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Optimization of Extraction Parameters, Total Polyphenols and Flavonoids Contents, and Antioxidant Activity of the Aqueous Extract of *Vernonia amygdalina* Leaves

Bedie Mbow, Aïssatou Alioune Gaye, Birane Diop, Abba Diarra Kone, Ibrahima Diallo, Mohamed Gaye*

Department of Chemistry, University Cheikh Anta Diop, Dakar, Senegal

Email address:

mohamedl.gaye@ucad.edu.sn (Mohamed Gaye)

*Corresponding author

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Abstract: *Vernonia Amygdalina* is a well-known and widely used plant in traditional African medicine. The decoctions of its leaves are used in the treatment of many pathologies including hepatitis, diabetes, cancer etc. It is a plant very rich in phenolic compounds, generally responsible for the biological activity of plants. In this study, we determined the optimal extraction conditions, including temperature, duration, and solvent mass/volume ratio. Subsequently, the total polyphenol contents are determined under these optimal conditions, then the antiradical activity evaluated. The Folin-Ciocalteu reagent was used to evaluate the content of phenolic compounds, with gallic acid as a reference. The radical DPPH· made it possible to measure the antioxidant power of the extract, with reference to Trolox. This study showed that the optimal conditions for extracting the bioactive molecules responsible for the antioxidant activity are obtained for a temperature of 80°C, for a period of 30 min in a ratio of 1%. Under these conditions, the total polyphenol and flavonoid contents are respectively equal to 10.536 ± 0.145 mg/g gallic acid equivalent and 1.930 ± 0.043 mg/g quercetin equivalent. Furthermore, the results show a maximum inhibition of 41.95% for a concentration of 0.044 mg/mL, for 30 minutes. The antioxidant properties were measured and demonstrated by the kinetics of reduction with DPPH·, thus showing an inhibitory power of 75% and a fairly slow reduction reaction. This high content of phenolic compounds and the fairly significant inhibitory power of *Vernonia amygdalina* justify the use of this plant in traditional medicine for the treatment of numerous pathologies.

Keywords: *Vernonia amygdalina*, Extraction, Polyphenols, Flavonoids, Antioxidant Activity

1. Introduction

The use of plants in herbal medicine is very old and is currently experiencing renewed interest among rural and urban populations [1–3]. According to the WHO, nearly 6377 species of plants are used in Africa, of which more than 400 are medicinal plants which constitute 90% of traditional medicine. In 2004, nearly 75% of the African population resorted to the plants around them for treatment and did not have access to so-called modern drugs [4, 5]. In the socio-economic context of developing countries, the study of plants can lead to obtaining adequate and accessible therapeutic responses, combining proven scientific effectiveness and optimal cultural acceptability [6–8]. The scientific promotion of traditional medicine should lead in particular to the development of herbal medicines.

Vernonia amygdalina is a plant widely used in West Africa and Asia due to its pharmacological effects [9–12]. She has proved to have an intense therapeutic and nutritional virtue and is commonly used in traditional medicine. Decoctions of its leaves are used to treat scabies, headaches, gastrointestinal disorders, malaria [13–16]. Aqueous extracts from the leaves of this plant containing peptides and edotides inhibit the growth of breast cancer cells [17–20]. These scientific results justify the choice made on this plant, in order to determine the nature of the

bioactive molecules it contains, its content of phenolic compounds and flavonoids and the antioxidant power of the organic extract of the leaves. The extraction time, the temperature and the mass/extract solvent volume ratio are very influential extrinsic factors on the quantity of secondary metabolites extracted and on the biological activity of organic plant extracts, which justifies the optimization of extraction conditions. To better characterize the antiradical power, kinetic parameters are introduced, such as the TEC50 time, the time required to reach equilibrium at CE50. Estimation of TCE50 makes it possible to establish the following classification: TCE50 < 5 min (fast reaction), 5-30 min (intermediate reaction) and TCE50 > 30 min (slow reaction) [21, 22]. The objective of this study is to propose a fast and reproducible method allowing to choose the most adequate conditions during the various stages of the valorization of *Vernonia amygdalina:* the extraction, the dosage of the phenolic compounds and the determination of the antiradical activity.

