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Artigo

The Effect of *Chlorophyll Degradation* (ECD) as Qualitative Antioxidant Assay in *Eruca sativa* Leaves

Colares, R. P.; de Lemos, T. L. G.; Monte, F. J. Q.; Alcócer, J. C. A.; de Oliveira, M. M.; Fonseca, A. M.*

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O Efeito da Degradação da Clorofila (EDC) como Ensaio Antioxidante Qualitativo em Folhas de *Eruca sativa*

Resumo: A atividade antioxidante de um composto é uma propriedade biológica que tem como função retardar as reações de degradação oxidativa, e desta forma, reduzir a velocidade da oxidação por um ou mais mecanismos, como a inibição de radicais livres ou a complexação de metais. O papel dos antioxidantes é proteger as células sadias do organismo contra a ação oxidante dos radicais livres. Este trabalho tem como objetivo contribuir com uma nova forma de determinar qualitativamente a capacidade antioxidante de substâncias orgânicas, usando uma metodologia simples e de baixo custo. Tanto o efeito do sequestro de radicais livres (DPPH) como o efeito da degradação da clorofila (EDC) mostraram que a atividade depende da concentração. A atividade antioxidante pelo DPPH variou de acordo com o material examinado, com valores de IC_{50} que foram $2{,}60x10^{-5}$ mg.mL^{-1} para a quercetina, em comparação com os padrões positivos $3{,}95x10^{-6}$ mg.mL^{-1} (trolox) e $6{,}29x10^{-6}$ mg.mL^{-1} (vitamina C). No entanto, revelou diferença significativa apenas para o β-sitosterol, mostrando que a quercetina, trolox e vitamina C são substâncias antioxidantes. De forma análoga, porém qualitativa, o efeito da degradação da clorofila mostrou resultados semelhantes, o que evidencia que essa nova metodologia pode ser usada com esse propósito.

Palavras-chave: Radicais livres; DPPH; Clorofila.

Abstract

The antioxidant activity of a compound is a biological property whose function is to retard oxidative degradation reactions thereby reduce the rate of oxidation by one or more mechanisms such as the free radicals inhibition or metal complexation. The role of antioxidants is to protect the body's healthy cells against the oxidizing action of free radicals. This paper intends to contribute with a new qualitative way to determine the antioxidant capacity of the compounds using a fairly simple methodology. Both the effect of scavenging of free radicals (DPPH) and the effect of chlorophyll degradation (ECD) have shown that the activity was concentration dependent. The antioxidant activity by DPPH presented a variation according to the material examined. The IC_{50} values were $2.60x10^{-5}$ mg.mL^{-1} for quercetin compared to the positive standards $3.95x10^{-6}$ mg.mL^{-1} (trolox) and $6.29x10^{-6}$ mg.mL^{-1} (vitamin C). However it revealed a statistical difference only for β-sitosterol, showing that quercetin, trolox and vitamin C are antioxidant substances. In an analogous but qualitative way the effect of chlorophyll degradation showed similar results which evidences that this new methodology can be used for this purpose.

Keywords: Free radicals; DPPH; Chlorophyll.

* Instituto de Ciências e da Natureza, Campus da Liberdade, Avenida da Abolição 3, Centro, CEP 62790-000, Redenção-CE, Brazil. Phone: +55 (85) 8811-2586.
aluisiomf@unilab.edu.br
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The Effect of Chlorophyll Degradation (ECD) as Qualitative Antioxidant Assay in *Eruca sativa* Leaves

Regilany P. Colares,[a] Telma L. G. de Lemos,[b] Francisco J. Q. Monte,[b] Juan C. A. Alcócer,[c] Mauro M. de Oliveira,[d] Aluísio M. Fonseca[a]

[a] Universidade da Integração Internacional da Lusofonia Afro-Brasileira (UNILAB), Instituto de Ciências e da Natureza, Campus da Liberdade, Avenida da Abolição 3, Centro, CEP 62790-000, Redenção-CE, Brazil.

[b] Universidade Federal do Ceará, Departamento de Química Orgânica e Inorgânica, Campus do Pici, s/n, Avenida Humberto Monte, Pici, CEP 60455-760, Fortaleza-CE, Brazil.

[c] Universidade da Integração Internacional da Lusofonia Afro-Brasileira (UNILAB), Mestrado Acadêmico em Sociobiodiversidade e Tecnologias Sustentáveis (MASTS), Campus das Auroras, Centro, CEP 62790-000, Redenção-CE, Brazil.

