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Antioxidant activity study of *Mareya micrantha* leaves extracts using Differential pulse voltammetry

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Abstract

The rapid evaluation of antioxidant activities of plant extracts is of interest to many researchers in the field of phytochemistry. Electrochemical methods appear to be an alternative to the classic 2,2-diphenyl-1picryl-hydrazyl (DPPH) method, which is considered complex and constraining to be easily implemented. Indeed, using cyclic voltammetry has already been proved to be relatively easy to implement to assess the antioxidant activity of plant extracts with no interference from solvents. For this study, the main objective is the use of Differential Pulse Voltammetry (DPV) to determine antioxidant activities as an alternative electrochemical method for evaluation of antioxidant activities of plant extracts. So, raw extracts of *Mareya micrantha* leaves, as well as their fractions were used. Under the optimal use conditions of both methods, antioxidant activity and particularly minimal concentrations of analyte inhibiting 50% of radicals (IC_{50}) were evaluated. For crude extract then hexane, dichloromethane, and ethyl acetate fractions, the IC_{50} of the DPV method are 0.23 ± 0.00 mg/mL, 0.30 ± 0.00 mg/mL, 0.28 ± 0.00 mg/mL and 0.25 ± 0.02 mg/mL respectively while those of DPPH method are 0.39 ± 0.01 mg/mL, 0.56 ± 0.00 mg/mL, 0.51 ± 0.00 mg/mL and 0.40 ± 0.02 mg/mL respectively. The two analysis methods are strongly correlated with $R^2 = 0.98$.

1. Introduction

During storage, foodstuffs tend to deteriorate, changing appearance and losing their taste, smell, and nutritional qualities [1, 2]. To minimize these phenomena, the food industry has resorted to synthetic compounds, some of which are currently criticized for their toxic effects on humans and environment [3, 4]. Although some of these antioxidant compounds help to trap free radicals responsible for the oxidation of food, they are also involved in the appearance of many diseases such as asthma, cataracts, joint disorders as well as cardiovascular and degenerative diseases [5]. It is therefore topical to search for new antioxidants of natural origin and mainly from biomass, which are the basis of many drugs.

In the literature, many works [6-8] are devoted to the study of medicinal plants and focused on the extraction, identification, and quantification of bioactive compounds, particularly antioxidants. So, controlling the phenomenon of oxidation of biological systems in general and, of food in particular, using new effective antioxidants, environment-friendly and inexpensive, becomes vital. However, the main difficulty when evaluating the antioxidant activity of plant extracts remains the complexity and cost of the analyses to be carried out. Indeed, the DPPH method, considered to be the reference method for the antioxidant activity evaluation has several technical constraints, including the decrease in the

absorbance of alcoholic solutions of DPPH over time. It is dependent on polar solvents which influence the mechanisms of radical inhibition [9, 10].
Use of electrochemical methods such as cyclic voltammetry has already shown that these methods are up for evaluation of antioxidant activity of plant extracts without interference with solvents [11]. The aim of this work is to establish the ability of differential pulse voltammetry (DPV) as well as chemical method (DPPH) to evaluate the antioxidant activity of plant extracts. For this purpose, the optimal conditions for implementing the DPV as well as a comparative study of the two methods are necessary.

2. Material and methods

2.1 Treatment of plant material before analysis

Plant material consists of leaves of *Mareya micrantha* harvested in May 2017 in the village "GUEZEM" located in Gagnoa region (West-Center of Cote d'Ivoire). *Mareya micrantha* is Euphorbiaceae used in traditional medicinal to treat malaria, cancer of stomach, cough, and leprosy [12-15]. Some research confirms *Mareya micrantha* antibacterial, antifungal, and antimalarial activities [16-18]. This plant has been selected in relation to several research studies which showed that this plant extract has a strong antioxidant activity [19, 20]. The plant identification was done by a botanist at the National Polytechnic Institute Felix HOUPHOUËT-BOIGNY in Yamoussoukro (6°53'04.7'' North and 5°13'54.9''West) in Center of Cote d'Ivoire – west Africa). In the laboratory, leaves were spread-dried in the shade for two months at temperature between 27°C and 32°C. The dry leaves were crushed using an electric grinder and the powder obtained was sieved at 0.5 mm and then stored at 4°C until being used.

