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Article

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

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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 planning for optimization of several parameters was carried out by a full factorial of two levels of three factors for secondary metabolite extraction from *Rauvolfia caffra* leaves by using water and hexane at 25 and 40 ºC and 200 rpm for 0 and 5 days of incubation/extraction. The best conditions for highest extraction of phenolic compounds (i.e 89.90 µmoles gallic acid equivalent/g leaves)) was obtained at 25ºC, in H_20 and 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 25ºC and for 5 days of incubation whereas leaves extraction with water at 40º 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 40ºC 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 peaks at 320, 530, 550, 590, 650, 675, 690, 700, 710 nm in hexane plant extracts whereas aqueous extracts revealed only peaks at 382, 430, 460 and 530 nm. On the other hand, intrinsic and non-destructive synchronous phosphorescence was also performed which exhibited peaks at 430, 500 and 540 nm in aqueous extracts whereas hexane extracts revealed peaks at 320, 530, 560, 655, 675, 690 and 710 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: *Rauvolfia caffra*; plant leaves extracts; experimental planning; characterization of secondary metabolites; antioxidant activity; intrinsic synchronous fluorescence and phosphorescence spectroscopy; 3 -D. 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 several 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]. 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 [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 [4–6]. The methanolic extract of this plant was found to exhibit antimalarial activity in vitro [2] and it has been widely used for malaria treatment. As far as biological and chemical composition of these plant extracts are concerned, they contain phenolic and flavonoids, terpenoids, proteins, carbohydrates alkaloids, flavonoids, tannins, saponins, glycosides as well as antioxidant and reducing power activities [4–7].

There are very few published reports about colorimetric data in the literature about phytochemical assays for reducing power and antioxidant activity from this plant extract as well as FTIR analysis [6,7]. Moreover, to our knowledge, we have not found any published report about experimental planning for extraction of secondary metabolites from these plant leaves. 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 [8]. 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 [9,10]. However, to author´s knowledge, there are no reports in the literature about fluorescence characterization of this plant extract. Therefore, the present work involved experimental planning for optimization of secondary metabolite extraction from plant leaves, and some phytochemical parameters were analyzed in these plant extracts. Subsequently, these secondary metabolites were investigated 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, deuterated water, gallic acid, catechin, Trolox, ascorbic acid, Folin & Ciocalteu, DPPH and ABTS (2,2'-Azino-di [3-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, *Rauvolfia caffra Sond.* 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°21'31.3"N 6°42'03.9"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 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, 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

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were prepared with 15 grams 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 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 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 representing sample 0 days. 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 which represents sample 5 days.

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 [11–13] 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 protected from light. Absorbances were read at 765 nm in a microtiter plate reader in triplicates (FLUOstar OPTIMA-BMG Labtec). 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 micromoles 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 [14], 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%). 7.5 µL aluminium chloride (10%) 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 and the reaction mixture 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 [13], 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 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. The IC_{50} values for each plant extract were calculated from the graph of DPPH scavenging effect against the concentration of extracts (mg/mL).

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2.3.5. ABTS Scavenging Activity

This assay was carried out as described previously [14,15] 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, plant extract (16 µL) and $ABTS^{\bullet+}$ radical solution (280 µL) was added, after manual homogenization, the microplate was incubated for 30 minutes, 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 $A_{control}$ - 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 described previously [14] with some modifications. Different concentrations of plant extracts (25µL) were added to sodium phosphate buffer (25µL25 µL, 0.2M pH 6.6), and 1% K_3FeCN_6 (25µL25 µL). The mixture was incubated at 50 ° C for 20 minutes. After incubation 80 µL trichloroacetic acid (10 % TCA) was added to the reaction mixture and finally 100 µL of demineralized water (H2O) and 20 µL FeCl3 (0.1%,) and the absorbance was read at 655nm. Trolox (0.3mg / 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.

2.3.7. Superoxide Radical Scavenging Activity

The superoxide radical scavenging activity was carried out as described previously [16] 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 measured at 550 nm (microplate reader Bio-Rad 680). All absorbance measurements were carried out in triplicate and ascorbic acid (0.3mg / mL) was used as standard. 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 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).

2.3.8. Superoxide Dismutase (SOD) Activity Assay

SOD activity was carried out by NBT method described previously [17] 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) 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) 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

 doi:10.20944/preprints202603.0379.v1

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 [18].

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 ($\Delta\lambda$) 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.

2.3.12. Intrinsic 3D Fluorescence Spectra Measurements of Plant Extracts

Three-dimensional intrinsic fluorescence spectra of secondary metabolites were performed across a 3D space (excitation λ, emission λ and fluorescence intensity). The samples containing 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 [18].

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.