[d] Faculdade Paraíso do Ceará (FAP), Rua da Conceição, 1228, S. Miguel, CEP 63010-465, Juazeiro do Norte-CE, Brazil.

* aluisiomf@unilab.edu.br

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1. Introduction

In the last decades, there has been considerable interest in finding sources of synthetic and natural antioxidants, which provide a measure of protection against the processes of oxidative damage.[1] Antioxidant compounds from plants have been reported to inhibit the propagation of free radical reactions, protecting the human body from diseases.[2]

An oxidation process that draws plenty attention is the degradation of chlorophyll by ethylene molecules. The result of chlorophyll degradation is the yellowing or brown green of tissues in a variety of leafy vegetables. This process was observed in several vegetables,

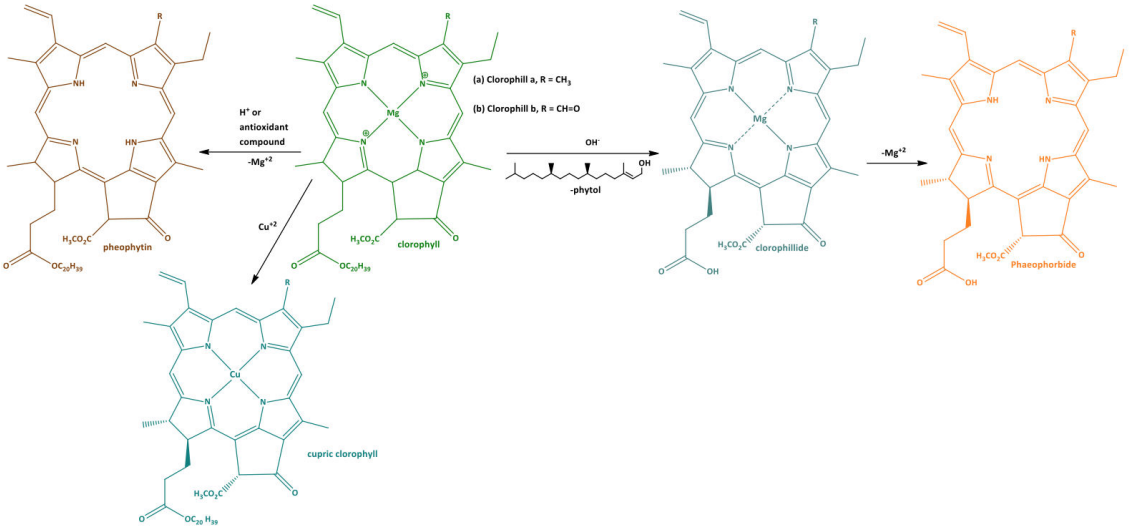
such as cabbage, parsley, coriander and broccoli.[3,4]

Rapid decline in quality due to post-harvest senescence is a serious problem for green leafy vegetables, for which the most common symptom is yellowing of the leaves. Rocket (*Eruca sativa* Mill.) is a very popular vegetable in many countries and is used for salads (Figure 1). This plant species, when undergoing senescence, changes colour from green to yellow. This change can be chemically attributed to the degradation of chlorophyll within the leaf. However, it is unclear whether ethylene is involved in the yellowing of leaves.[5-9]

Figure 1. Rocket (*Eruca sativa* Mill.)

A possible degradation of chlorophyll is evidenced in Scheme 1, that shows a porphyrin tetrapirrolic skeleton, which possess photossensibility, react to heat, oxygen, causing thermodynamics instability in your structure. Which also means that the instability of the porphyrin complex-Mg is associated with the excited state, suggesting that these structures are strong reducing agents and oxidize rapidly. The following diagram shows the possible reactions that happen with chlorophyll and its structures.[10,11]

Information concerning the composition of rocket leaves is surprisingly scarce. Much attention has been paid to *E. sativa* seeds, which previous phytochemical studies have shown contain thiocyanates, isothiocyanates and their precursors, glucosinolates.[12-19]



Scheme 1. Reaction of chlorophyll in several conditions[10,11]

Based on these data, a new test for antioxidants has been proposed with some of the rocket samples. This test is recommended to be monitored in pure substances, because in plant extracts, probably the presence of chlorophyll could interfere with absorption. This method, in comparison with the scavenging activity of DPPH radical, has the advantage of using only commonly used, relatively inexpensive reagents, such as ethanol and the leaves of the plants. In this work, the two methods are compared using as a positive control for both antioxidant methods: trolox and vitamin C.

