investigated in a spectrofluorometer with different Δλ for aqueous and hexane extracts at 25 °C and 0 days of incubation/extraction, respectively (Figure 2A,B). The aqueous extract revealed several fluorescence peaks at 382, 430, 460, 490 and 530 nm (Figure 2A), which exhibited an increase in fluorescence intensity as a function of Δλ. The hexane extracts also exhibited several fluorescence peaks at 315, 500, 550 and 675 nm (Figure 2B), which revealed a decrease in fluorescence intensity as a function of Δλ at 500, 550 and 675 nm. However, the fluorescent peak at 315 nm increased with increasing Δλ as shown in Figure 2B. 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, catechin, 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 [21,22]. However, it is important to analyze some analytical standards in fluorescence techniques to confirm full identification of these bioactive compounds. The data in Figure 2 also reveals that chlorophyll peaks were only found in organic extracts, suggesting that hexane was apparently able to extract this large and complex pigment molecule.



(**A**) (**B**)

Figure 2. Intrinsic synchronous fluorescence spectra with different secondary metabolites from plant extracts. (**A**) Aqueous plant extract at 25 °C and 0 days at high sensitivity as follows: ___ 10 nm; __ 15 nm; __ 20 nm; __ 25 nm and ___ 30 nm of Δλ. (**B**) Plant extract with hexane at 25 °C and 0 days at medium sensitivity as follows: ___ 25 nm: ___ 30 nm; ___ 20 nm; and ___ 5 nm of Δλ.

The data in Figure 3A,C reveals an increase in fluorescence intensity in all emission peaks as a function of Δλ, whereas hexane extracts exhibited a decrease in fluorescence at 675 nm as a function of Δλ. It is important to point out that aqueous extract exhibited a different emission pattern with fluorescence peaks at 320, 400, 430, 492 and 530 nm

(Figure 3A,C). The hexane extract revealed a specific emission pattern with fluorescence peaks at 320, 528, 577, 655, 675, 685 and 700 nm (Figure 3B). In a similar manner to the last figure, Figure 3B also exhibited several fluorescence peaks in the chlorophyll region (i.e., 650–700 nm) in organic extracts as opposed to aqueous extracts after incubation for 5 days at 25 °C. The hexane extracts also revealed chlorophyll peaks at 675 nm, 40 °C, and 0 days incubation (Figure 3D). The effect of temperature in aqueous plant extracts revealed a huge increase in fluorescence intensity from 25 to 40 °C, whereas there was a decrease in fluorescence intensity with hexane plant extracts (Figure 3).