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3. Results and Discussion

3.1. Phytochemical Assays of Plant Extracts

Several phytochemical parameters were analysed 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 25ºC and for 5 days of incubation whereas aqueous leaves extraction at 40º 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 40ºC for 5 days of incubation, respectively. The data of phytochemical assays from *Rauvolfia caffra* plant extracts published in the literature revealed only some data on DPPH antioxidant activity as well as reducing power in stem bark extracts [6,7]. 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 (mmoles gallic acid equivalent/g leaves)	TFC (mmoles catechin equivalent/g leaves)	Reducing power (mmoles TE equivalent/g leaves)	ABTS inhibition IC_{50} (mg extract/mL	DPPH inhibition IC_{50} (mg extract/mL)	Superoxide radical scavenging activity IC50 (mg extract/mL)	SOD activity (Units/g leaves)
H20,25ºC, 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]
H20,25ºC, 5 days	80.60±13.15[a]	48.05±2.70[a]	51.67±2.37[b]	0.95±0.[01c]	0.26±0.001[b]	2.07±0.02[a]	1083.92±21.40[a]
Hexane, 25ºC, 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,25ºC, 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]
H20,40ºC, 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]
H20,40ºC, 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, 40ºC, 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, 40ºC, 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 analyze several factors that affect the extraction of these compounds. 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).

 doi:10.20944/preprints202603.0379.v1

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	<0.0001significant
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 summarized the statistical analysis of variance (ANOVA). The F-value of 48.81 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 = 0.9771 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 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 exhibited the greatest effect. The following empirical equation was obtained to estimate the extraction of phenolic compounds from plant extracts:

$$y = 20,06-11,05xA+6,55xB-5,52xC-4,05xAB-0,77xAC-5,55xBC+3,91xABC$$

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.

 doi:10.20944/preprints202603.0379.v1

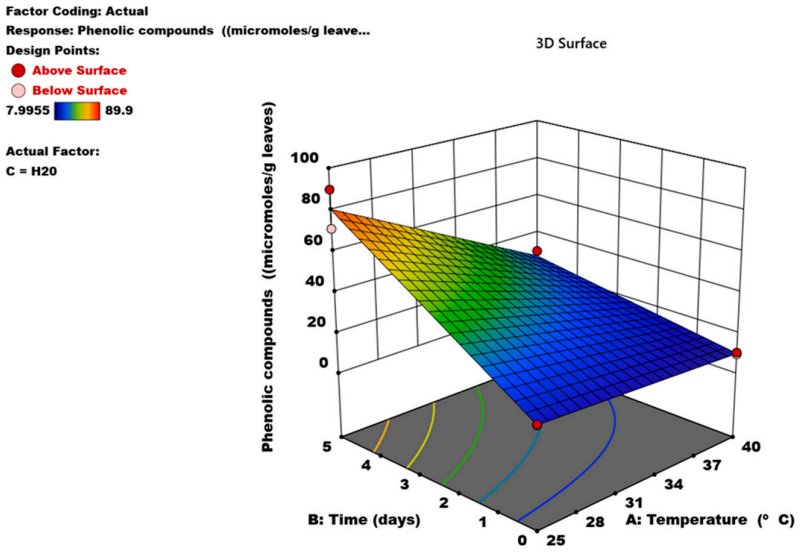

Figure 1. A- Three-dimensional representation of the interaction of extraction/incubation time and temperature of incubation on the extraction of phenolic compounds by using water as solvent.

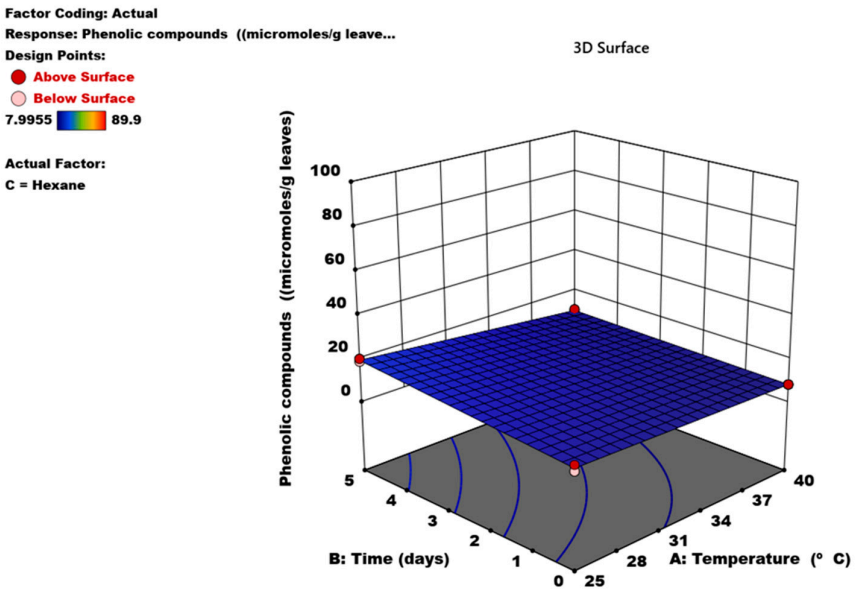

Figure 1. B- Three-dimensional representation of the interaction of extraction/incubation time and temperature of incubation on the extraction of phenolic compounds by using hexane as solvent.