2. Experimental section

2.1. Plant material

E. sativa leaves has been collected in a garden, situated on the farm "Piroás", at UNILAB (Universidade da Integração da Lusofonia Afro-Brasileira), in Redemption city (Ceará), Brazil, in April 2016. The specie was identified by Botanist Iracema B. Loiola. A voucher specimen #EAC0041628 is deposited at the Herbarium Prisco Bezerra (EAC), Universidade Federal do Ceará-Brazil.

2.2. Antioxidant assay to determine DPPH scavenging activity

The antioxidant activities of compounds were assayed based on the radical scavenging effect against the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical.[20-21] One millilitre of a 60 µM DPPH ethanol solution was added to sample solutions of different concentrations [C] and allowed to react at room temperature ($1,10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-6}$ mg.mL^{-1}). After 30 minutes, the absorbance values were measured at 520 nm, and the antioxidant activity was determined.[19] Each test was performed in triplicate. Data were plotted on the axes (%Activity *vs* p[C]) and evaluated through regression analysis, where p[C] is calculated by $-\log_{10}[C]$. From the regression line, the IC_{50} value was obtained by reading the value of p[C] corresponding to the percentage of free radical DPPH scavenging for 50% activity ($p[C_{50\%}]$), followed by conversion with Equation 1.

$$IC_{50} = 10^{-p[C_{50\%}]}$$ Equation 1

2.3. Antioxidant assay to determine ECD (Effect of Chlorophyll Degradation)

As part of this antioxidant testing methodology, positive controls of quercetin and the known commercial standards trolox and vitamin C were also tested. These are correlated with the intensity of extinction of chlorophyll in *E. sativa* leaves.[10-11] β-sitosterol was used as a negative control. It was added to 1 mL of material (*E. sativa* leaves) at a concentration (suspension) of either 1.0 g in 50 mL of ethanol, or 0.1 g in 50 mL of ethanol. The same volume of plant material was used with positive controls (trolox, vitamin C and quercetin), which were added at a concentration of 1.0 g·mL^{-1}, and the reaction mixtures were left to stand for 30 minutes at room temperature together with the negative control (β-sitosterol). The absorbance was measured at 664 nm after four days, of the mixture of chlorophylls (a) and (b). Positive controls and samples were dissolved in ethanol. A spectrophotometer UV model HP-8453 was used, and the absorbance was converted into percentage using the known procedure.

2.4. Statistical analysis

The results were expressed as the mean ± S.D. One-way analysis of variance (ANOVA) was used in the antioxidant activity assay (DPPH) followed by Tukey's test ($P < 0.01$). The regression analysis was carried out by the method of least squares.

3. Results and Discussion

The results of the free radical scavenging tests showed a concentration-dependent activity. This can be observed by the coefficient of determination (R^2) for the samples in Figure 2. The lowest proportion of the variance in the dependent variable that is predictable from the independent variable was 85.66% for vitamin C.