2. Material and Methods

The choice of *Vernonia amygdalina* is justified by its use by the medical practice Biokeneya, specializing in natural medicine for the treatment of certain pathologies. Indeed, after using the leaves of *Vernonia Amygdalina,* in the form of a decoction, patients with breast cancer are followed and spectacular results have been noted. The plant material was collected in Mbour [14° 24′ 20″ north, 16° 51′ 20″ west], a town located in western Senegal, about 80 km from Dakar. After harvest, the plant material is dried in the dark and then pulverized using an electric grinder. The powder obtained, put in condition, is used for the various chemical experiments.

2.1. Phytochemical Screening

Phytochemical screening is a qualitative analysis based on precipitation or coloring reactions. The latter make it possible to define the presence or absence of secondary metabolites which may be found in a plant sample. In this work, the screening concerns the search for: alkaloids, polyphenols, tannins, flavonoids, saponins, sterols and polyterpenes, leucoanthocyanins, catechols, mucilages. We tested the presence of these different chemical groups by referring to the techniques described in the work of Ronchetti and Russo [23].

The polyphenols and tannins were identified by the $FeCl_3$ test and Stiasny's reagent; flavonoids, leucoanthocyanins and catechols by reaction with cyanidin; saponins by the foam test; sterols and polyterpenes by the Liebermann- Burchard test; mucilages by the absolute ethanol test and alkaloids by Mayer's tests [24].

2.2. Determination of the Content of Total Phenolic Compounds

The content of total phenolic compounds was determined with the Folin-Ciocalteu reagent [25]. Indeed, 40 µL of each extract are taken and supplemented to 200 µL with distilled water. A volume of 150 µL of Folin-Ciocalteu reagent, 600 µL of a 20% Na_2CO_3 solution and 2.32 mL of distilled water are additionally added thereto. After 30 minutes of incubation in the dark, the absorbance is read at 760 nm using a UV/Visible spectrometer of the Perkin-Elmer Lamda 365. The measurement was compared to a gallic acid standard curve prepared from a stock solution of 0.1 mg/mL gallic acid.

2.3. Determination of Flavonoid Content

The flavonoid content was calculated by the method described by Dirar *et al*. [26]. This method consists of adding 2.5 mL of a 2% $AlCl_3$ ethanolic solution to 500 µL of each extract. The mixtures are incubated for 1 hour at room temperature and the absorbance is read at 425 nm. The flavonoid content is expressed in terms of Quercetin equivalent (EqQ) by reference to the calibration curve plotted with a concentration range obtained from a stock solution at 0.1 mg/mL of quercetin.

2.4. Antioxidant Activity by DPPH· Method

Firstly, the scavenging activity by $DPPH^{\bullet}$ were determined [27, 28]. At 0.2 ml of aqueous extract were added 3.8 ml of a 0.1014 mM $DPPH^{\bullet}$ methanolic solution. The mixes were incubated in the dark at room temperature for 30 minutes. The absorbance were read at 517 nm using a spectrophotometer UV/VIS Perkin-Elmer Lambda 365. TROLOX was used as a standard. With the operating conditions, IC50 could not be determine.

Secondly, the kinetic was also evaluated by reading the absorbance every 5 minutes for the next 360 minutes.

3. Results and Discussions

3.1. Phytochemical Screening

Table 1. *Phytochemical screening results of Vernonia amygdalina leaf solvent extracts.*

Solvent Metabolites	Hexane	Methanol	Water
Polyphenols	-	+	+
Flavonoids	-	+	+
Alkaloids	-	+	+
Sterols and polyterpenes	+	+	+
Catechic tannins	-	-	-
Gallic tannins	-	+	+
Leucoanthocyanins	-	+	-
Catechols	-	-	-
Saponosides	+		
mucilage	+		

Absent; + Present

The results of the phytochemical screening recorded in Table 1 reveal the presence of all the families of secondary metabolites tested in the leaves of *Vernonia amygdalina*, except for the case of catechols which are totally absent there. However, it should be noted that the presence is not uniform, it varies from one solvent to another. It is noted that the

sterols and polyterpenes are present in the three hexane, methanol, and aqueous extracts. Polyphenols, flavonoids, alkaloids, and gallic tannins are present in all three *Vernonia amygdalina solvent extracts.* All families of compounds are present in methanolic and aqueous extracts except catechin tannins and catechols. These experimental results show that the extraction of secondary metabolites depends on the polarity of the solvents used and that *Vernonia amygdalina* is a plant very rich in secondary metabolites.