The data in Figure 1A and 1B exhibited 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 25ºC and 5 days of incubation (Figure 1A). To authors' knowledge, there are no published reports about experimental planning for extraction of secondary metabolites either from leaves, stem bark or roots of this plant.

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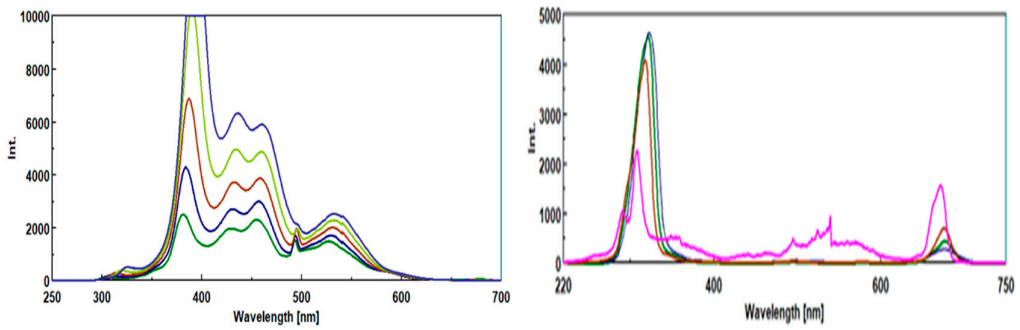
3.3. Synchronous Fluorescence Spectroscopy (SFS)

SFS involved simultaneous scans of both the excitation and emission wavelengths of a sample at a constant wavelength difference (Δλ) 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 Δλ 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 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 ºC and 0 days of incubation/extraction, respectively (Fig.2 A and B). The aqueous extract revealed several fluorescence peaks at 382, 430, 460,490 and 530 nm (Figure 2 A) which exhibited an increase in fluorescence intensity as a function of Δλ. The hexane extract also exhibited several fluorescence peaks at 315, 500, 550 and 675 nm (Figure 2 B) which revealed a decrease in fluorescence intensity as a function of Δλ at 500, 550 and 675 nm. However, the fluorescence peak at 315 nm increased with increasing Δλ as shown in Figure 2 B. 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 [19,20]. The data in Figure 2 also revealed that chlorophyll peaks were only found in organic extracts suggesting apparently that hexane was able to extract this large and complex pigment molecule.



A B

Figure 2. -Intrinsic synchronous fluorescence spectra with different Δλ of secondary metabolites from plant extracts. A Aqueous plant extract 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 25ºC and 0 days at medium sensitivity as follows: ___ 25 : ___ 30; ___ 20 nm; and ___ 5 nm of Δλ.

The data in Figure 3 A and C have revealed increase in fluorescence intensity in all emission peaks as a function of Δλ whereas hexane extracts have 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 3 A, C). The hexane extract revealed a specific emission pattern with fluorescence peaks at 320, 528, 577, 655, 675, 685 and 700 nm (Figure 3 B). In a similar manner to the last figure, Figure 3 B also exhibited several fluorescence peaks in chlorophyll region (i.e 650- 700 nm) in organic extracts as opposed to aqueous extracts after incubation for 5 days at 25 ºC. The hexane extracts also revealed chlorophyll peaks at 675 nm at 40 ºC at 0 days incubation (Figure 3 D).

