Antioxidant and Cytoprotective Properties of Three Egyptian *Cyperus* Species Using Cell-free and Cell-based Assays

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Abstract: Three Egyptian *Cyperus* species viz. *Cyperus esculentus*, *C. rotundus*, and *C. papyrus* that belong to Cyperaceae were used as food and medicines since ancient times. This study aimed to investigate the direct and indirect antioxidant properties of the selected species in two chemical assays (FRAP and DPPH) and a cell-based bioassay in hepa1c1c7 cells using *t*-butyl hydroperoxide (TBHP) as the inducer of cytotoxicity. Three different extracts were prepared from each *Cyperus* tuber. The results indicated that the 80% EtOH extract of *C. papyrus* was the most potent with direct antioxidant activity in both DPPH (EC_{50}: 5.1 µg/ml) and FRAP (FE: 48.7 µg/ml) assays and a significant full cytoprotection against TBHP at 100 µg/ml.

Keywords: Antioxidant, Cytoprotection, Egyptian *Cyperus*, FRAP, DPPH, TBHP.

INTRODUCTION

The damage caused by free radicals and/or their resulting oxidative stress on the living cells has been extensively studied in recent years. Free radicals have been demonstrated to be the main initiator for many diseases such as cancer, Alzheimer's disease, Parkinson's disease, and rheumatoid arthritis [1, 2]. For this reason, there has been intensive study of the antioxidant properties of plant extracts and isolated phytochemicals, with a view to identifying potentially useful antioxidant treatments.

Egypt is famous for the herbal medicine since ancient times [3]. It is still a confident way of remedy among Bedouins and folk communities. The verification of the medicinal uses of the traditional plants, of course will be great for their usefulness as a cheap and safe resource of natural medication.

Three Egyptian *Cyperus* L. (Cyperaceae) species viz. *Cyperus esculentus* L., *C. rotundus* L., and *C. papyrus* L. are claimed to cure and/or reduce many of the implicated symptoms that carried by diseases such as cancer [4]. An ayurvedic formulation containing *C. rotundus* was claimed as a remedy for some inflammatory disorders because of its significant inhibition against colitis and enterocolitis in rats. The same formulation was also found to be useful for ulcerative colitis in a clinical trial [5].

Our previous studies presented at conferences indicated that these species have hepatoprotective and antioxidant activities [6, 7]. Other investigations demonstrated that the hydroalcoholic extract of *C. Rotundus* had antidiabetic activity *in vivo* [8] and *in vitro* [9] due to its antioxidant properties. The same plant also showed to have antiviral [10], antibacterial, and antimutagenic activities [11]. In addition, the methanol extract of this herbal showed *in vitro* the potential as anti-inflammatory agent for inflammatory diseases mediated by overproduction of nitric oxide and superoxide [12].

The present study aimed to continue the investigation of the antioxidant properties of the selected species by employing an integrated two micro-scaled, cell-free antioxidant assays, namely the 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) and the ferric reducing antioxidant power (FRAP), and a developed cell-based anti-"oxidative stress'' assay in hepa1c1c7 cells using tertiary-butyl hydroperoxide (TBHP) as inducer of cytotoxicity.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and tissue culture reagents used in this work were purchased from Sigma-Aldrich Ltd. (Dorset, UK). Solvents used for the plant extraction were analytical grade.

Plant Materials

Dried tubers of *C. esculentus* were purchased from a legal Egyptian herbal market. Tubers of *C. rotundus* were provided from the National Research Centre Farm of Aromatic and Medicinal Plants. *C. papyrus* tubers were kindly provided from El-Karamos Village, El-Sharkia, Egypt. All species were identified by Prof. K. H. Batanouny, Department of Botany, Faculty of Science; Cairo University, Egypt. The voucher specimens were deposited at the herbarium of the Department of Botany, Faculty of Science; Cairo University, Egypt. The collected tubers from *C. rotundus* and *C. papyrus* were separately air dried in absence of direct sun light.

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Preparation of Plant Extracts

"The plant material was ground and extracted three times with a proper solvent to prepare three different extracts from each species. The solvents used for extraction were 80% ethanol (80% EtOH), a mixture of methylene chloride/methanol ($MeCl_2$/MeOH, 1:1) and hexane. The 80% EtOH extract was obtained after removal of ethanol under reduced pressure and then lyophilization. Other extracts were separately concentrated under *vacuo* at 40°C."

Cell-free Antioxidant Chemical Assays

DPPH Radical Scavenging Assay

Plant extracts/compounds were prepared in DMSO as 10× stocks from each test concentration and briefly sonicated when necessary in an ultrasonic water bath. In a preliminary screen, plant extracts producing radical scavenging activities equal to or higher than 80% at 1000 µg/ml were taken for further testing to determine the EC_{50} (concentration of the extract/compound producing 50% scavenging of the DPPH). Some known radical scavengers, quercetin (as dihydrate) and Trolox®, were tested in the assay as reference compounds. The method used in the present study was based on previously published methods [13, 14]. The plant extract/compound stock solutions (20 µl/well) were pipetted in triplicate onto 96-well plates (flat-bottomed, Nunc). The assay was started with the addition of DPPH reagent (0.004% wt/v in methanol, 180 µl/well). Appropriate blanks were prepared using the solvent only in addition to the same amount of DPPH reagent to get rid of any inherent solvent activity. The plate was immediately shaken in a Thermo® Multiscan Ascent plate reader for 30 sec and incubated in the dark for 30 min at room temperature. The remaining DPPH was measured in a plate reader at 540nm. The percentage of antioxidant activity (% *AA)* was calculated using the following equation:

$$\%\ Antioxidant\ activity\ DPPH\ (\%AA) = 100 \times \frac{[OD540\,(blank) - OD540\,(sample)]}{OD540\,(blank)}$$