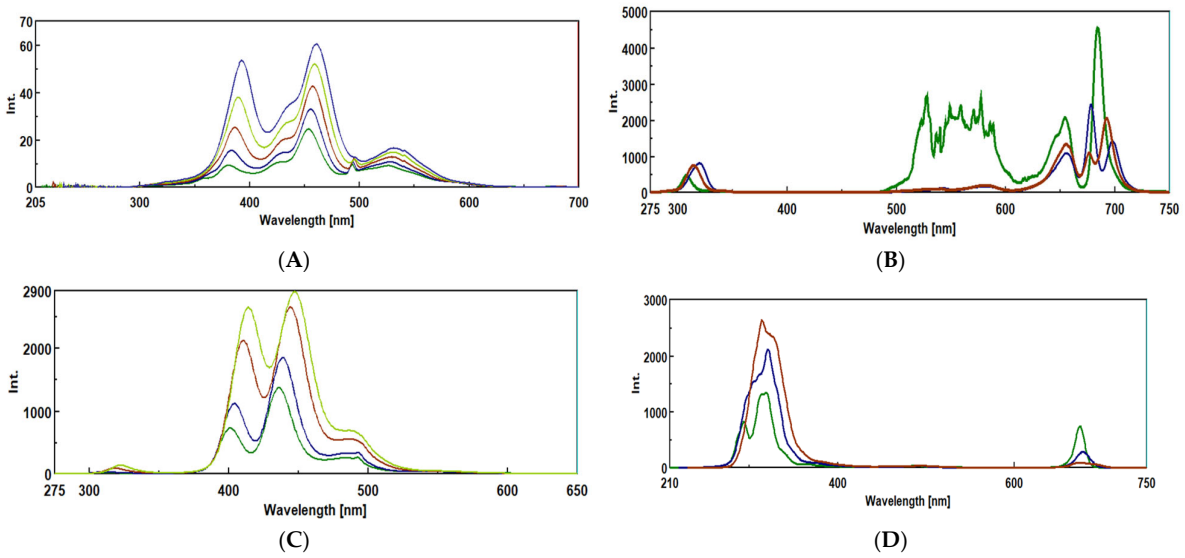


Figure 3. Intrinsic synchronous fluorescence spectra with different secondary metabolites from plant extracts. (**A**) Aqueous plant extract at 25 °C and 5 days at medium sensitivity as follows: ___ 10 nm; ___ 15 nm; ___ 20 nm; ___ 25 nm and ___ 30 nm of Δλ. (**B**) Plant extract with hexane at 25 °C and 5 days at medium sensitivity as follows: ___ 5 nm; ___ 20 nm; ___ 15nm. (**C**) Aqueous plant extract at 40 °C and 0 days at medium sensitivity as follows: ___ 10 nm; ___ 15 nm; ___ 25 nm; ___ 30 nm of Δλ. (**D**) Plant extract with hexane at 40 °C and 0 days at medium sensitivity as follows: ___ 10 nm; ___ 20 nm; ___ 30 nm Δλ.

The data in Figure 4 exhibit different fluorescence emission profiles of aqueous extracts compared with hexane extracts (Figure 4A,B), as emission peaks were observed at 320, 400, 430 and 492 nm for Figure 4A and 320, 528, 550, 650, 675, and 720 nm for Figure 4B. It is important to stress that there is a significant decrease in fluorescence intensity of all peaks, both for aqueous and hexane extracts after 5 days of incubation at 25 and 40 °C (Figures 3 and 4). This decrease in fluorescence intensity may be due to either a degradation of some phenolic substances and chlorophyll or a non-specific binding of these metabolites to insoluble components of the homogenate during this incubation period [23].

Although there are few reports in the literature about SFS of plant extracts in general, 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 [21–23].

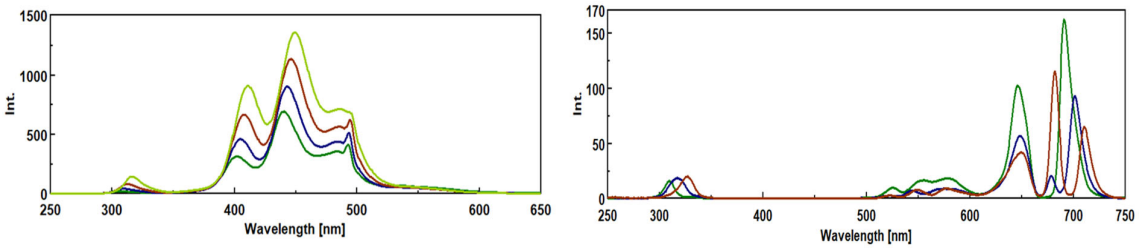


Figure 4. Intrinsic synchronous fluorescence spectra with different secondary metabolites from plant extracts. (**A**) Aqueous plant extract at 40 °C and 5 days at medium sensitivity as follows: ___ 10 nm; ___ 15 nm; ___ 20 nm; ___ 25 nm of Δλ. (**B**) Plant extract with hexane at 40 °C for 5 days at medium sensitivity as follows: ___ 10 nm; ___ 20 nm; ___ 30 nm of Δλ.