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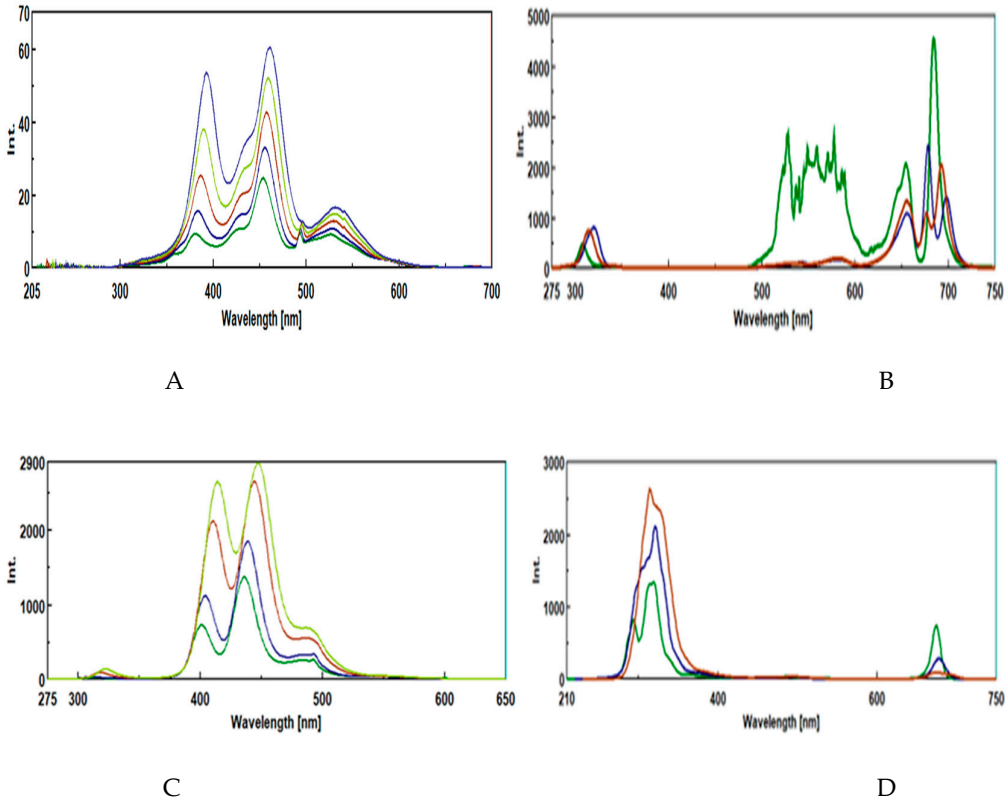

Figure 3. - Intrinsic synchronous fluorescence spectra with different Δλ of secondary metabolites from plant extracts. A- Aqueous plant extract at 25 ºC and 5 days at medium sensitivity as follows: ___ 10 nm; ___ 15 nm; ___ 20 nm; ___ 25 nm and ___ 30 nm of Δλ; B– Plant extract with hexane at 25ºC and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

The data in Figure 4 exhibited different fluorescence emission profiles of aqueous extracts compared with hexane extracts (Figure 4 A and B) as emission peaks were observed at 320, 400, 430 and 492 nm for Figure 4A and 320, 528, 550, 650, 675, 720 nm for Figure 4 B. 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 incubations at 25 and 40 º C (Figure 3 and 4).

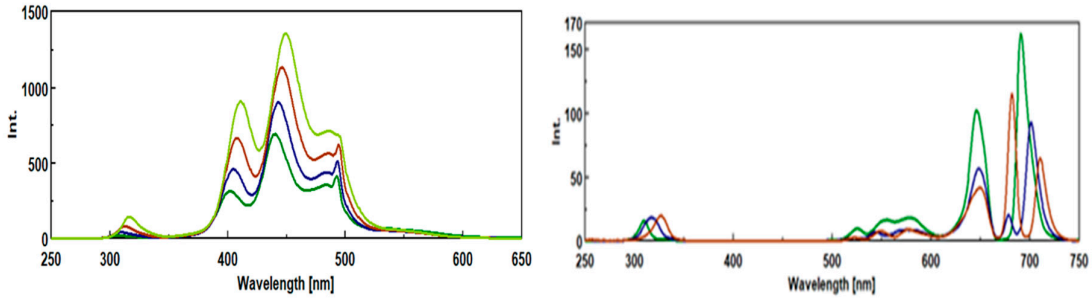

Figure 4. - Intrinsic synchronous fluorescence spectra with different Δλ of secondary metabolites from plant extracts. A- Aqueous plant extract at 40ºC and 5 days at medium sensitivity as follows: ___ 10 nm; ___ 15nm; ___ 20 nm; and ___ 25 nm ; B– Plant extract with hexane at 40 ºC, for 5 days at medium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

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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 [19,20].

3.3. 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. The data in Figure 5 A exhibited several SPF spectra at different $\Delta\lambda$ for aqueous plant extracts which revealed high fluorescence intensity at low $\Delta\lambda$ which was also observed in aqueous extract at 25 ºC and 5 days of incubation (Fig.5 B). In a similar manner to SFS, Figure 5 D also exhibited several fluorescence peaks in chlorophyll region (i.e 650- 700 nm) in organic extracts as opposed to aqueous extracts after incubation for 5 days at 25 ºC. On the other hand, the data presented in Figure 5 B revealed an increase in fluorescence at low $\Delta\lambda$ both at 390 and 560 nm. The data in Figure 5 also revealed that chlorophyll peaks were only found in organic extracts suggesting apparently that hexane was 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).

