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Kinetics of the reaction between *Moringa oleifera* leaf extracts and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical†

Cinética de la reacción entre extractos de hojas de *Moringa oleifera* y radical libre 2,2-difenil-1-picrilhidrazilo (DPPH)

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ABSTRACT

In this study, an antioxidant-rich extract was obtained by leaching *Moringa oleifera* leaves. The activity of the extract against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was measured by determining a second-order rate constant (k_2). The reaction between the mixture of antioxidants in the extract and the DPPH was monitored by measuring the absorbance at 517 nm in 30-second intervals for a period of 600 s. The absorbance exhibited a rapid decay within the first 100 s, followed by a slower decay. The absorbance data were fitted to a bi-exponential model. The curve fitting showed a linear relationship between the initial concentration of antioxidants and the reaction rate with the coefficient of determination values greater than 0.9979. The k_2 value was 0.0870 ± 0.0007 mg/mL.

Keywords: *Moringa oleifera*; Leaching; Bi-exponential model; Rate constant

RESUMEN

En este estudio, se obtuvo un extracto rico en antioxidantes mediante la lixiviación de hojas de *Moringa oleifera*. La actividad del extracto frente al radical libre difenil-1-picrilhidrazilo (DPPH) se midió determinando una constante de velocidad de segundo orden (k_2). La reacción entre la mezcla de antioxidantes en el extracto y el DPPH fue monitoreada midiendo la absorbancia a 517 nm en intervalos de 30 s durante un período de 600 s. La absorbancia exhibió un decaimiento rápido dentro de los primeros 100 s, seguido por un decaimiento más lento. Los datos de absorbancia se ajustaron a un modelo bi-exponencial. El ajuste de la curva mostró una relación lineal entre la concentración inicial de antioxidantes y la velocidad de reacción con valores del coeficiente de determinación superiores a 0.9979. El valor de k_2 fue de 0.0870 ± 0.0007 mg/mL.

Palabra claves: *Moringa oleifera*; Lixiviación; Modelo bi-exponencial; Constante de velocidad

† In honour and loving memory of *Prof. em. Dr.-Ing. habil. Luis Moreno* (1942-2022).

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1. INTRODUCTION

Moringa oleifera Lamarck (*Moringa oleifera* Lam.) is a deciduous tree of the family *Moringaceae* and is thought to be native to north-western India (Olson, 2017). *M. oleifera* is one of 13 species within the same genus and the most widely grown species, cultivated around the word for a variety of products. Almost all parts of the tree (e.g., leaves, fruits, flowers, immature pods, roots) contains bioactive constituents and nutrients.

M. oleifera is considered a significant source of *β*-carotene, vitamin A, B, and C, minerals, and phytochemicals that exhibit a demonstrated antioxidant activity. In addition, *M. oleifera* extracts exhibit significant pharmacological activities against inflammation, diabetes and hyperglycaemia, cancer, and neurodegeneration diseases, amongst others (Martínez *et al*., 2017; Nobossé *et al*., 2018; Xiao *et al*., 2020).

Generally, bioactive constituents and nutrients have been extracted from *M. oleifera* leaves using a variety of chemical processes. The most common process is the solid-liquid extraction that uses an ethanol-water mixture, followed by evaporation and drying (Valdés-Hernández *et al*., 2015). The *M. oleifera* extracts are employed for several applications within the food and pharmaceutical industries due to their potent antioxidant activity against free radicals (Falowo *et al*., 2017; Oswell *et al*., 2018).

Antioxidants are compounds or systems that retard auto-oxidation by inhibiting free radical formation or by disrupting free radical propagation in one (or more) of several mechanisms (e.g., scavenging of peroxidation-initiating species). The most employed synthetic antioxidants are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Villanueva *et al*., 2017).

Reaction kinetics of antioxidants is important since free radicals have a short half-life. Consequently, the kinetics of free radical degradation due to antioxidant action is useful for biotechnology and food industries.

