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RESEARCH ARTICLE

RAPID MICROTITRE PLATE BASED ASSAY PROTOCOL FOR HERBAL EXTRACTS

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Abstract:
DPPH assay has been practiced by researchers across the world for estimation of antioxidant activity. The reagents are costly and hazardous to environment. We describe a rapid and fast micro plate based protocol for DPPH assay of herbal extracts.

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Introduction:
DPPH (2,2-Diphenyl-1-picrylhydrazine) is used for free radical scavenger and antioxidant assay [1]. However, the need for an economical, microscale and quick protocol has been identified by researchers [2]. In the present paper, we describe a new, economical and quick method for DPPH assay.

Material and methods:

DPPH microplate assay protocol

1. DPPH radical scavenging activity (IC50) of extracts was estimated following a previously described procedure (Chen et al. 2013).
2. Hundred microliters 100 µL of DPPH (1 mg/mL) was mixed with 110 µL of each concentration of 1, 0.1, 0.01 and 0.001 mg/mL of extract. Note that the solution was prepared fresh.
3. After 15 min incubation at room temperature absorbance was measured at 517 nm using a spectrophotometer.
4. The effective dose of extract for 50 % inhibition of DPPH° (IC50) was obtained from a plot of percentage inhibition verses extract concentration.
5. Butylated hydroxyl toluene (BHT) can be used as positive control, as it itself is an antioxidant.

Procedure:

Preparation of DPPH stock solution (1 mg/ ml)

1. Accurately weighed quantity (10mg) of DPPH was transferred to a 10mL amber colored volumetric flask.
2. Approximately 5 mL methanol was added to the flask.
3. The flask was placed in ultrasonic water bath and ultrasonicated for 10 min.
4. The volume was made up to mark with methanol.
5. The solution was stored in refrigerator (4^{o}C) until use.
6. The solution was discarded and prepared freshly if it was not used for more than three weeks.

Preparation of extract stock solution (1 mg/mL)

1. Accurately weighed quantity (100mg) of extract was transferred to a 100 mL volumetric flask.
2. Approximately 50 mL methanol was added to the flask.
3. The flask was placed in ultrasonic water bath and ultrasonicated for 10 min.
4. The volume was made up to mark with methanol.
5. The solution was prepared fresh on each day of working.

Preparation of ascorbic acid stock solution (1 mg/mL)

1. Accurately weighed quantity (100 mg) of ascorbic acid was transferred to a 100 mL volumetric flask.
2. Approximately 50 mL methanol was added to the flask.
3. The flask was placed in ultrasonic water bath and ultrasonicated for 10 min.

4. The volume was made up to mark with methanol.
5. The solution was prepared fresh on each day of working.

Microplate assay:

Load the samples as shown in following diagram:

	1 Solvent control S1	**2 Solvent control S2**	**3 Solvent control S3**	**4 Positive control P1**	**5 Positive control P2**	**6 Positive control P3**	**7 Test T1**	**8 Test T2**	**9 Test T3**	**10 DPPH calibration curve**
A	MeOH 400 µL	MeOH 400 µL	MeOH 400 µL	AASS 400 µL 1 mg/mL	AASS 400 µL 1 mg/mL	AASS 400 µL 1 mg/mL	ESS 400 µL 1 mg/mL	ESS 400 µL 1 mg/mL	ESS 400 µL 1 mg/mL	DPPH 1 mg/mL 400 µL
B	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL
C	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL
D	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL
E	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL
F	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL

Table No.1: Sample loading

Note: MeOH can be replaced by Water if the sample is insoluble in alcohol.

DPPH is insoluble in water. However we found satisfactory results in concentrations up to 50% water in methanol.

One may use buffered methanol or aqueous buffer solution may be used (HPLC grade methanol with ammonium acetate buffer is best suited for this protocol).

Second step: with a multichannel pipette, take 200 µL from the first row of columns 2 to 10 and transfer them into the matching wells in the second row. Mix the contents with 4-5 times using pipette tip. After this, transfer exactly 100 µL to the thrid row and repeat the process until the lat one, discarding the last 200 µL. Each well contains 100 µL solution after 8 double dilutions in the columns 2 – 12.

Third step: DPPH (100 µL) is then added (0.2 mM solution) to each well of the microplate.

	1 Solvent control S1	**2 Solvent control S2**	**3 Solvent control S3**	**4 Positive control P1**	**5 Positive control P2**	**6 Positive control P3**	**7 Test T1**	**8 Test T2**	**9 Test T3**	**10 DPPH calibration curve**
A	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	AASS 200 µL 1 mg/mL	AASS 200 µL 1 mg/mL	AASS 200 µL 1 mg/mL	ESS 200 µL 1 mg/mL	ESS 200 µL 1 mg/mL	ESS 200 µL 1 mg/mL	DPPH 1 mg/mL 200 µL
B	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	AASS 200 µL 0.5 mg/mL	AASS 200 µL 0.5 mg/mL	AASS 200 µL 0.5 mg/mL	ESS 200 µL 1 mg/mL	ESS 200 µL 1 mg/mL	ESS 200 µL 1 mg/mL	DPPH 1 mg/mL 200 µL
C	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL
D	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL

E	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL
F	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL	MeOH 200 µL

Table No.2:

Step 4: Incubate the plates and 37^{o}C for 20 minutes. Scan the microplate using microplate scanner at 517 nm wavelength.

Result and Discussion:-

A rapid microplate based protocol for DPPH assay was described. The proposed protocol uses less amount of hazardous DPPH and is environment friendly. The protocol can be adapted to any microplate readers easily**.**

References:

[1] Medeiros de Morais, C. L.; de Lima, K. M. A colorimetric microwell method using a desktop scanner for biochemical assays. Talanta 2014, 126, 145-150.

[2] Soldat, D. J.; Barak, P.; Lepore, B. J. Microscale Colorimetric Analysis Using a Desktop Scanner and Automated Digital Image Analysis. Journal of chemical education 2009, 86 (5), 617-620.