3.4. Intrinsic Synchronous Phosphorescence Spectroscopy (SPS)

The Δλ in SPS is also a useful parameter to obtain spectra of an analyte with the best resolution and sensitivity. The data in Figure 5A reveal several SPF spectra at different Δλ for aqueous plant extracts which exhibited high fluorescence intensity at low Δλ, which was also observed in aqueous extract at 25 °C and 5 days of incubation (Figure 5B). In a similar manner to SFS, Figure 5D also exhibits several fluorescence peaks in the chlorophyll region (i.e., 650–700 nm) in organic extracts as opposed to aqueous extracts after incubation for 5 days at 25 °C. On the other hand, the data presented in Figure 5B reveal an increase in fluorescence at low Δλ both at 390 and 560 nm. The data in Figure 5 also reveal that chlorophyll peaks were only found in organic extracts, suggesting that hexane was apparently able to extract this large and complex pigment molecule. In a similar manner to SFS, there is a significant decrease in fluorescence intensity after incubation of plant extracts for 5 days (Figure 5).

The data in Figure 6A,B exhibit only two fluorescence peaks in aqueous extracts at 490 and 550 nm whose fluorescence intensity decreased with incubation for 5 days at 40 °C. On the other hand, the organic extracts reveal chlorophyll peaks at 675 nm as well as fluorescence peaks at 325, 450, 490 and 550 (Figure 6C,D).

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 is due to chlorophyll in chloroplasts [19,20]. 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 [24,25]. 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, although the fluorescence peaks are sharper in SFS than in SPS.

