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Kinetics of the Antioxidant Activities of *Solanum macrocapon* and *Crassocephalum rubens* by DPPH Radical Scavenging Method

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ABSTRACT

Kinetics of the antioxidant activities of two edible vegetables; *Solanum macrocapon (S.macrocapon)* and *Crassocephalum rubens* (*C.rubens*) was investigated. The methanolic extracts of the samples were tested with 2,2-diphenyl-1-picrylhydrazyl (DPPH) at different times (30, 50, 70 and 90 minutes). UV-Visible Spectroscopy technique was employed to evaluate the ability of the plant extracts to scavenge DPPH radicals by measuring the absorbance at the various experimental times. The concentration of sample required to inhibit 50 % of the DPPH free radical (IC_{50}) and kinetic parameter (rate constant k_2) were determined from the absorbances values. *S.macrocapon* and *C.rubens* had DPPH scavenging potency with IC_{50} values of 2.18 x 10^{-2} $mgml^{-1}$ and 6.27 x 10^{-2} $mgml^{-1}$ respectively. This implied that *S.macrocapon* is a more potent antioxidant than *C.rubens*. The rate constant for the hydrogen atom abstraction by DPPH (k_2) in the presence of *S.macrocapon* is 2.70 x 10^{-3} ± 0.0006 $mlmg^{-1}min^{-1}$ with R^2 value of 0.709 while for *C.rubens*, the rate constant is 6.89 x 10^{-4} ± 0.03 x 10^{-4} $mlmg^{-1}min^{-1}$ with R^2 value of 0.987 using Pseudo-first order kinetics model. However, under second order kinetics, the rate constant, k_2, for *S.macrocapon* is 4.73 x 10^{-1} ± 0.020 $mM^{-1}min^{-1}$ with R^2 value of 0.993 while *C.rubens* has k_2 value of 5.55 x 10^{-2} ± 0.00236 $mM^{-1}min^{-1}$ with R^2 value of 0.795. Thus, the depletion of DPPH by *S.macrocapon* followed a second order kinetics while that of *C.rubens* followed a Pseudo first order kinetics.

Keywords: Kinetics, DPPH, Antioxidant, UV-Vis spectroscopy, IC_{50}

Introduction

Free radicals (known as electrically charged molecules) have unpaired electrons which make them attractive to electrons from other substances to neutralize themselves. These free radicals in the presence of reactive oxygen species play a vital role in the pathogenesis of many diseases such as ischemic heart disease, atherosclerosis, cancer, etc [1, 2]. A free radical is defined as a molecule or molecular fragments containing one or more unpaired electrons in its outermost atomic or molecular orbital and is capable of independent existence [3, 4]. Most biologically relevant free radicals are derived from oxygen and nitrogen and their corresponding reactive oxygen species (ROS) and reactive nitrogen species (RNS). Common examples of ROS are hydrogen peroxide, singlet oxygen, hydroxyl radical, nitric oxide radical, superoxide anion, hypochlorite radical and various lipid peroxides while peroxynitrite radical, nitrogen dioxide radical and nitrogen trioxide radical are examples of RNS [5, 6].

Living organisms are capable of producing various compounds as a part of their antioxidant defence. Naturally occurring antioxidants include a range of enzymes (for example superoxide dismutase, gluthatione peroxidase), iron-binding proteins (for example transferrin, lactoferrin), vitamins C, and E; carotenoids, flavonoids and other plant phenolics [7]. There exist also numerous synthetic antioxidants such as gluthatione donors, superoxide dismutase and catalase mimetics, derivatives of vitamins C and E, N-acetyl-cysteine, xanthine oxidase inhibitors and various lipid-soluble chain breaking and transition metal binding compounds [8].

Many plants including vegetables have been categorized as sources of natural antioxidants that can protect against oxidative stress, thus, play an important role in the chemoprevention of diseases that have their aetiology and patho-physiology in reactive oxygen species [9 – 13]. Some compounds possessing antioxidant activity are in clinical use for the treatment of various conditions, although they were not primarily developed as antioxidants [7]. For example; sulphasalazine and its active metabolite, 5-aminosalicylic acid used in the treatment of inflammatory bowel diseases exert free radical scavenging activity [14]. Reactive species formed from sulphasalazine and 5-aminosalicylic acid oxidation can be scavenged by vitamin C.