2.2 Raw extracts of Mareya micrantha leaves

The powder of leaves of *Mareya micrantha* was extracted three times, by stirred maceration for 24 hours of 100 g of powder in 500 mL of 70% ethanol [21]. After thorough filtration, the filtrates were evaporated under reduced pressure using a Büchi brand rotary evaporator at 50°C. The residue constitutes the crude extract (E_{HA}).

2.3 Fractionation of the raw extract of Mareya micrantha

For fractionation, 5g of the crude extract was dissolved in 100 mL of distilled water at 50°C. The cold aqueous solution underwent series of three cycles of liquid - liquid extraction using 30, 30 and 40 mL of different organic solvents. Fractionation was carried out with increasing polarity in the following order: hexane (H), dichloromethane (DM) and acetate ethyl (AE). Residue from organic fraction of each solvents constitutes the corresponding fraction codified respectively F_H, F_{DM} and F_{AE}.

2.4 Evaluation of antioxidant activity by the DPPH method

The determination of the antioxidant activity of the crude extract as well as of its fractions by DPPH was carried out according to the methodology described by Sene and al. [22]. A stock solution of DPPH was prepared by dissolving 25 mg of DPPH in 100 mL of methanol. In parallel, a range of solutions of concentrations 0.1 mg/mL; 0.01 mg/ml; 0.001 mg/mL and 0.0001 mg/mL for each analyte is prepared. For the evaluation of the antioxidant activity, 100 µL of analyte was added to 3.9 mL of stock solution of DPPH. After 30 min of incubation in the dark and at room temperature, the absorbance was measured using a UV / visible JASCO V-530 spectrophotometer at 517 nm, against a corresponding blank solution. Antioxidant activity (PI) is expressed using the following equation:

$$PI = \frac{A_O - A_1}{A_O} * 100$$

- A_0 is the absorbance of the solution of pure DPPH,
- A_1 is that of DPPH mixed with the analyte.

The IC_{50} , corresponding to the concentration of analyte essential for the inhibition of 50% of DPPH, was determined using a graph of evolution of the inhibition rate as a function of the analyte concentration.

2.5. Evaluation of antioxidant activity by the electrochemical method (DPV)

The use of an experimental device composed of a glassy carbon electrode, a platinum counter electrode, a saturated silver chloride reference electrode (Ag/AgCl/KClsat) and a potentiostat (910 PSTAT mini) of Metrohm type was required for the evaluation of the antioxidant activity by the DPV method. The manipulations were carried out at 25°C using an electrolyte composed of 15 mL of N, N-dimethylformamide (DMF) containing 0.1 M tetra-N-butylammonium hexafluorophosphate (TBuNPF6) as the carrier salt.

The tests were carried out according to the slightly modified protocol of Litwinienko and Ingold [10]. For this analysis, a range of electrolytes containing corresponding analyte (plant extract or ascorbic acid) with concentration of 0.073 g/L; 0.147 g/L; 0.220 g/L; 0.294 g/L was prepared for the tests. For each test, oxygen was bubbled through the electrolyte solution for 10 min before the voltammogram got recorded. When recording voltammograms, the slew rate was at 100 mV / s, the pulse amplitude at 17 mV, and the pulse duration at 30 ms.

The antioxidant activity is determined using the following equation:

$$I = \frac{I_{pa0} - I_{pas}}{I_{pa0}} * 100$$

- Ipa_0 is the intensities of the oxygen oxidation current before antioxidant added
- Ipas is the intensities of the oxygen oxidation current after the addition of the antioxidant.

The IC_{50}, corresponding to the concentration of analyte, essential for the inhibition of 50% of radicals, was determined using a graph of evolution of the inhibition rate as a function of the analyte concentration Y=ax +b, where Y is inhibition percentage and x represent analyte concentration.

2.6 Optimal conditions for the evaluation of antioxidant activity by the DPV

Carole B [23] demonstrated that three key factors influence the interfacial phenomena of electrodes during the implementation of electrochemical methods. In this study these factors were assessed for their influence. A full factorial design with the establishment of a factorial matrix using the Yates algorithm was used. Thus, a series of experiments was carried out with variation of the values of the three factors as indicated in **Table 1** below.

Table 1. Code and factor values

Code	FACTORS	VALUE (-1)	VALUE (+1)
X_1	Concentration in analyte	73 mg/L	400 mg/L
X_2	Oxygen bubbling time	5 min	15 min
X_3	Sweep speed	50 mV/s	100 mV/s

For conducting experiments, a **Table 2** of matrix has been established as indicated below. Basing on results from these experiments a mathematic model was developed and optimized by the Simplex

method using the Microsoft Office software Excel spreadsheet [24].