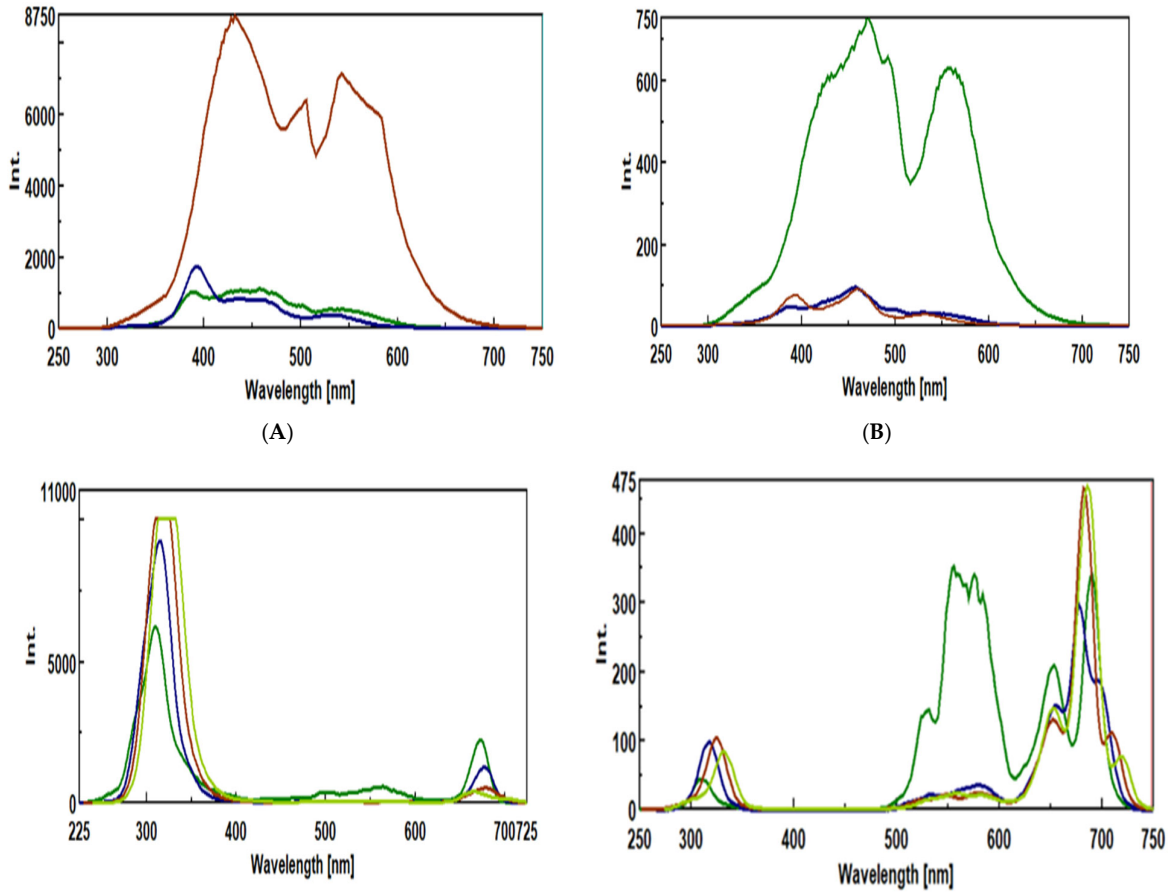


Figure 5. Intrinsic synchronous phosphorescence spectra of secondary metabolites from plant extracts. (**A**) Aqueous plant extracts at 25 °C and 0 days at low sensitivity with different Δλ as follows: ___ 20 nm; ___ 30 nm; ___ 10 nm. (**B**) Aqueous plant extracts at 25 °C and 5 days at very low sensitivity as follows: ___ 10 nm; ___ 20 nm; ___ 30 nm of Δλ. (**C**) Plant extracts with hexane at 25 °C and 0 days at low sensitivity as follows: ___ 10 nm; ___ 20 nm; ___ 30 nm; ___ 40 nm of Δλ. (**D**) Plant extracts with hexane at 25 °C and 5 days at low sensitivity as follows: ___ 10 nm; ___ 20 nm; ___ 30 nm; ___ 40 nm of Δλ.

SFS and SPS are sensitive photoluminescence techniques used to characterize plant extracts by decreasing spectral complexity as well as separating overlapping peaks. The main difference is related to the excited state involved, which is singlet for fluorescence and triplet for phosphorescence. This difference is responsible for their speed, sensitivity to environmental oxygen as well as the type of compounds detected. Therefore, SFS captures rapid, short-lived emission (i.e., <10 ns) whereas SPS captures longer-lived emission (milliseconds to seconds). SFS is routinely used for identification and quantification of aromatic substances, chlorophylls and polyphenols in plant extracts, whereas SPS is used for investigation of some specialized secondary metabolites in non-destructive studies of specific leaf extracts such as some phenolic compounds not found in SFS. As far as sample matrix influence is concerned, SFS exhibits high-intensity fluorescence peaks at 675 nm whereas SPS revealed some peaks at 430, 500 and 540 nm in aqueous extracts and fluorescence peaks at longer wavelength in hexane extracts, which suggest that it is useful for characterization of substances in specific microenvironments of solvents.