Although, there have been several investigations into the radical scavenging activities of some plants as natural sources of antioxidants [15-19]. However, there are still large gaps to be filled in the studies and the understanding of the kinetics and mechanisms of action of these natural sources of antioxidants. Phenolics from red sweet potato and purple corn have been reported to exhibit faster reaction kinetics than phenolics from blue berries and that phenolics from red sweet potato also react faster to stabilize DPPH radicals as compared to Trolox, which is a powerful antioxidant [20]. Phenolic antioxidants have also been shown to react slowly with DPPH reaching steady state in one to six hours or longer [21]. This suggests that antioxidant activity using DPPH should be evaluated over time. In a study carried out on tropical fruits, it was reported that all the ten fruits presented different kinetic periods [22].

This present study investigates the kinetics of the free radical scavenging capacity of rag leaf (*c.rubens*) and egg plant (*S.macrocapon*) using 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH) to evaluate their effectiveness as source of antioxidant and to understand the kinetics of their antioxdant activities.

Materials and Methods

Plant collection

S.macrocapon and *C.rubens* leaves were sourced and purchased in their fresh form from a popular market in Ilorin East Local Government Area of Kwara State, Nigeria at their commercially marketable maturity. These plants were then verified and authenticated in the herbarium of Plant Biology Department, University of Ilorin with voucher number UILH/001/1063 and UILH/001/1062 respectively.

Preparation of plant extracts

The samples were rinsed with distilled water; air dried at room temperature to contant weight and then pulverized. 100 g of powdered leaf material was macerated with 1.0 litre of methanol for 5 days. The methanol extract was then filtered using Whatman filter paper and concentrated under vacuum at 40 °C using a rotary evaporator (Buchi rotavapor R-124). Finally, the crude extracts obtained were stored at 4 °C prior to antioxidant tests.

Calibration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

0.001 g of DPPH was dissolved in 100 ml of methanol to give a stock solution of 2.54 x 10^{-2} mM. Serial dilutions were made to obtain concentrations of 1.27 x 10^{-2}, 6.35 x 10^{-3}, 3.18 x 10^{-3}, 1.59 x 10^{-3}, 7.95 x 10^{-4} and 3.98 x 10^{-4} mM. Absorbance of the various concentrations was measured at wavelength

of 517 nm as the wavelenght of maximum absorbance using a UV-Vis Spectrophotometer (Beckman coulter). A graph of absorbance against concentration was plotted and the slope of the graph represents the molar absorptivity of DPPH.

DPPH free radical scavenging assay

The antioxidant potential of the methanolic crude extracts was determined on the basis of their scavenging activity of the stable DPPH free radical using the method described in literature with modifications [23]. The stock solutions of the extracts were prepared to the concentration of 1.00 $mgmL^{-1}$. Dilutions were made to obtain 5.0 x 10^{-1}, 2.5 x 10^{-1}, 1.25 x 10^{-1}, 6.25 x 10^{-2}, 3.13 x 10^{-2}, 1.56 x 10^{-2}, 7.81 x 10^{-3}, 3.91 x 10^{-3} and 1.95 x 10^{-3} $mgmL^{-1}$. 2 mL each of the diluted solutions, was mixed with 2 mL of DPPH methanolic solution of concentration of 2.542 x 10^{-2} mM. The mixtures were then vigorously shaken and allowed to stand at room temperature for 30, 50, 70 and 90 minutes in the dark. The absorbances of the mixtures were measured at 517 nm using a UV–Vis spectrophotometer (Beckman coulter). A mixture of the DPPH solution and methanol only was used as the control. The results were obtained in triplicate and mean values were used for further calculation. The scavenging activity of the DPPH radical was expressed as percentage inhibition using the following equation (19):

$$\%\ Inhibition = \left[\frac{A_B - A_S}{A_B}\right] \times 100 \text{ ----- (1)}$$

Where A_B is the absorbance of the control reaction (containing all reagents except the test compound), and A_S is the absorbance of the test compound.

The IC_{50} value of the samples, which is the concentration of sample required to inhibit 50 % of the DPPH free radical, was calculated from the graph of percentage inhibition plotted against extract concentration.

Kinetics analysis

The concentrations of DPPH at various time intervals were calculated using the relation from Beer-Lambert law:

$A = \varepsilon cl$ ---------- (2)

Where A = Absorbance, ε = Molar absoptivity of DPPH, c = Concentration and l = Path length.