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay is the most common method used for the assessment of antioxidant capacities. This method is stable, easy to perform, and commercially feasible compared to other methods. The assay is based on the reduction of DPPH radical by receiving hydrogen atoms from a scavenger compound, which changes the sample colour from violet to yellow (Ahmad *et al*., 2018). The reaction can be represented as follows:

$$\left[\mathrm{DPPH}^{\bullet}\right]+\left[\mathrm{AOH}\right]\rightarrow\left[\mathrm{DPPH}-\mathrm{H}\right]+\left[\mathrm{AO}^{\bullet}\right] \quad (1)$$

Kinetic data are obtained by measuring the decrease in absorbance (515-520 nm) as a function of time to subsequently calculate the rate constant (Guija-Poma *et al*., 2015). Each antioxidant group has different reaction kinetics. In the case of the *M. oleifera* leaves extract, vitamin C reacts faster than vitamin E and other constituents. Therefore, it is possible to verify a rapid decay (related to vitamin C), followed by a slower decay (corresponding to compounds with slow kinetics) in the concentration versus time curve. In most cases, reaction kinetics of $DPPH^{\bullet}$ and antioxidants can be adjusted by a mono-exponential model (Guija-Poma *et al*., 2015). However, in other cases, reaction kinetics of $DPPH^{\bullet}$ and some antioxidants follows a bi-exponential model (Foti *et al*., 2016). Bi-exponential behaviour can be caused by many factors, such as the existence of two steps in the dimerization reaction or a mixture of compounds.

In this study, the DPPH free radical degradation kinetics due to the action of antioxidants from *M. oleifera* leaves was investigated. The activity of the antioxidants against DPPH free radical was measured by determining a second-order rate constant.

2. MATERIALS AND METHODS

2.1 Material

M. oleifera leaves were collected from trees grown within the National University of Engineering (UNI), Nicaragua. The collected leaves were healthy and uninfected. Leaves, excluding petioles, were washed with distilled water to eliminate dust and other foreign particles and to cleanse the leaves thoroughly and drained before experimentation.

2.2 Chemicals

All the chemicals, reagents, and solvents used in the study were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Co. (St Louis MO, USA). Ethanol absolute was purchased for analysis and leaching step from EMD Millipore Co. (Billerica MA, USA) and J.T. Baker® Chemical Co. (Center Valley PA, USA), respectively.

2.3 Experimental apparatus

Solid-liquid extraction of leaves was performed in a Julabo® Shaking Water Bath model SW-23. Vacuum evaporation was performed in a Büchi Rotavapor model R-124 connected with a Büchi Vac® model V-500 Vacuum Pump.

A Hach® DR 5000™ UV-Vis Laboratory Spectrophotometer was used to measure absorbance for the free radical inhibition method employing DPPH at 517 nm to determine kinetic data.

2.4 Experimental procedure

100 grams of leaves were introduced into one flask containing 1000 mL each of a mixture of 35% ethanol (v/v) in distilled water. Solid-liquid extraction of leaves was performed for two hours at 50 °C. After the extraction process, the leaves extract was separated from the solids using a Büchner flask and funnel along with a vacuum pump. For the leaves extract, the concentration of the solids in the liquid extract was calculated, and the obtained value was 10.40 mg/mL after vacuum evaporation of the samples in the rotary evaporator.

2.5 Kinetic data

Several studies have shown that the reaction between DPPH and antioxidants occurs under first-order conditions. Kinetic analysis was performed by measuring the absorbance as a function of time and fitting the data to a mathematical model. After determining the fitting parameters, the second-order rate constant (k_2) can be calculated (Guija-Poma *et al*., 2015).

For obtaining the kinetic data, free radical and leaves extract solutions in ethanol were prepared as follows:

A 0.025 mg/mL solution of DPPH in ethanol was prepared by adding 2.5 mg of DPPH to an aliquot of 100 mL of an ethanol solution. Then, four solutions of leaves extract were prepared. The solution concentrations were: 0.2300, 0.1370, 0.0920, and 0.0462 mg/mL.