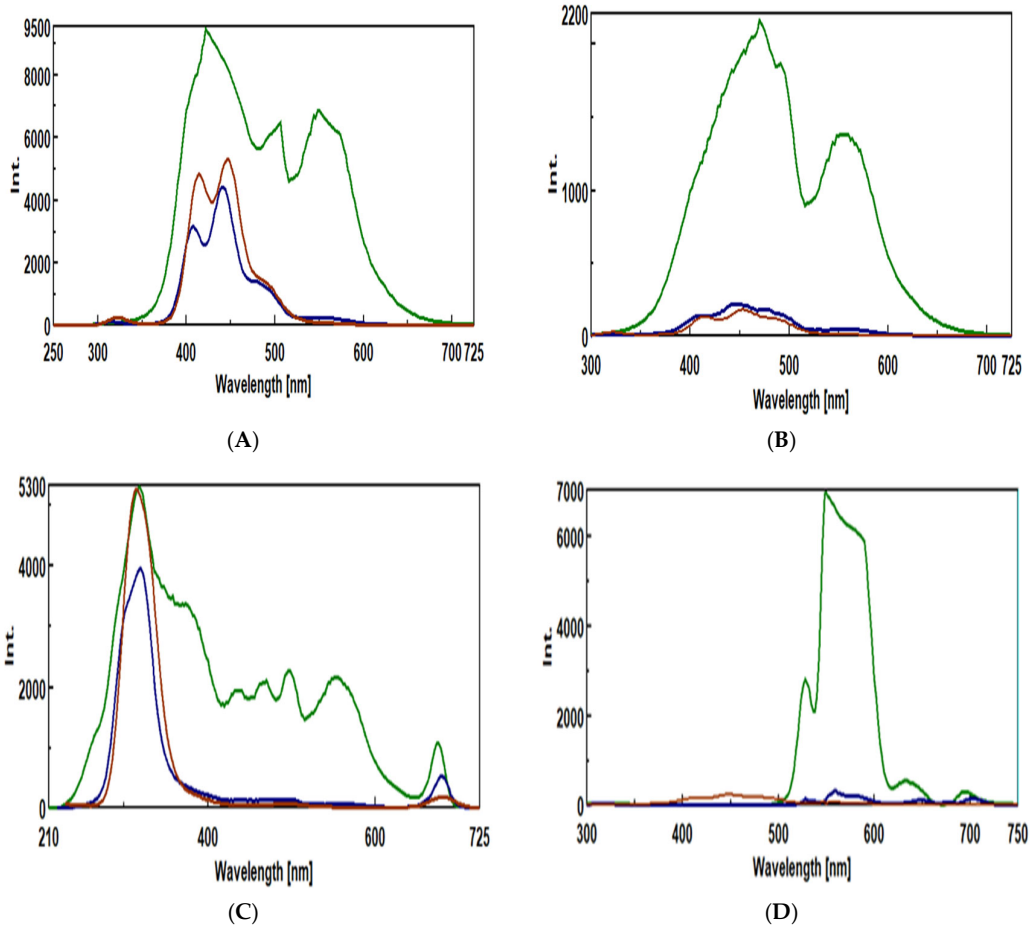


Figure 6. Intrinsic synchronous phosphorescence spectra of secondary metabolites from plant extracts. (**A**) Aqueous plant extracts at 40 °C and 0 days at low sensitivity as follows: ___ 10 nm Δλ; ___ 20 nm of Δλ; ___ 30 nm of Δλ. (**B**) Aqueous plant extracts at 40 °C and 5 days at very low sensitivity as follows: ___ 10 nm Δλ; ___ 20 nm of Δλ; ___ 30 nm of Δλ. (**C**) Plant extracts with hexane at 40 °C and 0 days at low sensitivity as follows: ___ 10 nm of Δλ; ___ 20 nm of Δλ; ___ 30 nm of Δλ. (**D**) Plant extracts with hexane at 40 °C and 5 days at low sensitivity as follows: ___ 10 nm Δλ; ___ 20 nm Δλ; ___ 30 nm of Δλ.

3.5. Intrinsic 3D Fluorescence Spectroscopy

The 3D fluorescence spectrum exhibited a fluorescence map of all fluorophores present in a sample for their characterization [20]. The data in Figure 7A–F reveal 3D spectra in different formats for secondary metabolites from plant extract with hexane at 25 °C and 0 days of extraction, as well as a synchronous 2D spectrum which exhibits fluorescence peaks at 290 and 675 nm, as shown in Figure 7F. The data in Figure 7 related to 3D spectra exhibit 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 is subdivided into five peaks with different excitation λ (Figure 7A,B,D,E), which are due to complex, overlapping contributions from different pigment–protein complexes, photosynthetic photosystems, as well as their degradation products [26]. Therefore, the 3D spectra of plant extracts behave as a fingerprint region of the photosynthetic system's state, subdividing the main emission peak at 675 nm into various components which represent different functional and structural parts of the chloroplast [26].