The integrated form of Second-order reaction equation was applied to the experimental results for *S.macrocapon* and *C.rubens*.

$$\frac{1}{[DPPH]_t} = k_2 t + \frac{1}{[DPPH]_0} \text{----- (3)}$$

Where $(DPPH)_0$ and $(DPPH)_t$ are the DPPH concentrations at time zero and any time 't' respectively. A plot of $\frac{1}{[DPPH]_t}$ against time gives a slope, k_2 [24]. Second order anti-radical kinetics determination was adapted from a reported work [25] using DPPH and antioxidants of the extracts:

$$\frac{-d[DPPH]}{dt} = k_2[DPPH][Antioxidant] \text{ --- (4)}$$

The second order rate constant (k_2) was determined with the anti-radical compound (Antioxidant) in large excess as compared with the radical compound (DPPH), thus forcing the reaction to behave as first order in DPPH.

$$\frac{-d[DPPH]}{dt} = k_1[DPPH] \text{ ---------- (5)}$$

Where $k_1 = k_2$ (Antioxidant) ------- (6)

(Antioxidant) is assumed to remain constant throughout the reaction and can be modified to obtain different k_1 values. The change in (DPPH) by change in time will give k_1 values. Determination of k_1 was repeated using different antioxidant concentration for each sample and the mean gave the overall k_1.

Therefore, DPPH was depleted from the medium under pseudo first-order conditions following the equation:

$$[DPPH] = [DPPH]_0\, e^{-k_1 t} \text{ -------- (7)}$$

Where (DPPH) is the radical concentration at any time (t), $(DPPH)_o$ is the radical concentration at time zero and k_1 is the pseudo first order constant. This constant (k_1) is linearly dependent on the concentration of the antioxidant and from the slope of these plots, k_2 was determined.

The percentage of DPPH remaining at anytime t can be determined as:

$$\%\ DPPH_{remaining} = \frac{A_t}{A_0} \times 100 \text{ ----- (8)}$$

Where A_0 is the initial absorbance and A_t is the absorbance at time t, both measured at 517 nm respectively.

plots of percentage DPPH against time showed the disappearance pattern of DPPH with time in the presence of each extract [26].

Results and Discussion

Calibration of DPPH

The measure of how well the DPPH absorbs light at a wavelength of 517 nm (The Molar absorptivity (ε)) was obtained from a plot of Absorbance against concentration. The plot obtained is presented in figure 1. From the plot, a linear regression from the origin was obtained with $R^2 = 0.99898$. The slope of the graph which corresponds to the Molar absorptivity of DPPH is obtained as 100.02 ± 1.06 $(mM)^{-1}$. This value will be used to calculate the concentration of DPPH after an incubation time (t) using equation 2.

Antioxidant activity

The % Inhibitions of DPPH of *S.macrocapon* and *C.rubens* are shown in Table 1 and Table 2 respectively.

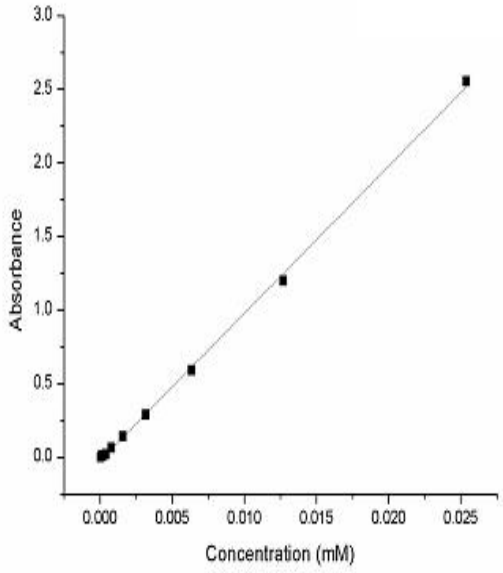

Figure 1: DPPH Calibration Curve

Table 1: % Inhibition of *S.macrocapon*

Concentration (mg/ml)	%Inhibition (30 mins)	% Inhibition (50 mins)	% Inhibition (70 mins)	% Inhibition (90 mins)
1.00	94.43	94.64	94.95	95.20
5.00×10^{-1}	93.60	93.78	94.13	94.38
2.50×10^{-1}	77.72	77.97	78.28	78.53
1.25×10^{-1}	57.58	57.84	58.15	58.44
6.25×10^{-2}	51.06	51.37	51.64	51.91
3.13×10^{-2}	46.53	46.81	47.01	47.30
1.56×10^{-2}	42.84	43.10	43.37	43.63
7.81×10^{-3}	32.72	33.09	33.40	33.69
3.91×10^{-3}	30.65	30.98	31.23	31.57
1.95×10^{-3}	25.61	25.96	26.28	26.52