DPPH reacted with the leaves extract in a square glass cuvette of 10 mL with a ratio of 9.20:1, 5.48:1, 3.68:1 and 1.85:1 (g of DPPH per g of leaves extract).

The absorbance was monitored at 517 nm by measurements at 30-second intervals for 600 seconds. All measurements were performed in triplicate at room temperature.

2.6 Calculation of k_2 constant

The kinetic analysis was performed to observe the reaction behaviour that occurs between the extract of *M. oleifera* leaves and DPPH, as well as to determine the reaction rate. According to Leal Alturo (2012), the rate law for the reaction between an antioxidant and DPPH follows a second-order reaction kinetics as shown in Eq. (2):

$$-\frac{d\left[\mathrm{DPPH}^{\bullet}\right]_t}{dt} = k_2\left[\mathrm{AOH}\right]_t\left[\mathrm{DPPH}^{\bullet}\right]_t \tag{2}$$

where k_2 corresponds to the second-order rate constant. [DPPH$^{\bullet}$] and [AOH] are the molar concentrations of DPPH and the mixture of antioxidants, respectively.

The concentration of antioxidants was considered constant over time, i.e. [AOH] >> [DPPH$^{\bullet}$]. Therefore, based on the fundamentals of chemical kinetics, it is possible to assume that the reaction follows a pseudo-first-order kinetics as indicated in Eq. (3):

$$-\frac{d\left[\mathrm{DPPH}^{\bullet}\right]_t}{dt} = k_{obs}\left[\mathrm{DPPH}^{\bullet}\right]_t \tag{3}$$

where k_{obs} corresponds to the pseudo-first-order rate constant observed for the reaction at temperature T.

Data fitting of absorbance versus time and their respective statistical analyses were performed using MATLAB®. Subsequently, the rate constant (k_2) was calculated according to Foti *et al*. (2016), in which they used a bi-exponential model to adjust the data. The modified model is shown in Eq. (4) as follows:

$$A_t = \alpha\exp\left(k_{obs1}\cdot t\right) + \beta\exp\left(k_{obs2}\cdot t\right) \tag{4}$$

where A_t is the absorbance as a function of time (t), α and β are constants, and k_{obs1} and k_{obs2} are pseudo-first-order rate constants.

3. RESULTS AND DISCUSSION

3.1 Absorbance

The absorbance data (absorbance versus time) are shown in Fig. 1. As observed, there is a rapid decay in the first 100 seconds, followed by a slower decay. This fact occurs due to the mixture of different antioxidant compounds and the reaction rate for each one.

Martínez *et al*. (2017) quantified the presence of vitamin C in the extract and reported that its antioxidant efficiency was higher than the other compounds. This fact indicates that the initial decay is caused mainly by the reactivity of vitamin C.

Initially, the absorbance data were fitted to the mono-exponential model without reaching a good fit. Secondly, the absorbance data were fitted to the bi-exponential model, represented in Eq. (4), and a good fit was obtained. The average fitting parameters for the three replicates are shown in Table 1.

Table 1 shows the four constants of the bi-exponential model together with the coefficient of determination (r^2). It is important to highlight that the initial absorbance is represented in the model by the sum of α and β. In addition, all the coefficients of determination for the different concentrations of the extract, including replicates, were greater than 0.9983, thus indicating a good data fit. Besides, it can be observed that the values of the pseudo-first-order constants k_{obs1}, responsible for the initial decay, decrease with the decrease of the initial concentration. According to this model, k_{obs1} is always greater than k_{obs2}.