3.2. Optimization of Vernonia Amygdalina Extraction Parameters

Temperature, time, and solvent mass/volume ratio are determining factors for the extraction of secondary metabolites in plant material samples, as well as for the determination of their biological activity. In this study, it is a question of determining the conditions for which the contents of polyphenols, flavonoids, and the antioxidant activity at DPPH· are maximal. To do this, one of the analytical parameters is varied, and by fixing the other two parameters, the value for which the polyphenol content and the antiradical activity are maximum is noted.

3.2.1. Optimization of the Temperature

In this part, the temperature is varied while fixing the extraction time to 20 min and the mass of solvent to 500 mg in a constant volume of solvent. The variations in the content of polyphenolic compounds and in the antiradical activity with DPPH· as a function of temperature are recorded (Table 2).

The antiradical activity of the various extracts was evaluated by their inhibitory activity on a methanolic solution of DPPH· at 40 mg/L, by measuring the absorbance at the wavelength 517 nm (Table 3). The standard used is Trolox, a water-soluble analogue of tocopherol.

Table 2. *Evolution of polyphenol content as a function of temperature.*

Temperature	50			60			70		
Mass (mg)	500.9			500.4			500.3		
Absorbance at 760nm	0.111	0.111	0.114	0.131	0.133	0.131	0.121	0.121	0.123
Temperature	80			90			100		
Mass (mg)	500.1			500.1			500.3		
Absorbance at 760nm	0.129	0.134	0.132	0.146	0.149	0.143	0.146	0.150	0.148

Table 3. *Evolution of the antiradical activity with DPPH· as a function of temperature.*

Temperature	50			60			70		
Mass (mg)	500.9			500.4			500.3		
Absorbance at 517 nm	1.038	1.038	1.035	1.035	1.035	1.034	1.027	1.025	1.030
Temperature	80			90			100		
Mass (mg)	500.1			500.1			500.3		
Absorbance at 517 nm	1.020	1.023	1.016	1.022	1.025	1.020	0.999	0.999	0.997

The evolution of the contents of total polyphenols and of the antiradical activity with DPPH· is represented in Figure 1.

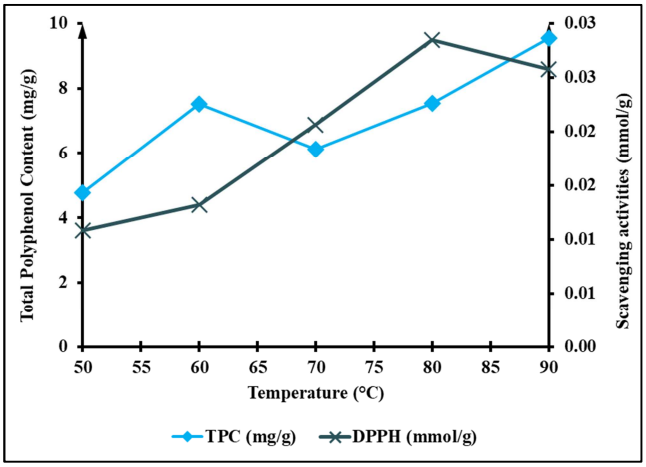

Figure 1. *Evolution curve of the total polyphenol content and of the scavenging activity with DPPH· of the aqueous extract of Vernonia Amygdalina as a function of temperature.*

The experimental results show an increase in the polyphenol content and in the antiradical activity as a function of the temperature. However, a decrease in this content is noted between 60°C and 70°C while the activity continues to increase more markedly. Beyond this temperature, the content of total polyphenols increases according to the temperature without reaching its maximum, even at 90°C. As for the antiradical activity, it reaches its maximum at 80°C, before starting to decrease. under the effect of temperature. This decrease may be due to the degradation of certain heat-sensitive compounds endowed with antiradical activity. According to these experimental results, it can be concluded that the optimal extraction temperature of the bioactive molecules, responsible for the antioxidant activity, contained in the leaves of *Vernonia amygdalina* is 80°C.