Table 2: % Inhibition of *C.rubens*

Concentration (mg/ml)	% Inhibition (30 mins)	% Inhibition (50 mins)	% Inhibition (70 mins)	% Inhibition (90 mins)
1.00	84.27	84.56	84.80	85.11
5.00×10^{-1}	71.48	71.81	72.05	72.30
2.50×10^{-1}	57.84	58.13	58.37	58.67
1.25×10^{-1}	50.67	50.99	51.21	51.52
6.25×10^{-2}	41.24	41.53	41.79	41.99
3.13×10^{-2}	40.86	41.19	41.41	41.67
1.56×10^{-2}	33.44	33.74	33.98	34.31
7.81×10^{-3}	32.43	32.75	33.01	33.21
3.91×10^{-3}	31.64	31.86	32.10	32.45
1.95×10^{-3}	26.28	26.61	26.81	27.05

The IC_{50} values were obtained from the plot of % Inhibition against concentration. The results are presented in Figure 2 for both samples at 30, 50, 70 and 90 minutes incubation time respectively. In addition, Table 3 showed the corresponding IC_{50} for the two samples at various times.

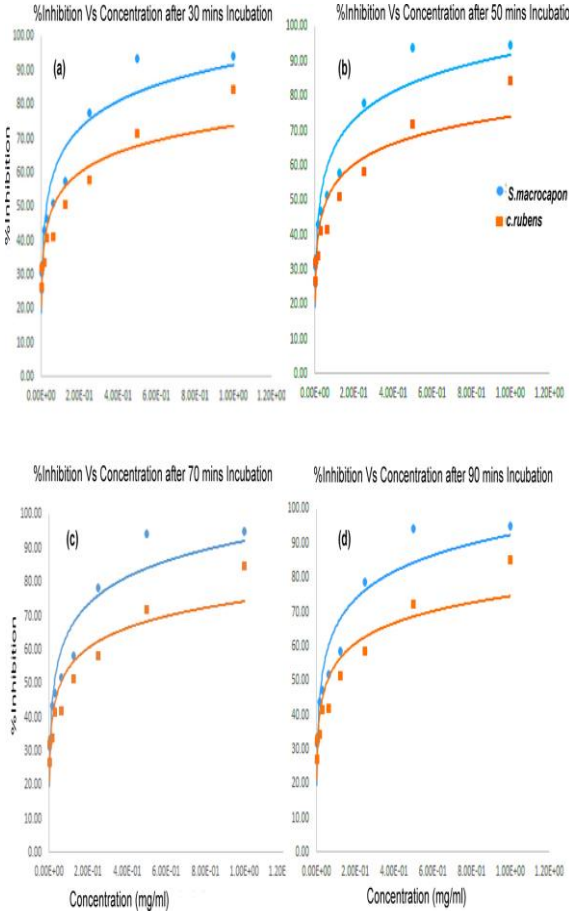

Figure 2: % Inhibition curve of *S.macrocapon* and *C.rubens* after (a). 30 mins. (b). 50 mins, (c). 70 mins and (d). 90 mins of incubation

Table 3: IC_{50} for *S.macrocapon* and *C.rubens*

Time (mins)	**IC_{50} (X 10^{-2} mg/ml)**	
	S.macrocapon	*C.rubens*
30	2.18	6.27
50	2.74	6.04
70	2.67	5.88
90	2.61	5.69

From the results obtained, the IC_{50} is inversely related to the antioxidant capacity of the compounds. The lower the IC_{50} value, the higher the antioxidant activity of the compound as reported by Villano *et al.* [26]. *S.macrocarpon* is a more potent vegetable that could scavenge free radicals than *C.rubens* as shown by the lowest IC_{50} value across all the time (Table 3). This is in close agreement with Bondet *et al.* [21], which indicate that most antioxidants react slowly with DPPH and suggest that antioxidant activity using DPPH should be evaluated over time. The antioxidant activity obtained in this study is comparable with those obtained by Tailor & Goyal [27] but less than that of Olajire & Azeez [28]. This could be due to methods used for the analysis and the medium of extraction as pointed out by Li *et al.* [29].