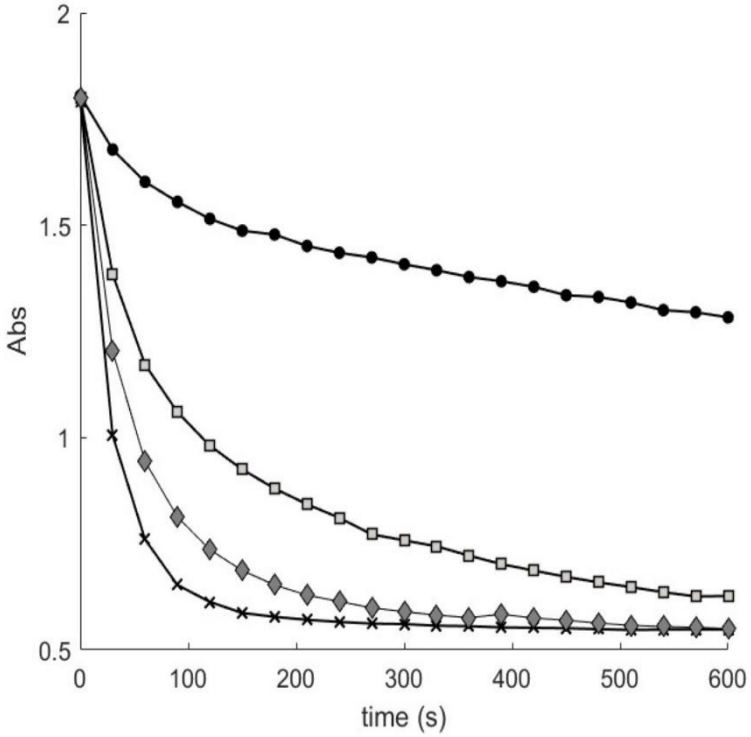

Fig. 1 Kinetic curve for DPPH free radical vs. leaves extract at different antioxidant concentrations: (×) 0.2300 mg/mL; (◊) 0.1370 mg/mL; (□) 0.0920 mg/mL; (•) 0.0462 mg/mL

Table 1 Results of data fitting

Coefficient	Concentration (mg/mL)			
	0.2300	0.1370	0.0920	0.0462
α	1.1950	1.1183	0.8099	0.2592
β	0.5930	0.6727	0.9810	1.5450
k_{obs1}	-0.0339	-0.0227	-0.0198	-0.0184
k_{obs2}	-0.0001	-0.0004	-0.0008	-0.0003
r^2	0.9993	0.9984	0.9984	0.9993

According to Fig. 1, it is assumed the existence of two groups of antioxidants. The first group causes the first decrease with the rate constant k_{obs1}. The second group controls the reaction for a longer time with the rate constant k_{obs2} that is less than k_{obs1}. Considering that $k_{obs1} >> k_{obs2}$ and the second term of Eq. (4) tends to one, k_{obs} can be calculated as shown in Eq. (5):

$$k_{obs} = k_{obs1} \tag{5}$$

3.2 Calculation of k_2 constant

The second-order constant k_2 can be estimated by combining Eqs. (2) and (3). Considering that the concentration of antioxidants remains constant throughout the reaction (i.e., $[AOH]_t = [AOH]_0$) and equalling the right terms of Eqs. (2) and (3), the Eq. (6) was obtained

$$k_{obs}\left[\mathrm{DPPH}^{\bullet}\right]_t = k_2\left[\mathrm{AOH}\right]_0\left[\mathrm{DPPH}^{\bullet}\right]_t \quad (6)$$

which can be simplified to Eq. (7) as follows

$$k_{obs} = k_2\left[\mathrm{AOH}\right]_0 \quad (7)$$

Equation (7) represents a solution for the determination of k_2. The four k_{obs} values corresponding to the four initial concentrations of leaf extract were plotted, as shown in Fig. 2, and fitted to the following linear model, represented by Eq. (8) as follows:

$$k_{obs} = \mathrm{p}_1\left[\mathrm{AOH}\right]_0 + \mathrm{p}_2 \quad (8)$$

The k_{obs} and $[AOH]_0$ data showed a good fit concerning the linear model represented by Eq. (7). The coefficients of determination (r^2) for all replicates were greater than 0.9393.