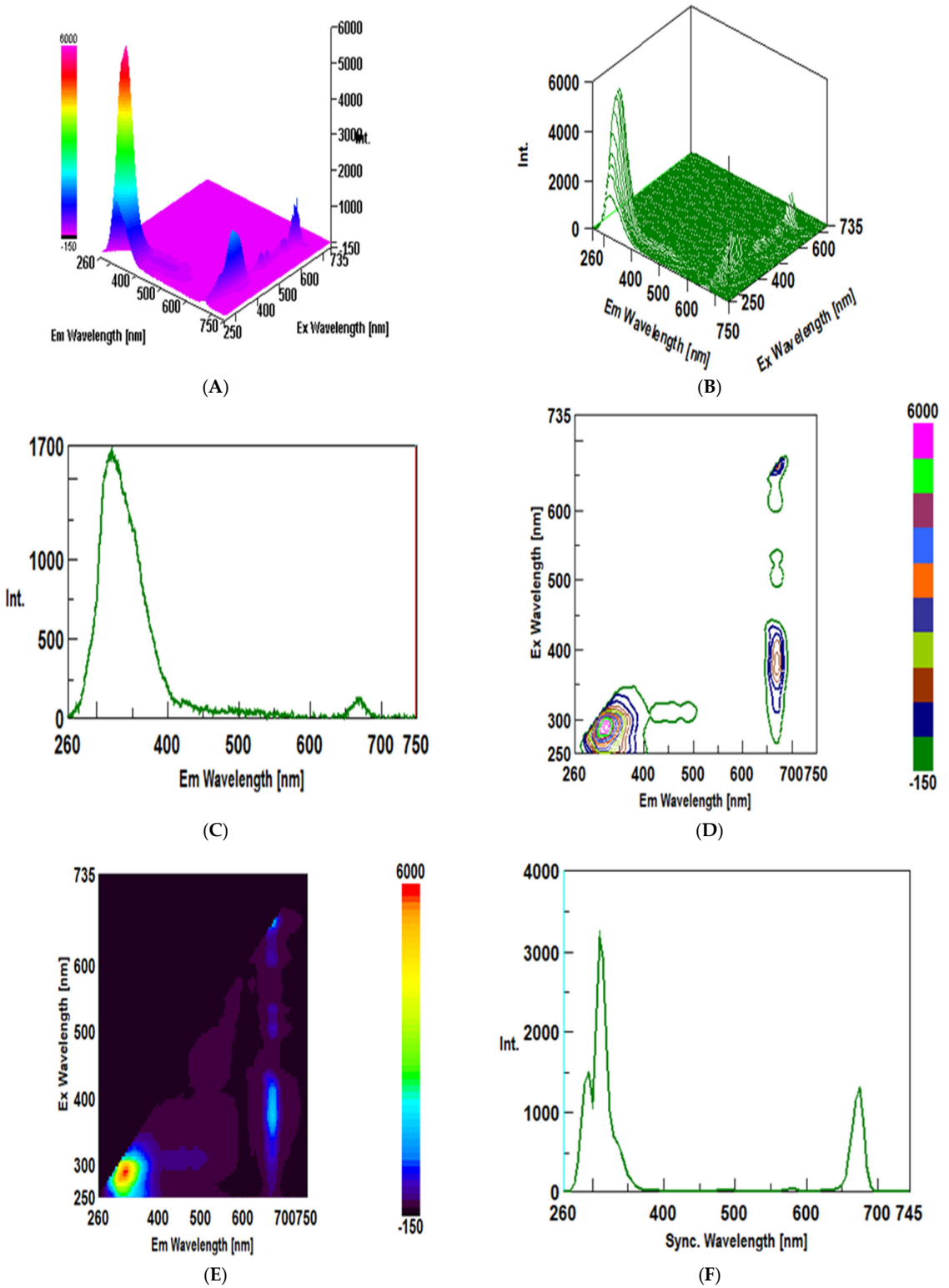


Figure 7. Secondary metabolites of plant extracts with hexane at 25 °C and 0 days with medium sensitivity. (**A**) Color 3D view; (**B**) 3D spectrum view; (**C**) 2D spectrum view; (**D**) contour view; (**E**) color view; (**F**) synchronous 2D spectrum with 10 nm of Δλ.

In a similar manner to Figure 7, the data in Figure 8 related to 3D spectra exhibit several fluorescence peaks (i.e., 320, 400, 450 and 490 nm) which are in agreement with SFS data. However, these data in Figure 8 are due to the aqueous extract at 40 °C for 5 days of incubation/extraction which confirm the absence of a chlorophyll peak at 675 nm compared to hexane extract at 25 °C for 0 days (Figure 7).