3.2.2. Optimization of the Time

In this part, the extraction time is varied while fixing the temperature at 80°C as found above and the mass of solvent at 500 mg in a constant volume of solvent. The variations in the polyphenol content and in the antiradical activity with DPPH· are thus noted.

Table 4. *Evolution of the polyphenol content as a function of time.*

Time (min)	5			10			15		
Mass (mg)	500.5			500.4			500.7		
Absorbance at 760nm	0.136	0.139	0.141	0.148	0.142	0.145	0.141	0.140	0.142
Time (mins)	20			30			60		
Mass (mg)	500.5			500.2			499.6		
Absorbance at 760nm	0.151	0.146	0.153	0.167	0.163	0.169	0.146	0.146	0.142

Table 5. *Evolution of absorbance as a function of time.*

Time (min)	5			10			15		
Mass (mg)	500.5			500.4			500.7		
Absorbance at 517 nm	1.019	1.012	1.022	1.013	1.010	1.008	1.010	1.010	1.008
Time (mins)	20			30			60		
Mass (mg)	500.5			500.2			499.6		
Absorbance at 517 nm	0.991	0.991	1.002	0.979	0.979	0.978	0.992	0.989	1.006

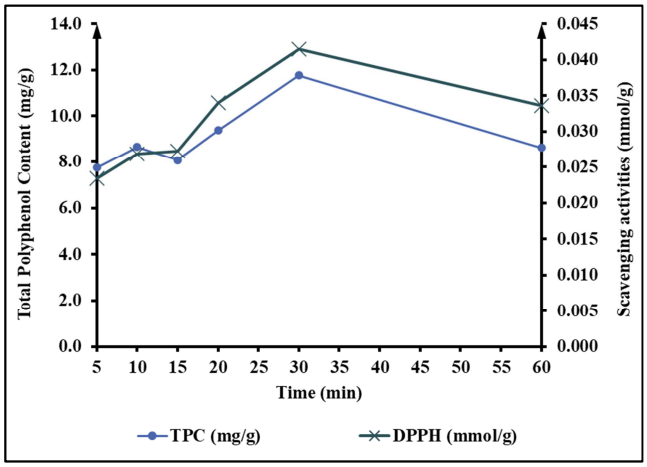

Figure 2. *Evolution curve of total polyphenol content and scavenging activity with DPPH· of the aqueous extract of Vernonia Amygdalina as a function of time.*

The optimum temperature being set at 80°C. By varying the extraction time, an increase in the polyphenol contents and in the antiradical activity with DPPH· as a function of time is observed ((Tables 4 and 5). These two indicators reach their maximum values after 30 min. Beyond this 30 minutes, they begin to decrease and steadily. From these experimental results, it can be concluded that the optimal extraction time of the bioactive molecules responsible for the antioxidant activity, contained in the aqueous extract of *Vernonia amygdalina* is 30 min.

3.2.3. Optimization of the Ratio Vegetal Mass/Solvent Volume

In this part, the solvent mass/volume ratio is varied while setting the temperature at 80°C and the extraction time at 30 min. The variations in the polyphenol content and in the antiradical activity with DPPH· are thus noted.

Table 6. *Evolution of the polyphenol content as a function of ratio.*

Mass	0.5			1.0			2.0		
Mass (mg)	0.5002			1.0003			2.0008		
Absorbance at 760nm	0.129	0.130	0.132	0.122	0.124	0.120	0.164	0.164	0.164
Mass (mg)	3.0			4.0			5.0		
Mass (mg)	3.0006			4.0004			5.0000		
Absorbance at 760nm	0.180	0.184	0.182	0.194	0.195	0.192	0.220	0.222	0.217