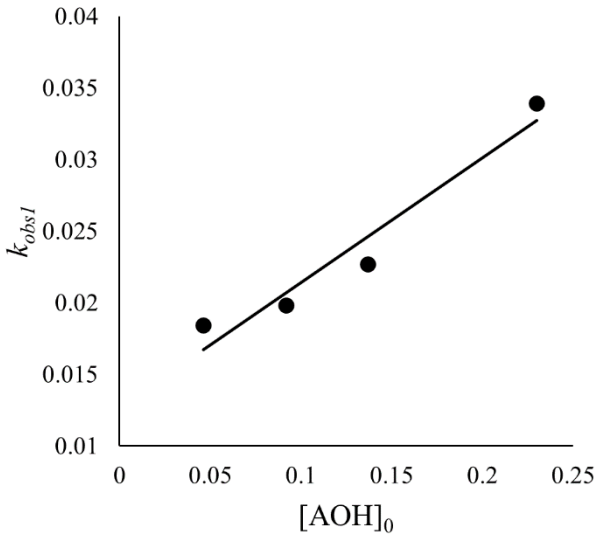

Fig. 2 Pseudo-first-order rate (k_{obs}) constants vs. initial concentrations of antioxidants from *M. oleifera* leaves extract

Figure 2 shows a good correlation between the k_{obs} values and the initial antioxidant concentrations, and according to Eq. (8), p_1 is the angular coefficient of the straight line shown in Fig. 2, which is equal to k_2 with a value of 0.0870 ± 0.0007 mg/mL. It is important to highlight that this result was the average of three values.

Table 2 shows the coefficients of the linear and bi-exponential models calculated with a confidence level of 95%. According to the lower and upper limits of each value, it is known that the coefficients of both the bi-exponential and linear models are different from zero, which is significant. It is important to note that

Table 2 shows α, β, k_{obs1}, and k_{obs2} coefficients for the reaction between DPPH and the antioxidant concentration at 0.2300 mg/mL. In addition, p_1 and p_2 values showed within the same table belong to one of the three replicates.

Table 2 Confidence bounds for fitted coefficients

Coefficient	Value	Lower bound	Upper bound
α	1.1950	1.1740	1.2150
β	0.5936	0.5816	0.6055
k_{obs1}	-0.0339	-0.0353	-0.0325
k_{obs2}	-0.0001	-0.00021	-0.00010
p_1	0.0853	0.0227	0.1478
p_2	0.0132	0.0042	0.0222

Table 3 shows the statistical evaluation of the data fits for the two models. The r^2 values for both models indicate a good fit of the data. Besides, the sum of squared estimate of errors (SSE) and the root-mean-squared error (RMSE) values are quite close to zero, thus indicating that the model is quite useful for predicting new values.

Table 3 Goodness of fit for linear and bi-exponential models

Parameter	Linear model	Bi-exponential model
SSE	7.792×10^{-6}	1.148×10^{-3}
r^2	0.9451	0.9993
RMSE	1.974×10^{-3}	8.219×10^{-3}

According to Villanueva-Tiburcio *et al*. (2010), k_2 value for BHT was 0.05 mg/mL, less than present *M. oleifera* extract k_2 value. These results and other antioxidant k_2 values can be sorted from highest to lowest, depending on the reactivity of the compounds: semi-ripe camu-camu peel > green tea > vitamin E > ripe camu-camu peel > green camu-camu peel > flavones > BHA > *Moringa oleifera* leaf extract > BHT.

4. CONCLUSIONS

The kinetic analysis of the reaction between the DPPH free radical and the antioxidant mixtures, extracted from *Moringa oleifera* leaves, was performed by determining a second-order rate constant (k_2). The analysis demonstrated that the decrease in DPPH concentration can be represented by a bi-exponential model, which includes two pseudo-first-order rate constants associated with two groups of antioxidants with different reaction rates. The k_2 value was 0.0870 ± 0.0007 mg/mL. This result indicates that *Moringa oleifera* leaves can be used as an antioxidant source, which exhibits a good reactivity profile, with a high potential within the food and pharmaceutical industries, such as BHT and BHA.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

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