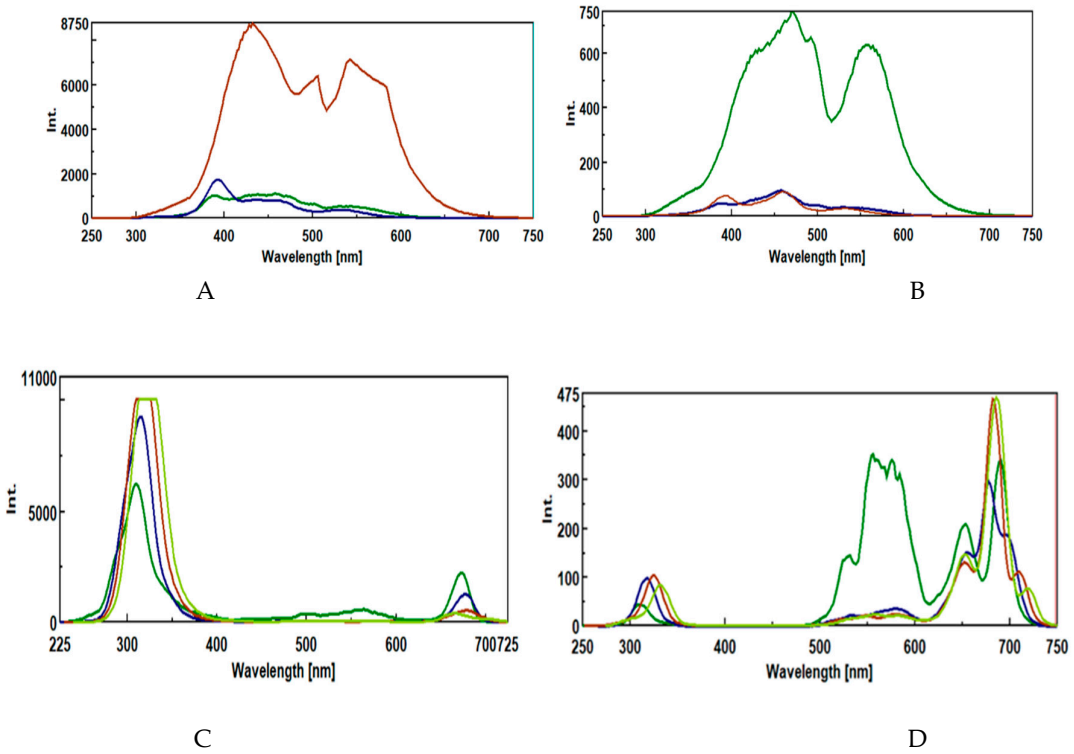

Figure 5. - Intrinsic synchronous phosphorescence spectra of secondary metabolites from plant extracts. A – Aqueous plant extracts at 25 ºC and 0 days at low sensitivity as follows: ___ 20 nm; ___ 30 nm; ___ 10 nm; B- Aqueous plant extracts at 25 ºC and 5 days at very low sensitivity as follows: ___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