Kinetic analysis

Considering the depletion of *S.macrocapon* extracts under Pseudo-first order condition (Equation 7), the results obtained are presented in Figure 3. The rate constant k_1 for the reaction of the antioxidant in the extract and DPPH was obtained from the slopes of the plots. Second order rate constant k_2 was obtained by linear regression fitting of k_1 and antioxidant concentration following Equation 6.
The average value obtained for k_2 was 0.0027 ± 0.0006 $mlmg^{-1}min^{-1}$ with a linear regression coefficient of 0.709.

However, considering the depletion of *S.macrocapon* extracts under Second order condition (Equation 3), the results obtained are as presented in Figure 4.

The Second-order rate constant k_2 was calculated from the slopes of the plots with the average to be the overall k_2. From the plots, an average k_2 value of 0.473 ± 0.020 $(mM)^{-1}min^{-1}$ was obtained with a linear regression coefficient of 0.993. Also of note is that the k_2 value decreased with reduction in the concentration of extract. This implies that samples at high concentration reacted within a short time and also slow kinetic behaviour implies longer period before steady state is attained. These results are in agreement with those obtained by Sladjana *et al.* [30] and Nikolaos & Maria [31]. The results indicate that when extracts of *S.macrocapon* were added to DPPH solution, the DPPH radicals were better depleted under Second-order condition due to the higher R^2 value obtained when compared with that of pseudo first order condition

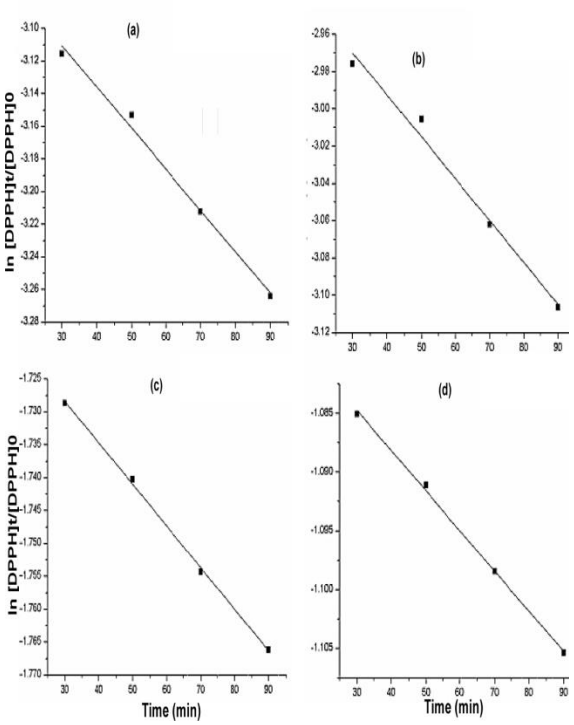

Figure 3: Plots of ln $(DPPH)_t/(DPPH)_0$ versus time for *S.macrocapon* at concentrations of (a) 1.00 mg/ml, (b) 0.50 mg/ml (c) 0.25 mg/ml and (d) 0.125 mg/ml