Table 2. Matrix of experiments

N° TEST	X_1 (mg/L)	X_2 (min)	X_3 (mV/s)
1	73	5	50
2	400	5	50
3	73	15	50
4	400	15	50
5	73	5	100
6	400	5	100
7	73	15	100
8	400	15	100

2.7. Correlation between DPV and DPPH methods

The correlation between the two methods was established graphically using a graph of evolution of IC_{50} obtained from the DPV method in function of the IC_{50} from the DPPH method. The linear regression coefficient estimated permit to judge the degree of correlation between the two methods.

2.8. Expression of results and statistical analysis

For the comparison of the two methods (DPPH and DPV), a Bland-Altman analysis was performed. For this, the difference between the two methods was compared to the average obtained from each of the two analysis techniques. Bias and limits of agreement provided the variation from one technique to another.

The method of Pareto was performed to determine the significant parameters which influence results of the DPV method. A factor is significant if its cumulative effect is less than or equal to 80%.

3. Results and Discussion

3.1 Antioxidant activity using DPPH method

The results of the antioxidant activity of the crude extract (E_{HA}) and the fractions (F_H, F_{DM}, F_{AE}) of *Mareya micrantha* as well as ascorbic acid (AA) using DPPH method are presented in **Table 3**. According to this table, crude extract and its hexane, dichloromethane and ethyl acetate fractions inhibit the DPPH radical at different concentrations. However, these inhibition rates are low compared to that of ascorbic acid. Moreover, at concentrations between 0.0001 and 0.01 mg/mL, only analyte from ethyl acetate fraction has an inhibition rate closed to that of the crude extract. But, at 1 mg/mL of concentration, the inhibition rates of all analytes are close to that of ascorbic acid what suggests that at high concentrations all analytes (crude and fractions) of *Mareya micrantha* are radical inhibitors like ascorbic acid. For a better comparison of the antioxidant activity of plant extracts to that of ascorbic acid, the concentration of analyte, essential for the inhibition of 50% of DPPH radicals (IC_{50}) was determined graphically. The IC_{50} of 0.39 ± 0.01 mg/mL; 0.56 ± 0.00 mg/mL; 0.51 ± 0.00 mg/mL; 0.40 ± 0.02 mg/mL and 0.24 ± 0.02 mg/mL were obtained for the crude extract, analytes of hexane, dichloromethane, ethyl acetate and ascorbic acid, respectively. According to the IC_{50} values, ascorbic acid remains the more powerful analyte to reduce DPPH radical than all the analytes from leaves.

Table 3. DPPH radical inhibition rate by the analytes

Analyte	DPPH radical inhibition rate according to analyte concentration				
	0.0001mg/mL	**0.001 mg/mL**	**0.01mg/mL**	**0.1mg/mL**	**1mg/mL**
E_{HA}	13.30±0.01	25.08±0.03	31.42±0.01	42.75±0.02	86.29±0.00
F_H	3.22±0.02	5.14±0.01	8.04±0.01	22.68±0.00	83.22±0.00
F_{DM}	8.22±0.02	10.61±0.02	15.57±0.01	28.42±0.03	84.54±0.00
F_{AE}	10.83±0.00	23.84±0.03	28.61±0.02	40.13±0.01	88.13±0.01
AA	18.77±0.03	32.77±0.03	40.32±0.01	60.75±0.02	93.69±0.00

The inhibition rate of ascorbic acid is 1.65 times stronger than the most powerful analyte from leaves of *Mareya micrantha*. The modest difference observed between antioxidant activities of crude extract and then fractions of *Mareya micrantha* leaves extracts suggests that the fractionation method used is not appropriate because it does not allow to correctly segregate the phenolic compounds from others, as they are generally responsible for the antioxidant activities of the plant extracts [25]. Moreover, depending on the substituents or on the phenolic systems (mono or polyhydroxylated), different organic solvents can extract various polyphenols as reported in divers works devoted to the antioxidant activities of phenolic compounds [26, 27]. The results of antioxidant activities of extracts from leaves of *Mareya micrantha* are similar to those of plant extracts used in the African pharmacopoeia such as *Palisota hirsuta, Mallotus oppositifolius, Ocimum gratissimum, Glyphaea brevis* [28]; *Nigella sativa L.* [29]; *Mitragyna ciliata, Trichilia prieuriana, Chrysophyllum perpulchrum* and *Disthemonanthus benthamianus* [30].