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

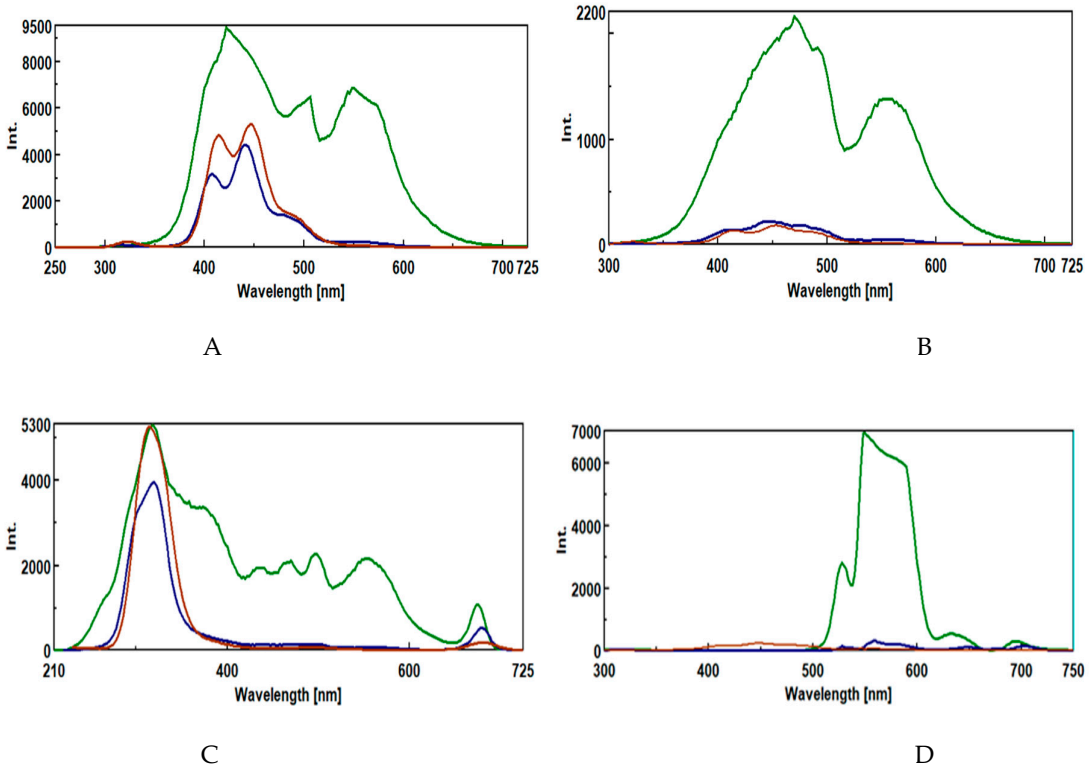

Figure 6. - Intrinsic synchronous phosphorescence spectra of secondary metabolites from plant extracts. A- Aqueous plant extracts at 40 ºC and 0 days at low sensitivity as follows: ___ 10 nm Δλ ; ___ ;20 nm Δλ ___ 30 nm of Δλ. B- Aqueous plant extracts at 40 ºC and 5 days at very low sensitivity as follows: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

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 [21,22].

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

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plant extract with hexane at 25 ºC and 0 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 [23]. Therefore, the 3D spectra of plant extracts behaved as 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 [23].