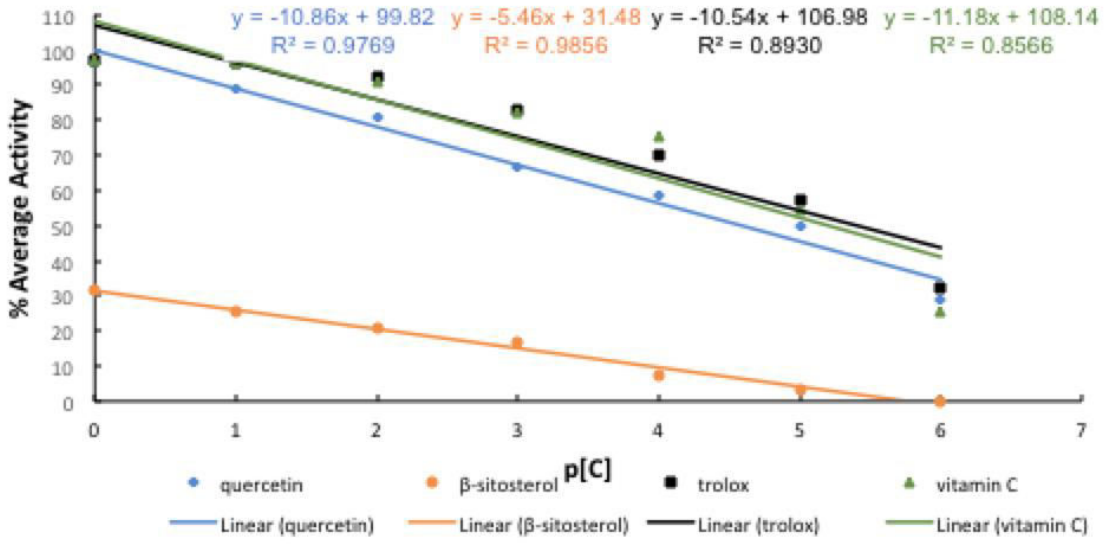


Figure 2. Antioxidant assay to determine DPPH scavenging activity

The IC_{50} values representing the concentrations of trolox and vitamin C required to scavenge free radical DPPH down to 50% activity were $3.95x10^{-6}$ mg·mL^{-1} and $6.29x10^{-6}$ mg·mL^{-1}, respectively. An IC_{50} value with the same order of magnitude was observed for the flavonoid quercetin, which showed similar activity (IC_{50} $2.60x10^{-5}$ mg·mL^{-1}). However, for β-sitosterol, which has been shown to lack significant antioxidant activity, the IC_{50} value was >1.0 mg·mL^{-1}. One-way ANOVA showed a difference in the mean IC_{50} values once the calculated statistic F (94.74) was higher than F critical (7.59) for □*=0.05*. Tukey's pairwise comparisons indicated that the mean IC_{50} for β-sitosterol was significantly different from the other treatments. This result can be interpreted as a conformation of β-sitosterol's lack of antioxidant capacity. The results of the free radical scavenging effect of two samples and the positive control (trolox and vitamin C) in a DPPH free radical system can be seen in Table 1.

To analyse the data of the ECD absorbance, initial readings were made by sweeping the spectrophotometer from 200 to 1000 nm. This analysis showed that the maximum absorbance, and thus the best wavelength for readings, was at 664 nm. Where, (B1) and (B2) means blank, (E1) and (E2) mean the negative pattern, steroid, (T1) and (T2) means the default positive, and trolox (F1) and (F2) are the flavonoids, both at concentrations (1) and (2), respectively (see Figures 3a-3b).

Table 1. Antioxidant assay to determine DPPH scavenging activity

Treatment	quercetin	β-sitosterol	trolox	vitamin C
$\overline{IC_{50}}\ mg.mL^{-1}$	$2.60x10^{-05}$	$2.51x10^{03}$	$3.95x10^{-06}$	$6.29x10^{-06}$
S.D	$2.34x10^{-06}$	$4.46x10^{02}$	$2.86x10^{-07}$	$3.50x10^{-07}$
Variance	$5.46x10^{-12}$	$1.99x10^{05}$	$8.18x10^{-14}$	$1.22x10^{-13}$

S.D = Standard deviation. $\overline{IC_{50}} = Average\ from\ triplicate$. p-Value = 0.0000014

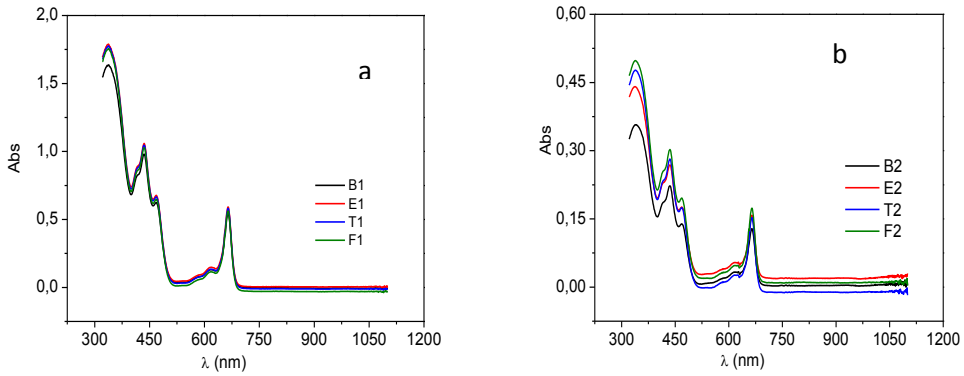


Figure 3. The absorbance was measured at 664 nm because it was appear better comprehension in data analysis for the test (a) (in 1.0 g of leaves in 50 mL of ethanol); and (b) (in 0.1 g of leaves in 50 mL of ethanol)