3.2 Evaluation of antioxidant activity by the DPV technique

The voltammograms recorded at 100 mV/s (**Figure 1**) show the inhibition of oxygen radicals by the crude extract in electrolytic solution in function of concentration. On the voltammograms, it can be noted that the intensity of the current decreases with the increase in the quantity of analyte introduced into the electrolyte solution.

This shows an inverse proportionality between the intensity of the current flowing through the electrolyte solution and the concentration of analytes in the electrolyte. **Table 4** shows the rate of radical inhibition as a function of the analyte concentration in the electrolyte.

According to table 4, crude extract and its hexane, dichloromethane and ethyl acetate fractions inhibit oxygen radical at different concentrations. DPV method can be used to measure the inhibition capacity at different concentration of different analytes.

The decrease in current intensity reflects the ability of analyte present in the electrolytic medium to trap or inhibit the oxygen radical. Indeed, the superoxide anion radical ($O_2^{-\bullet}$) Was generated by reduction of oxygen at the working electrode according to this reaction: $O_2 + e^- \rightleftharpoons O_2^{-\cdot}$

According to Mickaël and al [31], the superoxide anion radical ($O_2^{-\cdot}$) is oxidized during the backwash (reverse reaction) and the intensity of the oxidation current is proportional to the concentration of oxygen in the electrolytic medium. The superoxide anion radical is therefore responsible for the initiation of oxidation [32].

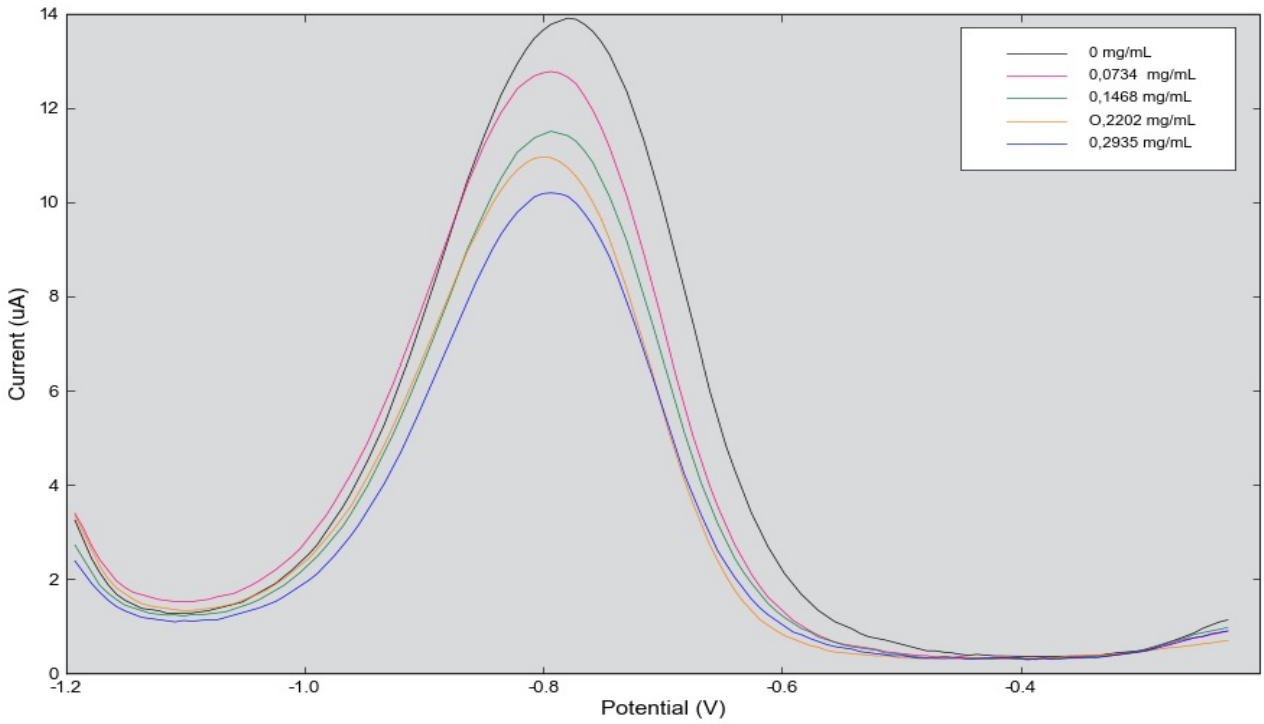

Figure 1. Voltammograms of inhibition of oxygen radicals by the crude extract

So, the decrease in intensity of the current reflects the trapping or inhibition of this radical in the medium according to reaction: $O_2^{-\cdot} + AH \rightarrow O_2H^- + A^{\cdot}$

Table 4. Oxygen radical inhibition rate by analytes using DPV method.