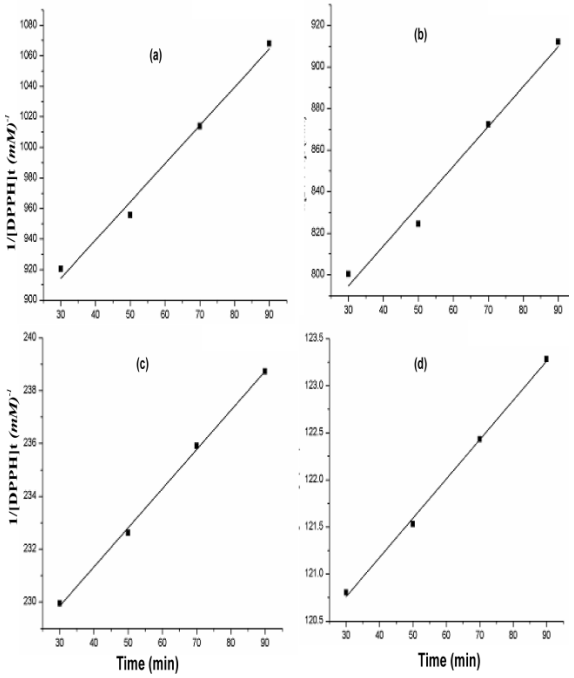

Figure 4: Plots of $1/(DPPH)_t$ versus time for *S.macrocapon* at concentrations of (a) 1.00 mg/ml, (b) 0.50 mg/ml (c) 0.25 mg/ml and (d) 0.125 mg/ml

For *C.rubens* extracts depleted under Pseudo-first order condition (Equation 7), the results obtained are presented in Figure 5. When the experimental data was plotted, a linear fit curve was employed and an average regression coefficient of 0.99 was obtained. The rate constant k_1 for the reaction of the antioxidant in the extract and DPPH was obtained from the slopes for the plots. Second order rate constant k_2 was obtained by linear regression fitting of k_1 and antioxidant concentration following Equation 6.

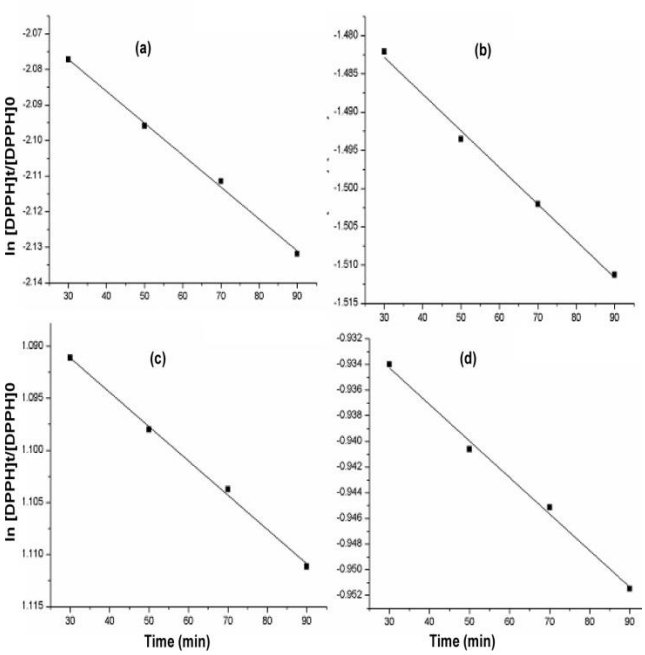

Figure 5: Plots of ln $(DPPH)_t/(DPPH)_0$ versus time for C.rubens at concentrations of (a) 1.00 mg/ml, (b) 0.50 mg/ml (c) 0.25 mg/ml and (d) 0.125 mg/ml.

The average value of $6.89 \times 10^{-4} \pm 0.03 \times 10^{-4}$ $mlmg^{-1}min^{-1}$ with a linear regression coefficient of 0.987 was obtained for k_2. However, considering the depletion of *C.rubens* extracts under Second order conditions (Equation 3), the results obtained are as presented in Figure 8.

From the plots, an average k_2 value of 0.05551 ± 0.00236 $(mM)^{-1}min^{-1}$ was obtained with a linear regression coefficient of 0.795. Based on the R^2 value, *C.rubens* extracts were better depleted under pseudo first order condition with a k_2 value of $6.89 \times 10^{-4} \pm 0.03 \times 10^{-4}$ $mlmg^{-1}min^{-1}$ having a linear regression coefficient of 0.987.