The results of the studies on the degradation of chlorophyll in *E. sativa* leaves proved to be quite satisfactory. Four effect curves were plotted to compare the absorbance of the solutions containing the sample at the two concentrations, the

control solution, and another without a sample (corresponding to the blank), as shown in Figures 4a and 4b. At both concentrations, the absorbance curve for the samples decreased more than the blank or the negative control. For the known antioxidant compounds (trolox and quercetin), their absorbance curves decreased less than the blank, which means they tended to oxidize spontaneously instead of the substances in the leaf solution being oxidized as was observed in the blank. We should have compared the absorbances of the test compounds to the absorbance of the leaf solution treated with vitamin C, but the vitamin C test mixture had degraded by the third day because of this we decided to only compare the test samples with trolox to determine viability with this technique.

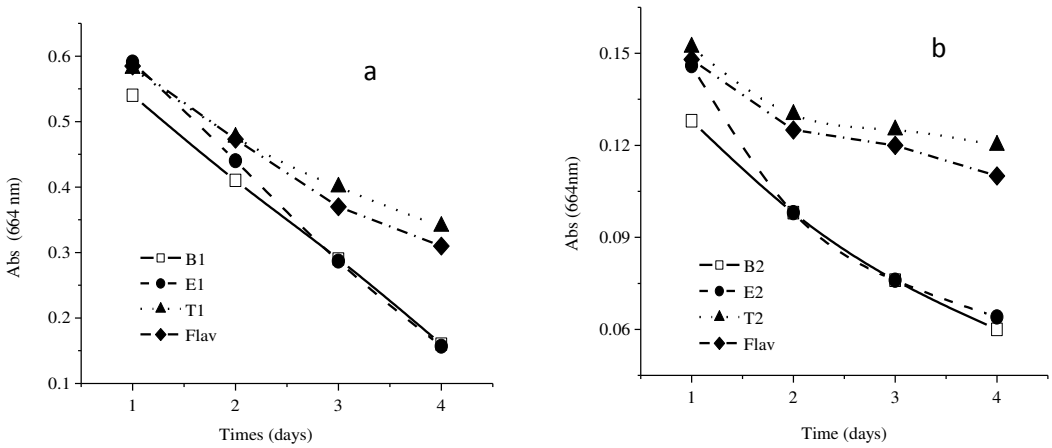


Figure 4. The absorbance was measured at 664 nm for four days. The degradation of the chlorophyll effect was measured by the absorbance of solution pattern at 664 nm in a reaction containing the test sample (a) (in 1.0 g of leaves in 50 mL of ethanol) and (b) (in 0.1 g of leaves in 50 mL of ethanol). B1 (blank); E1 (steroid β-sitosterol); T1 (trolox); Flav (flavonoid quercetin)

4. Conclusion

This research has focused on the design of a methodology to determine the presence of antioxidant substances. In summary, a qualitative method was developed. This method serves to determine only whether the compound has antioxidant activity. However, the materials were inexpensive, and the methodology was quite simple. The results were very promising. Furthermore, additional studies are required for optimization of methodology, suggesting a new perspective toward the test.

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References

[1] Jacob, R. A.; Burri, B. J. Oxidative damage and defense. *American Journal of Clinical Nutrition* **1996**, *63*, 985S. [PubMed]

[2] Kinsella, J. E.; Frankel, E.; German, B.; Kanner, J. Possible mechanism for the protective role of antioxidants in wine and plant foods. *Food Technology* **1993**, *47*, 85. [Link]

[3] Koukounaras, A.; Siomos, A. S.; Sfakiotakis, E. 1-Methylcyclopropene prevents ethylene induced yellowing of rocket leaves. *Post harvest Biology and Technology* **2006**, *41*, 109. [CrossRef]

[4] Yamawo, A.; Suzuki, N. Concentration and retention of chlorophyll around the extrafloral nectary of *Mallotus japonicas*. *Ecology and evolution* **2017**, *7*, 3987. [CrossRef] [PubMed]

[5] Lers, A.; Jiang, W. B.; Lomaniec, E.; Aharoni, N. Gibberellic acid and CO_2 additive effect in retarding postharvest senescence of parsley. *Journal of Food Science* **1998**, *63*, 66. [CrossRef]