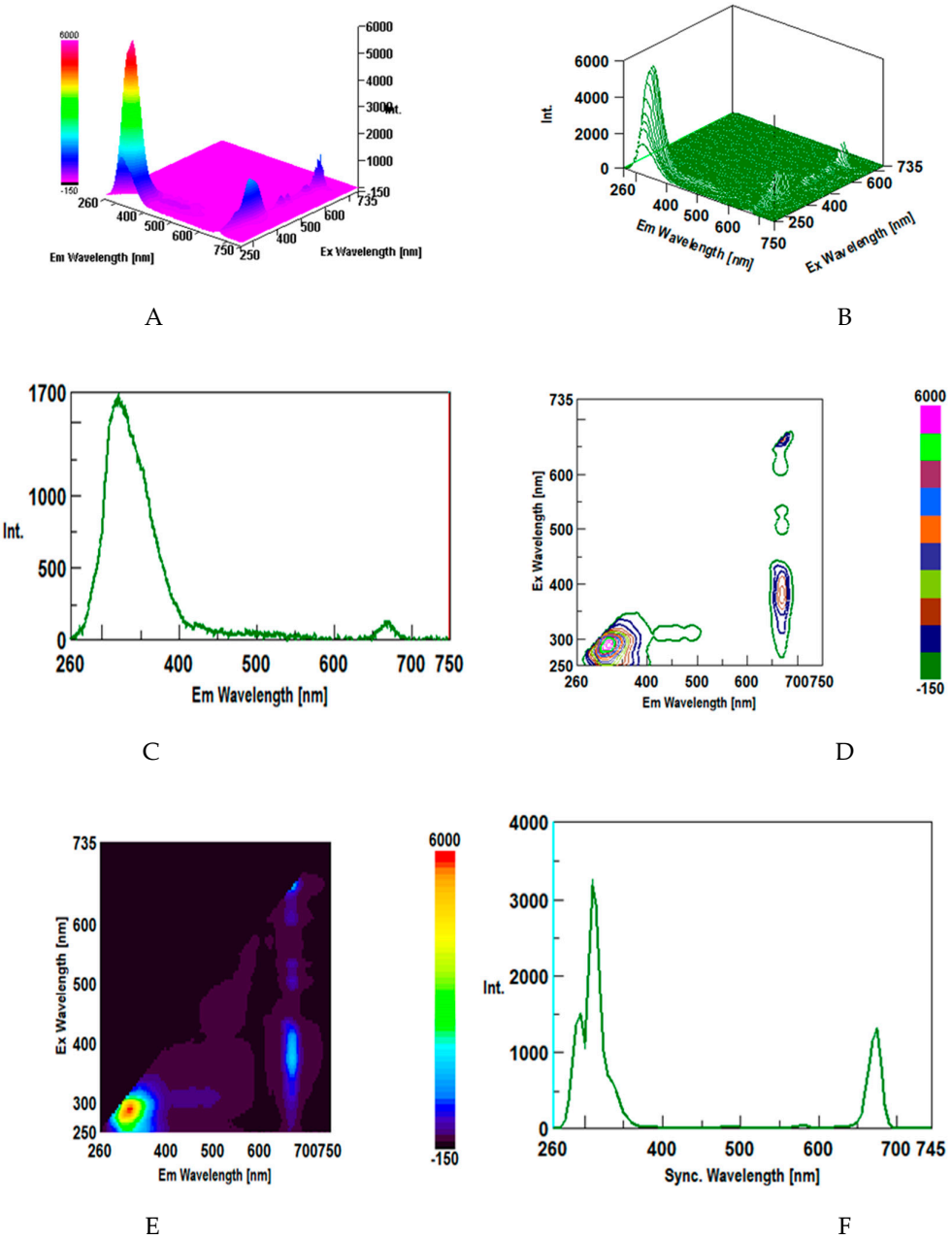

Figure 7. Secondary metabolites of plant extract with hexane at 25ºC and 0 days with medium sensitivity. (A)-Colour 3D view; (B) Three-dimensional spectrum view; (C) 2-D spectrum view; (D) Contour view; E- Colour view; F- Synchronous 2D spectrum with 10 nm of $\Delta\lambda$.

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In a similar manner to Figure 7, the data in Figure 8 related to 3- D spectra exhibited 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 40ºC for 5 days of incubation/extraction which confirmed the absence of chlorophyll peak at 675 nm compared to hexane extract at 25ºC for 0 days (Figure 7).