Analyte	Oxygen radical inhibition rate according to analyte concentration			
	0.0734mg/mL	**0.1468 mg/mL**	**0.2202mg/mL**	**0.2935 mg/mL**
E_{HA}	23.48±0.01	32.35±0.03	50.59±0.00	60.19±0.00
F_H	0.91±0.00	17.23±0.00	30.18±0.00	40.00±0.01
F_{DM}	11.18±0.01	19.84±0.00	38.11±0.01	54.22±0.01
F_{AE}	18.10±0.00	27.66±0.00	45.81±0.01	58.19±0.00
AA	24.19±0.01	38.06±0.01	60.22±0.01	75.11±0.00

The IC_{50} in each case is 0.23 ± 0.00 mg/mL; 0.30 ± 0.00 mg/mL; 0.28 ± 0.00 mg/mL; 0.25 ± 0.02 mg/mL and 0.19 ± 0.00 mg/mL respectively for the crude extract, the hexane, dichloromethane, ethyl acetate and ascorbic acid fractions. According to these numbers, all analytes from leaves of *Mareya micrantha* possess an inhibition capacity. However crude extract has antioxidant activities closed to those of ascorbic acid. This can be correlated to the degree of purity of these analytes as different organic solvents can extract various polyphenols systems that could possess different antioxidant activities [27].

At high concentration, the antioxidant activities measured by the DPV method do not tend to be close to those of ascorbic acid as has been shown when using the DPPH method. This may be due to the difference in sensitivity of these methods.

3.3 Comparative study of chemical and electrochemical methods

To compare both methods: electrochemical method (DPV) and chemical method (DPPH) for evaluation of antioxidant activity, a correlation curve was plotted between the IC_{50} of these methods (**Figure 2**).