Table 7. *Evolution of the antiradical activity as a function of ratio.*

Mass (mg)	0.5			1.0			2.0		
Mass (mg)	0.5002			1.0003			2.0008		
Absorbance at 517 nm	0.966	0.964	0.961	0.966	0.968	0.969	0.935	0.935	0.936
Mass (mg)	3.0			4.0			5.0		
Mass (mg)	3.0006			4.0004			5.0000		
Absorbance at 517 nm	0.917	0.915	0.914	0.891	0.892	0.890	0.867	0.866	0.868

By varying the mass/volume ratio of solvent used, a reduction in the polyphenol content and in the antiradical activity beyond 1% is observed (Tables 6 and 7). These results show a better ratio at 1%, *ie*. it is necessary to dissolve 0.5 g of plant material in 50 mL of solvent for an optimal polyphenol content and antiradical activity.

The results obtained, illustrated in Figures 1, 2 and 3 reveal that the content of total polyphenols and the antioxidant activity of *Vernonia amygdalina* are optimal by dissolving the plant material in a solvent mass/volume ratio of 1%, for a period of 30 min and at a temperature of 80°C.

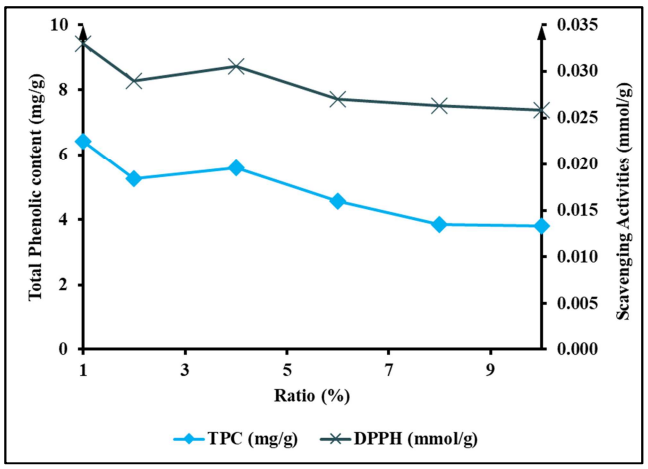

Figure 3. *Evolution curve of total polyphenol content and scavenging activity with DPPH· of the aqueous extract of Vernonia Amygdalina as a function of the solvent mass/volume ratio.*

3.3. Application in Optimal Conditions

3.3.1. Assay of Total Polyphenols and Flavonoids Contents in Aqueous Extract of Vernonia Amygdalina

In this part, the contents of polyphenols and flavonoids are determined, then the antiradical activity with DPPH· under the optimal extraction conditions determined previously. The extraction solvent used is water.

The results obtained are recorded in Table 8 below.

Table 8. *Content of total polyphenols and flavonoids in the aqueous extract of Vernonia Amygdalina.*

Total polyphenol content	10.536 ± 0.145mg/g
Flavonoid content	1.930 ± 0.043mg/g
Amount of antioxidants at DPPH·	4.356 ± 0.145mg/g

The results obtained show a very high content of polyphenolic compounds in the aqueous extract of *Vernonia amygdalina.* The work of Atangwho *et al.* [29] showed that the extracts of this plant inhibit the activity of DPPH· in a dose-dependent manner, with higher potencies in polar extracts compared to non-polar ones. These results are confirmed by our study which reveals a very high value in terms of the quantity of antioxidants with 4.356 ± 0.145 mg per gram of vegetable matter, with reference to Trolox.

3.3.2. Determination of Antiradical Activity by the DPPH· Method

The 50% inhibitory concentration noted IC_{50} indicates the concentration at which an active substance is capable of inhibiting a particular biological process by 50%. It gives a precise indication of the effectiveness of a drug. Indeed, the lower its value, the more active the substance. For this, we vary the concentration of the sample, and the corresponding absorbance, measured using the spectrophotometer, is noted. The values obtained, given in Table 9, and illustrated in Figure 4, make it possible to graphically determine the inhibitory power which is intrinsically linked to the IC_{50}.