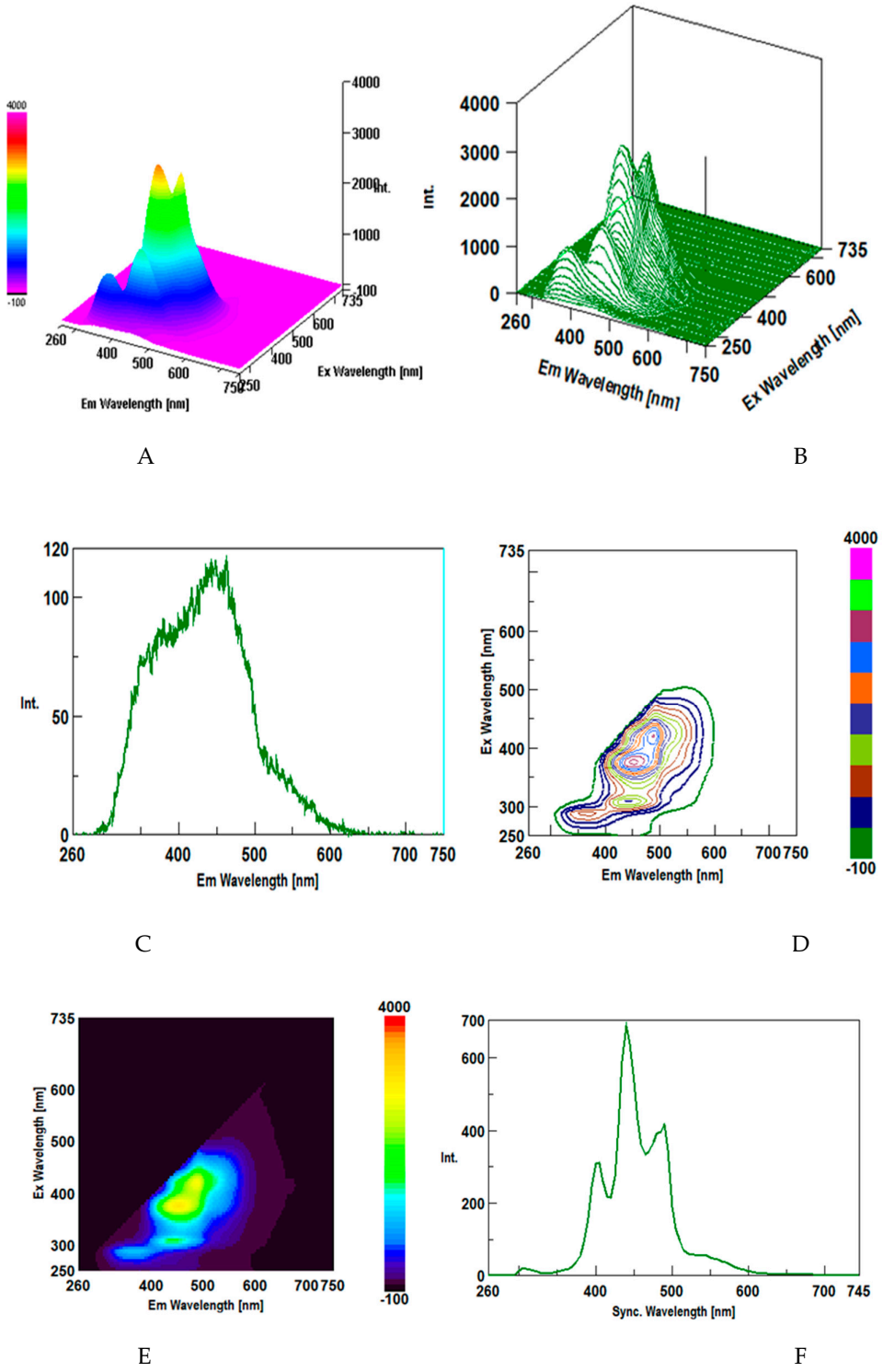

Figure 8. - Secondary metabolites of plant extract with H_20 at 40ºC and 5 days with medium sensitivity. (A)- Colour 3D view; (B) Three-dimensional spectrum view; (C) 2-D spectrum view; (D) Contour view ; (E) - Colour view; (F)- Synchronous 2D spectrum with 10 nm of $\Delta\lambda$.

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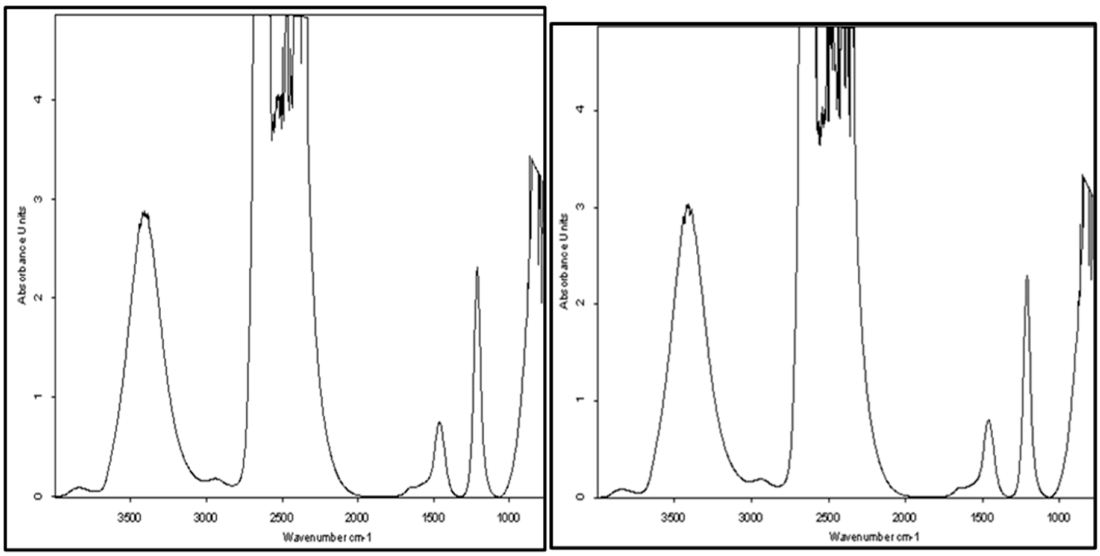
Although the data on the 3D spectra measurement for secondary metabolites from this plant extract have not been reported in the literature, this analytical technique of 3D spectra has been widely used in research areas such as smart agriculture, identification of key bioactive substances, geographical origin of plants , evaluation of anti-oxidant capacity of plant extracts and quality control and authentication of plant-based products in terms of adulteration [23–26].

3.6. FTIR Analysis

FTIR spectra of some aqueous plant extracts at 25 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 H20 for 0 days of incubation and B- 25 ºC in H20 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 [27]

4. Conclusions

To the author's knowledge, this is the first report about fluorescence properties of plant leaves extracts of *Rauvolfia caffra*, 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 by analysing three factors (i.e , temperature, nature of solvent and extraction/incubation time). Some phytochemical assays were performed in

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these plant extracts to investigate their antioxidant, TPC, TFC, reducing power and SOD levels. 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.

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, 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 further investigation of fluorescence properties of secondary metabolites from other sources in terms of selectivity and specificity. Moreover, fluorescence, phosphorescence and time-resolved fluorescence techniques must be used to fully characterize secondary metabolites from several sources in conjunction with chemometric approach. However, the full structural characterization of purified secondary metabolites should be complemented by using NMR, FTIR and fluorescence spectroscopy as well as by HPLC, GC-MS and ELISA combined with a chemometric approach.

Author Contributions: Conceptualization, Amin Karmali; Methodology, Karla Ramos and Amin Karmali; Software, Karla Ramos and Amin Karmali; Validation, Karla Ramos; Formal analysis, Karla Ramos; Investigation, Karla Ramos and Amin Karmali; Resources, Karla Ramos; Writing – original draft, Amin Karmali; Supervision, Amin Karmali. 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

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TCA	Trichloroacetic acid
TPC	Total phenolic content
TFC	Total flavonoids content

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