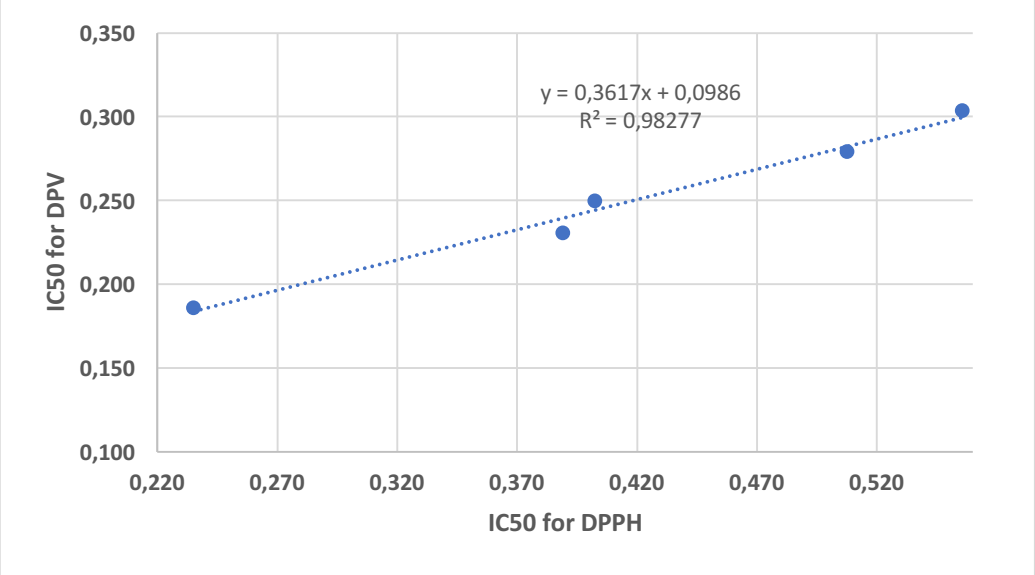

Figure 2. Correlation between the IC_{50} of both methods

According to this figure, a correlation between the two analytical methods can be established with a coefficient of determination of $R^2 = 0.98$. The statistical analysis of the concordance of the antioxidant activities given by the two techniques was done by the test of Bland and Altman. The standard deviation of 0.079 showed that at 95% the limits of concordance are evaluated by 0.01g/L and 0.32g/L. Therefore, there is a similarity of responses between chemical (DPPH) and electrochemical (DPV) methods when assessing antioxidant activity. In addition, the IC_{50} of the electrochemical method are significantly lower than those of the chemical method. This result could be linked to the absence of complexation phenomena observed in chemical method. This constitutes a serious advantage for the DPV method for determining the antioxidant activity of plant extracts as the extraction yields of plants materials are often low. The use of small quantities of plant extracts for analyses is in evidence a factor to boost studies of active substances from plants.

In electrochemistry, the interfacial phenomena constitute the main difficulty in the repeatability of the results. It is therefore essential to determine the optimal conditions for implementing the electrochemical method. For this, a complete factorial plan was chosen to conduct the experiments.

3.4. Optimal conditions for DPV method

According to the literature, three factors have a potential influence on the efficiency of electrochemical methods, in particular cyclic voltammetry. These factors were evaluated for the DPV method. The results of this assessment are summarized in **Figure 3**. It appears that depending on the different analytical conditions responses of the antioxidant activity vary between 31% and 58%, what confirms that the DPV method is strongly influenced by external factors. However, the best responses were obtained when the oxygen solution enrichment time was 15 minutes. Thus, the first factor that governs the response of the DPV method appears to be the oxygen content in electrolytic solution.

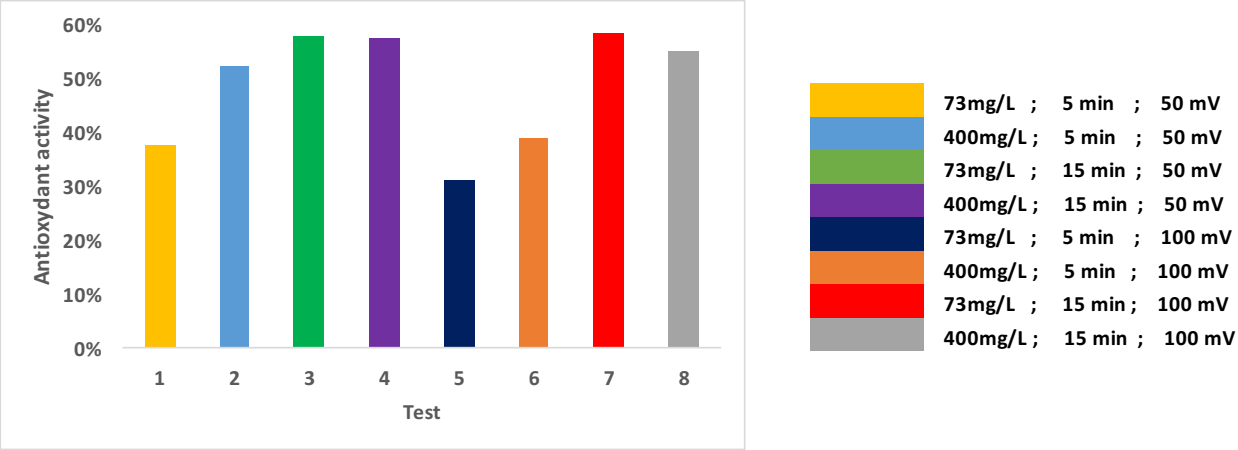

Figure 3. Inhibition of radicals by the crude extract depending on the operating conditions

3.4.1. Estimation of the effects of factors on the electrochemical method

Using the Microsoft Office Excel and setting a confidence level at 95%, α = 0.05 a multiple linear regression of the results obtained was carried out. **Table 5** presents the effects of the factors on the expected responses using the electrochemical method and their significance.

It can be seen from the table that the results of the antioxidant activity are influenced by the factors as the concentration of extract to be analyzed, the oxygen bubbling time in the solution and the scanning speed of the potentials. Also, the combined effect of the concentration of plant extract and the duration of oxygen bubbling (X_1X_2) in the solution remain the most important factors to control. The mathematical model describing the response to the use of the DPV method is described by equation: $Y = 0{,}486 + 0{,}023X_1 + 0{,}086X_2 - 0{,}027X_3 - 0{,}032X_1X_2$. But it must be validated before its use.