Table 9. *Change in absorbance as a function of concentration.*

Concentration (mg/mL)	FD	Absorbance 1	Absorbance 2	Absorbance 3
0.004	10	0.931	0.932	0.933
0.009	5	0.889	0.892	0.895
0.011	4	0.873	0.876	0.879
0.015	3	0.843	0.841	0.843
0.022	2	0.790	0.790	0.790
0.044	1	0.588	0.589	0.590
White	0.059		control	0.913

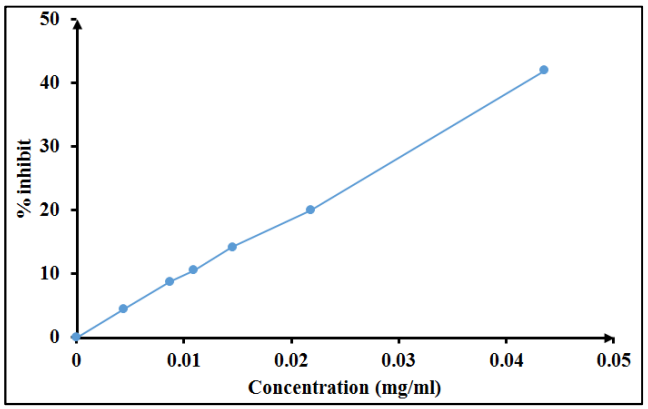

Figure 4. *Evolution curve of the percentage of inhibition according to the concentration.*

For a 30 minutes incubation, it is not possible to determine the IC_{50}. Indeed, the maximum inhibition is 41.95% during this time, for a concentration of 0.044 mg/mL. We proceeded to the kinetic study of the evolution of the reduction of DPPH·

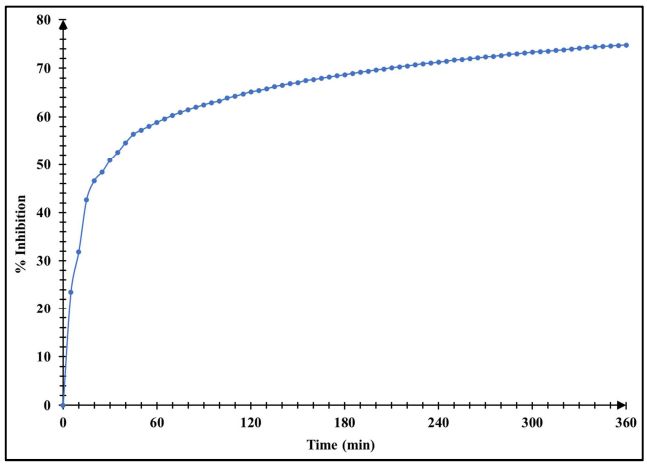

Figure 5. *Evolution of kinetics by DPPH·*

The results of the evolution of the kinetics of reduction illustrated in Figure 5 show that the power of inhibition increases as a function of time. However, the maximum value

of the inhibition is obtained at 300 min where the percentage of inhibition is almost constant and is equal to 74%.

The evolution of the inhibition power shows that the kinetics of reduction by DPPH· is slow because the IC_{50} is obtained for a duration of the order of 30 min.

4. Conclusion

In this work, we studied the impact of physico-chemical parameters such as extraction time, temperature, and vegetal mass/solvent volume ratio on the total polyphenols and flavonoids contents and on the antioxidant activity of the aqueous extract of *Vernonia amygdalina* leaves. The experimental results revealed that the optimal values are obtained at a temperature of 80°C, for 30 min and for a ratio of vegetal mass/solvent volume 1%. Under these conditions, the contents of total polyphenols and flavonoids are respectively equal to 10.536 ± 0.145 mg/g gallic acid equivalent and 1.930 ± 0.043 mg/g quercetin equivalent. The antioxidant activity study shows a maximum inhibition of 41.95% for a concentration of 0.044 mg/mL, for 30 minutes. The DPPH· reduction kinetics reveals an inhibitory power of 75% and a fairly slow reduction reaction. This antioxidant activity of *Vernonia amygdalina*, which increases with the content of phenolic compounds up to 80°C and for 30 min of extraction, constitutes a good indicator for the use of this plant in the management of numerous pathologies.

In view of the results obtained, we plan to deepen the study of the anticancer and antidiabetic activities of the aqueous extract of this plant by isolating the active principles by organic fractionation in order to test them in their pure state.

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