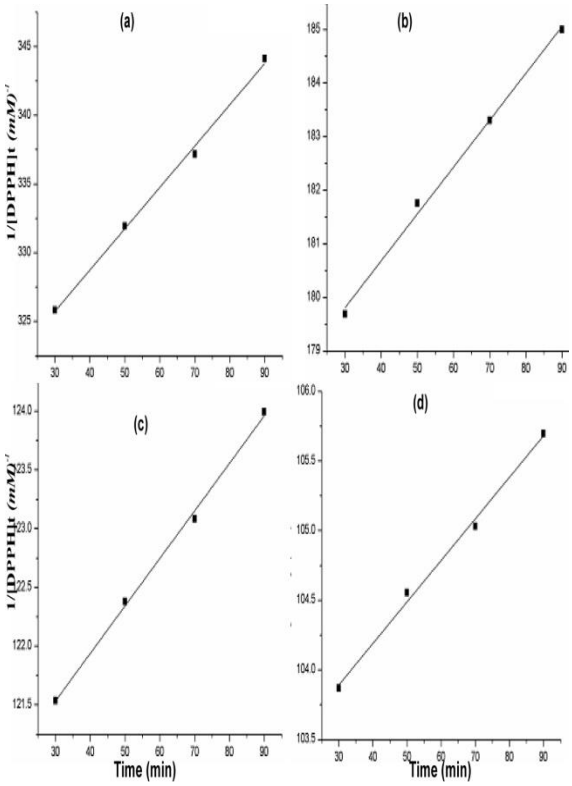

Figure 6: Plots of $1/(DPPH)_t$ versus time for C.rubens at concentrations of (a) 1.00 mg/ml, (b) 0.50 mg/ml (c) 0.25 mg/ml and (d) 0.125 mg/ml.

Also, the results show that the k_2 values for extracts from *S.macrocapon* were higher than that from *C.rubens* extracts. This implies that the antioxidants from *S.macrocapon* have faster reaction kinetics than the antioxidants of *C.rubens*. Figure 7 presents the disappearance pattern of DPPH free radicals with time in the presence of the extracts within a time of 90 minutes.

Figure 7 presents the disappearance pattern of DPPH free radicals with time in the presence of methanol extracts of *S.macrocapon* and *C.rubens* within a time of 90 minutes. The free radical scavenging pattern presented is monophasic, characterised by a slow step of degradation of the antioxidant concentration as the time of reaction increases for all concentrations of the extracts used. This result is in close agreement with results obtained by Bondet *et al*. [21] which indicate that most antioxidants react slowly with DPPH and suggest that antioxidant activity using DPPH should be evaluated overtime.

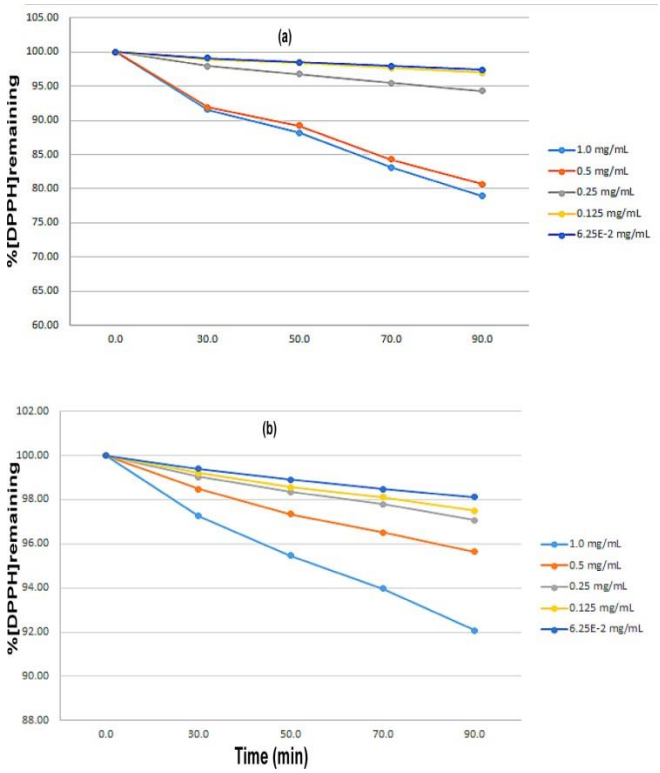

Figure 7: Disappearance pattern of DPPH Free radicals with Time in (a) *S.macrocapon* and (b) *C.rubens*

Conclusions

The results from this study confirmed their potency of the two edible vegetables as a natural source of antioxidant and revealed that *S.macrocapon* has higher free radical scavenging activity than *C.rubens*. It was also shown that under the experimental conditions, *S.macrocapon* has a higher DPPH radicals scavenging potential as reflected in its higher rate constant (k_2) for the process and is best described by the second order reaction kinetics. However, the DPPH scavenging potential of *C.rubens* has lower rate constant value and its best described by a pseudo first order kinetics

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