Table 5. Estimation of the effects of significant coefficients according to Pareto

Coefficient	Value	Effect	Cumulative effect	%	% cumulative	Decision
(X_2)	0,086	0,086	0,086	41,56	41,56	Significant
($X_1.X_2$)	-0,032	0,032	0,118	15,61	57,18	Significant
(X_3)	-0,027	0,027	0,145	13,19	70,36	Significant
(X_1)	0,023	0,023	0,168	11,22	81,58	Significant
($X_2.X_3$)	0,022	0,022	0,190	10,63	92,21	Insignificant
($X_1.X_3$)	-0,012	0,012	0,202	5,63	97,84	Insignificant
($X_1.X_2.X_3$)	0,004	0,004	0,206	2,16	100,00	Insignificant

3.4.2. Validity of experimental model

Based on the results which led to the mathematical model, the optimal condition of using the DPV method to evaluate the antioxidant activity depends on three factors, the main two being the content of extract of the electrolyte solution (73 mg/L) and the oxygen bubbling time (15 min). To evaluate the validity of this model, a correlation between experimental data and those from model (**Figure 4**) was revealed.

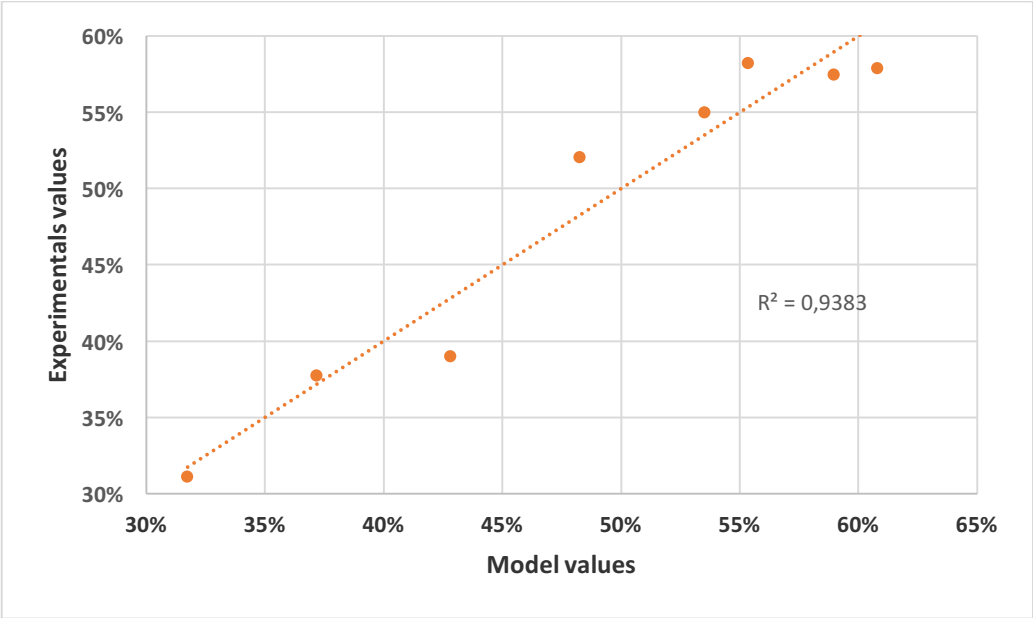

Figure 4. Correlation line between the experimental data and the model

Figure 4 shows that a linear regression between the theoretical and experimental inhibition rates can be established with the coefficient of $R^2 = 0.94$. Such a model is quite suitable and can be a model to a good prediction of the results of analysis by the DPV method [33, 34]. These results confirmed those of the comparative study of the two methods to assess the antioxidant power of plant extracts. This makes the DPV method a potential reliable alternative in predicting the antioxidant activity of plant extracts.

Conclusion

The 2,2-diphenyl-1picryl-hydrazyl (DPPH) and pulsed differential voltammetry (DPV) methods permitted measuring the antioxidant activity of the crude extract and fractions of leaves of *Mareya micrantha*. A linear correlation ($R^2 = 0.98$) was established between the two methods thus implying that there is a similarity of responses obtained by the chemical method (DPPH) and the electrochemical method (DPV) when evaluating antioxidant activities. But, based on the IC_{50} values, the electrochemical method (DPV) seems to be more suitable than that of DPPH for analyzing natural substances with low extraction efficiency.

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