[6] Able, A. J.; Wong, L. S.; Prasad, A.; O'Hare, T. J. 1-MCP is more effective on a floral brassica (*Brassica oleracea* var. *italica* L) than a leafy brassica (*Brassica rapa* var. chinensis). *Postharvest Biology and Technology* **2002**, *26*, 147. [CrossRef]

[7] Jiang, W.; Sheng, Q.; Zhou, X.; Zhang, M.; Liu, X. Regulation of detached coriander leaf senescence by 1-methylcyclopropene and ethylene. *Postharvest Biology and Technology* **2002**, *26*, 339. [CrossRef]

[8] Able, A. J.; Wong, L. S.; Prasad, A.; O'Hare, T. J. The effects of 1-methylcyclopropene on the shelf life of minimally processed leafy Asian vegetables. *Postharvest Biology and Technology* **2003**, *27*, 157. [CrossRef]

[9] Wakagi, M.; Taguchi, Y.; Watanabe, J.; Ogita, T.; Goto, M.; Arai, R.; Ujihara, K.; Takano-Ishikawa, Y. Determination of the antioxidative activities of herbs harvested in Japan by oxygen radical absorbance capacity methods. *Food Science and Technology Research* **2016**, *22*, 301. [CrossRef]

[10] Schiozer, A. L.; Barata, L. E. S. Stability of Natural Pigments and Dyes: A review. *Revista Fitos* **2007**, *3*, 6. [Link]

[11] Schoefs, B. Chlorophyll and carotenoid analysis in food products. Properties of the pigments and methods of analysis. *Trends in Food Science & Technology* **2002**, *13*, 361. [CrossRef]

[12] Madkour, S. A.; Laurence, J. A. Egyptian plant species as new ozone indicators. *Environmental Pollution* **2002**, *120*, 339. [CrossRef] [PubMed]

[13] Hamence, J. H.; Taylor, D. The composition of rapeseed meals, part I: the determination of the isothiocyanate present in *Eruca sativa*, a cruciferous seed, present in some rapeseed meals. *Journal of the Association of Public Analysts* **1978**, *16*, 49. [Link]

[14] Adhikari, J.; Adhikari, S.; Achaya, A.T. Glucosinolates in the seeds of Indian brassicas and *Eruca sativa*. *Journal of Oil Technologists Association of India* **1989**, *21*, 13. [Link]

[15] Mahran, G. H.; Kadry, H. A.; Thabet, C.K.; Olemy, M. M.; Al-Azizi, M. M.; Schi, P. L.; Wong, L. K. GC/MS Analysis of volatile oil from *Eruca sativa* seeds. *International Journal of Pharmacognosy* **1992**, *30*, 135. [Link]

[16] Weckerle, B.; Michel, K.; Balázs, B.; Schreier, P.; Tóth, G. Quercetin 3,3',4'-tri-*O*-β-D-glucopyranosides from leaves of *Eruca sativa* (Mill.). *Phytochemistry* **2001**, *57*, 547. [CrossRef] [PubMed]

[17] Sadiq, A.; Hayat, M. Q.; Mall, S. M. Qualitative and quantitative determination of secondary metabolites and antioxidant potential of *Eruca sativa*. *Natural Product Chemistry Research* **2014**, *2*, 137. [CrossRef]

[18] Gotoh, M.; Nakayama, Y.; Nagai, K. Chemical sensor for total antioxidant capacity assay. *Japanese Chemistry Sensors* **2015**, *31*, 13. [Link]

[19] Dijkstra, D. D.; Longo, U.; Guilherme, I. H.; Ferreira, R. V.; Dias, L. N. S.; Buso, W. H. D. Cultivo de Eruca sativa sob diferentes manejos nutricionais. *Agrarian* **2017**, *10*, 61. [Link]

[20] Hegazi, A. G.; El Hady, F. K. A. Egyptian Propolis: 3. Antioxidant, Antimicrobial Activities and Chemical Composition of Propolis from Reclaimed Lands. *Zeitung Naturforsch* **2002**, *57*, 395. [CrossRef] [PubMed]

[21] Oliveira, G. L. S. Determinação da capacidade antioxidante de produtos naturais in vitro pelo método do DPPH•: estudo de revisão. *Revista Brasileira de Plantas Medicinais* **2015**, *17*, 36. [CrossRef]