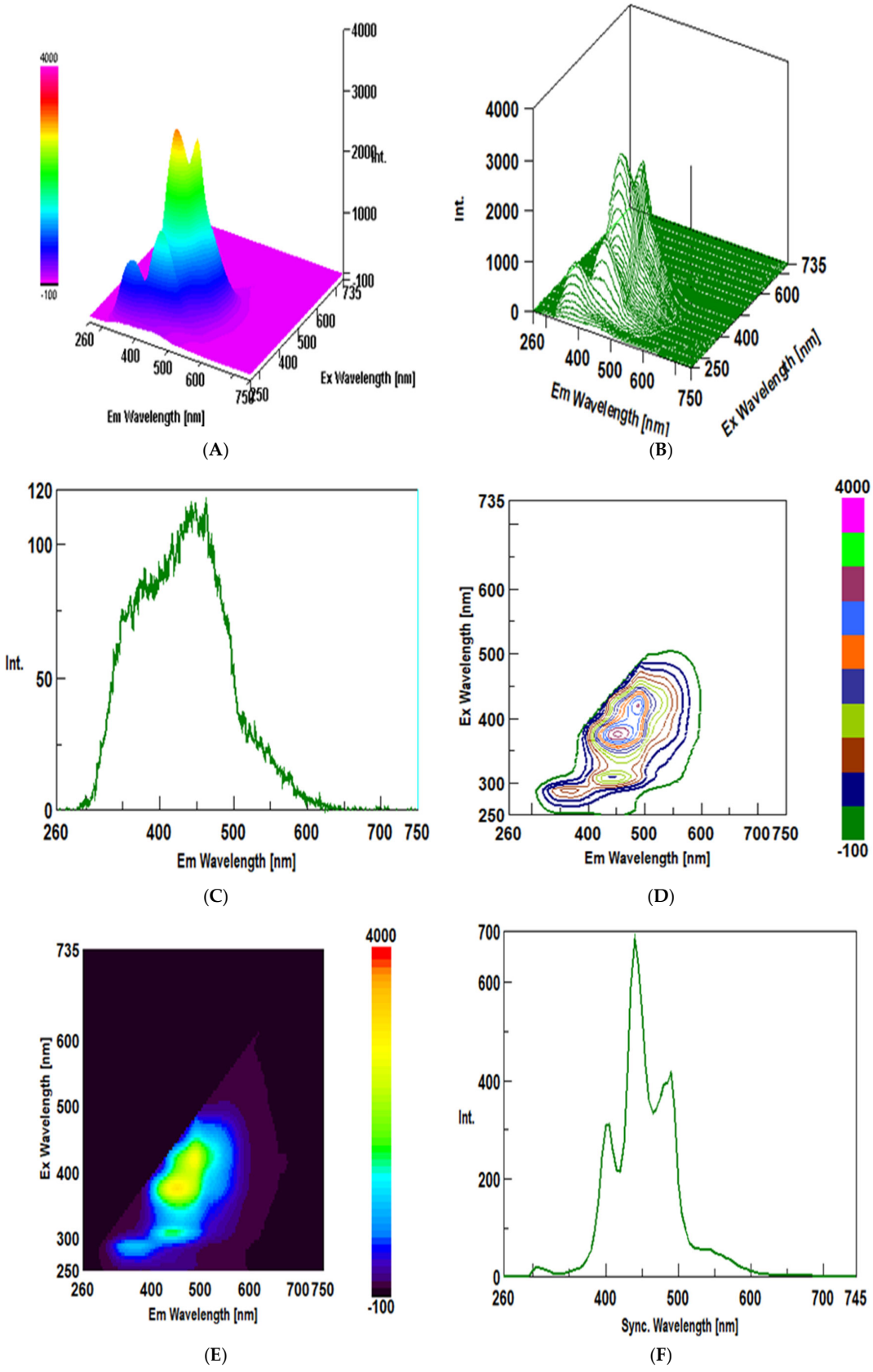


Figure 8. Secondary metabolites of plant extract with H_2O at 40 °C and 5 days with medium sensitivity. (**A**) Color 3D view; (**B**) 3D spectrum view; (**C**) 2D spectrum view; (**D**) contour view; (**E**) color view; (**F**) synchronous 2D spectrum with 10 nm of Δλ.

