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Antioxidant activity by DPPH assay: *in vitro* protocol

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ABSTRACT

Considering the role of oxidative stress in the pathology of several diseases and the use of antioxidants as treatment and/or adjuvants in these conditions. Here we propose a protocol to evaluate the antioxidant capacity of compounds by the DPPH method through the scavenging capacity of free radicals by reducing the DPPH radical. This protocol was standardized at LAPCOM (Psychopharmacology and Behavior Laboratory at UFRGS) to assess biochemical parameters *in vitro.*

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MATERIALS TEXT

Gloves **Contributed by users**

96 well plate **Contributed by users**

1.5 mL Eppendorf tubes **Contributed by users**

Surgical mask **Contributed by users**

Micropipette (0.5 - 10 µL) **Contributed by users**

Micropipette (100 - 1000 µL) **Contributed by users**

pH meter **Contributed by users**

Synergy™ HTX Multi-Mode Microplate Reader **Contributed by users**

Multichannel pipette (5 µL; 30- 300 µL) **Contributed by users**

22-Diphenyl-1-picrylhydrazyl **Sigma-aldrich Catalog #D9132** Step 1.1

Ethanol **Merck Millipore Catalog #100983** Step 1.1

SAFETY WARNINGS

Use personal protective equipment (including lab coat, masks, and gloves) whenever manipulating chemical and biological samples. Make sure to read all Safety Data Sheets for the reagents.

Preparing the reagents

1 The first step is to prepare the reagents to be used in this protocol;

1.1 **2,2-Diphenyl-1-picrylhydrazyl (DPPH)** [M]**0.24 mg/mL** :

1.1.1 Weigh **0.024 g** of DPPH in a piece of aluminum foil;

22-Diphenyl-1-picrylhydrazyl **Sigma-aldrich Catalog #D9132**

1.1.2 Add **50 mL** of absolute ethanol to the beaker to dissolve the salt;

Ethanol **Merck Millipore Catalog #100983**

1.1.3 Transfer your solution to a **100 mL** volumetric flask;

1.1.4 Using absolute ethanol, complete the solution's volume to reach **100 mL** ;

1.1.5 Read the absorbance of the solution at **517 nm** in a microplate reader;

If the absorbance of the solution is above 1.12, dilute the solution by adding absolute ethanol;
If the absorbance of the solution is lower than 1.08, add DPPH;

Incubation of the samples 1d

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To optimize the reaction, an incubation step is needed.

2.1 Prepare **1.5 mL** microtubes, to be used to store the samples, with the correct information. Wrap the microtubes in aluminum foil. The number of microtubes depends on the number of samples. You should provide at least five replicates (n = 5) of each sample with at least one control tube per sample. Perform the test at least two times to ensure the results are as correct as possible.

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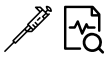
2.2 For each sample, fill the plastic microtubes as described below. Using a micropipette fill the tubes in this order: Absolute ethanol + DPPH + Sample. Mix the solution with the pipette tip to homogenize the content;

A	B	C	D
Microtubes	**EtOH (µL)**	**DPPH (µL)**	**Sample (µL)**
Control	20	280	-
Sample	10	280	10

1d

2.3 Incubate all your samples in the dark at **25 °C** for **24:00:00** ;

Reading your samples

3 

Prepare to read the absorbance of your samples in a microplate reader;

3.1 Use a conventional 96-well microplate to run your samples. Before start pipetting, each well of the microplate should be marked for sample identification.

3.2 

Add **250 µL** of the content of each microtube to the wells needed. Fill two additional wells with **250 µL** of absolute ethanol to work as the negative control for the absorbance reading of all of your samples;

3.3 Read the absorbance of the samples at **517 nm** in a microplate reader;

Calculating data and determinig results

4 Prepare to analyze the results obtained after reading the absorbance of the samples;

4.1 Calculate the mean absorbance of your replicates;

4.2 Determine the percentage of inhibition of the DPPH radical:

$$\%\ \text{of inhibition of the DPPH radical} = \left[\frac{Abs_{control} - (Abs_{sample} - Abs_{blank})}{Abs_{control}}\right] \times 100$$

$Abs_{control}$ = The absorbance of DPPH
Abs_{sample} = The absorbance of your sample
Abs_{blank} = The absorbance of ethanol negative control

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4.3 Results should be expressed as % of inhibition of the DPPH radical.

5 Determine the median effective concentration (EC50) for each sample, which expresses the minimum amount of the extract capable of reducing the initial concentration of DPPH radical by 50%. Calculated in a nonlinear regression model using GraphPad Prism.

Prism 8.0
by GraphPad

5.1 On an XY graph, plot the concentrations of the samples in column X and the results of the DPPH assay (% inhibition) in column Y.

5.2 Transform the values to logarithmic scale:

Analyze -> Transform -> Ok -> transform X values using = X = Log (x) -> Ok.

5.3 Perform a nonlinear regression analysis:

Analyze -> XY analyzes -> Nonlinear regression -> Ok -> Dose responds stimulation– Log (agonist) VS. Normalized response -> variable slope -> Ok.

5.4 Results should be expressed as EC50.

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