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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 [24–27].

3.6. FTIR Analysis

FTIR spectra of some aqueous plant extracts at 25 °C and incubated at 0 and 5 days were analyzed 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.

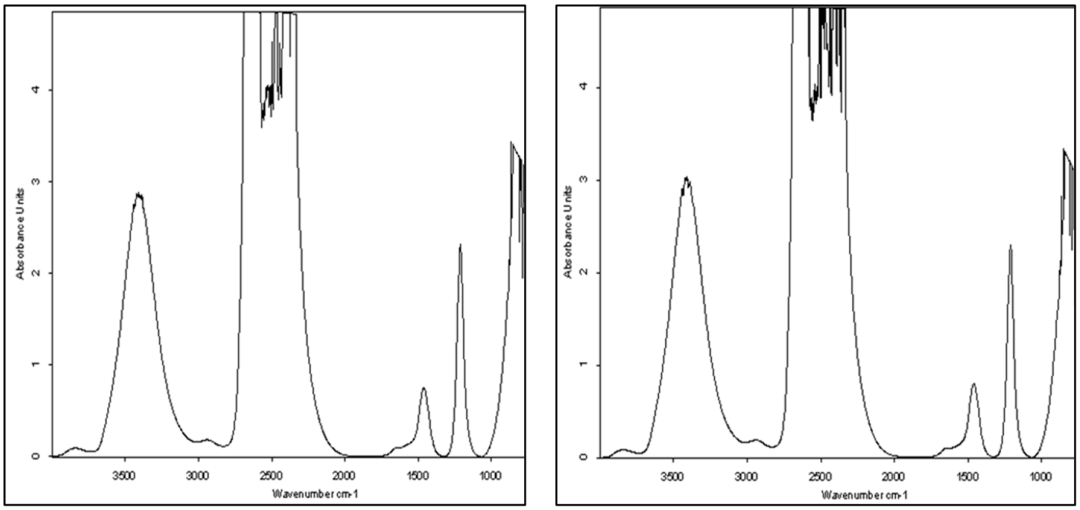


Figure 9. FTIR spectra of selected plant extracts. (**A**) 25 °C in H_2O for 0 days of incubation and (**B**) 25 °C in H_2O for 5 days of incubation.

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 *Rauvolfia caffra* plant extracts from FTIR spectra [12].

4. Conclusions

To the authors' 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 from *Rauvolfia caffra* plant leaves extracts. The fluorescence characterization of secondary metabolites involved the use of SFS and SPS as well as the fingerprint of 3D spectra of secondary metabolites. Moreover, experimental design 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 indicates that SPS revealed higher fluorescence intensity for secondary metabolite levels than SFS for these plant extracts. For complex sample matrices of secondary metabolites, SPS and 3D-SFS may provide valuable 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.

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. The quantitative data of secondary metabolites can be obtained from spectral measurements at specific wavelengths for some secondary metabolites such as chlorophyll as well as catechin (675 and 450 nm, respectively). Moreover, additional information can be generated by using chemometric techniques with these fluorescence data. These analytical techniques are cheap, fast, non-destructive, intrinsic, and do not require exogenous fluorophores; they also exhibit high sensitivity and selectivity. The use of a fluorescence reader coupled to the spectrofluorometer can process huge sample numbers with only 50 µL sample volume 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 approaches by running analytical standards.

Author Contributions: Conceptualization, A.K.; Methodology, K.R. and A.K.; Software, K.R. and A.K.; Validation, K.R.; Formal Analysis, K.R.; Investigation, K.R. and A.K.; Resources, K.R.; Writing—Original Draft, A.K.; Supervision, A.K. All authors have read and agreed to the published version of the manuscript.

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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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