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP values of plant extracts/compounds were determined based on previously published method [15] with some modifications. The FRAP reagent was prepared by mixing solution A [300 mM acetate buffer prepared by dissolving sodium citrate trihydrate (3.1 g/l) and glacial acetic acid (16 ml/l) in distilled water], solution B [31.2 mg of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) dissolved in 10 ml of 40 mM HCl] and solution C [540 mg of ferric chloride hexahydrate dissolved in 100 ml distilled water] in a volume ratio of 10:1:1, respectively. The FRAP reagent was warmed to 37 °C before being used. The plant extract concentrations (10×, 20 µl) were added in triplicate onto wells of a 96-well plate (flat-bottomed, Nunc). The assay was started by adding 180 µl of FRAP reagent to each well. Appropriate blanks were prepared from the solvent only and run simultaneously with the samples. The plate was immediately shaken in a Thermo® Multiscan Ascent plate reader for 30 sec and the reaction was allowed to run for 10 min after which the plate was read on a plate reader (595 nm). A reference ferrous compound, ferrous sulphate ($FeSO_4$, 0-250 µM in distilled water), was run simultaneously and used to generate the calibration curve by linear regression.

Cell-based Antioxidant Assay (Anti-TBHP)

Cell Culture

Murine hepatoma cell line hepa1c1c7 was obtained from the European Collection of Animal Cell Culture (UK) and maintained as monolayer culture grown in 175 cm^2 flasks in a humidified atmosphere (5% CO_2/95% O_2) in α-MEME supplemented by 10% foetal bovine serum (FBS), 2 mM L-glutamine, 2 µg/ml Fungizone™ and 50 µg/ml gentamicin.

Bioassay Procedure

TBHP was used as an oxidant in the cell-based assay in the present study. After routine sub-culture, hepa1c1c7 cells were seeded onto 24-well plates (0.4×10^6 cells/well). Confluent monolayers were pre-treated with either the plant extracts of *Cyperus* species (50 and 100 µg/ml) or 0.5% DMSO (vehicle control) for 20 h after which the medium was discarded and monolayers washed briefly with Dulbecco's modified phosphate buffered saline (DPBS) (400 µl/well). TBHP-containing culture medium was then added (2% FBS and 200 µM TBHP as final concentrations) and incubated for 5 h. Following TBHP challenge, medium was discarded and the monolayers were washed once with DPBS as above and cell viability was assessed using neutral red uptake (NRU) assay, based essentially on the method of Borenfreund and Puerner [16]. Some reference protective compounds (quercetin, sulforaphane and β-NF) were tested in parallel in the assay.

Statistical Analysis

The data were analysed using GraphPad Prism® software package (version 4). In terms of DPPH assay, the means of the values of the calculated %AA obtained for plant extracts/compounds were plotted against their corresponding concentrations and the data were analyzed using non-linear regression (concentration-response) curve fit from which the EC_{50} values were obtained. For the FRAP assay, the standard curve of concentration-OD (595 nm) for $FeSO_4$ was fitted by linear regression. From the $FeSO_4$ linear regression, the activities of plant extracts/compounds were represented as concentrations (µg/ml) equivalent to the OD (595 nm) of 100 µM $FeSO_4$ (Ferrous equivalents, FE). All data presented were based on means of triplicate absorbance determinations. For the anti-TBHP assay, data were analyzed using One-way ANOVA followed by Dunnett's test to compare all groups including the control to the TBHP group. Data were considered significantly different when $P< 0.05$.

RESULTS

DPPH Assay

Table **1** displayed the DPPH assay results. The data were expressed as EC_{50} in µg/ml as derived from the non-linear regression analysis of the concentration-response relation for each plant extract/compound. As representatives, the sigmoidal non-linear regression of the concentration- response of quercetin is demonstrated in Fig. (**1**). The produced scavenging activity for *Cyperus* extracts ranked in the following order: 80% EtOH extract of *C. papyrus* (EC_{50} = 5.1 µg/ml) > 80% EtOH extract of *C. rotundus* (EC_{50} = 44 µg/ml) > $MeCl_2$/MeOH extract of *C. papyrus* (EC_{50} = 44.8 µg/ml) > $MeCl_2$/MeOH extract of *C. rotundus* (EC_{50} = 222.2 µg/ml).

Table 1. Results of the Antioxidant Cell Free Assays of *Cyperus* Plant Extracts and Reference Antioxidant Compounds

Sample	Extract Yield (%)	FRAP FE (µg/ml))	DPPH EC_{50} (µg/ml)
C. esculentus: 80% EtOH	21.9	>1000	>1000
C. esculentus: $MeCl_2$/MeOH	25.1	>1000	>1000
C. esculentus: Hexane	19.2	>1000	>1000
C. rotundus: 80% EtOH	7.5	160.8	44.0
C. rotundus: $MeCl_2$/MeOH	5.3	n.d.	222.2
C. rotundus: Hexane	0.75	>1000	>1000
C. papyrus: 80% EtOH	14.0	48.7	5.1
C. papyrus: $MeCl_2$/MeOH	12.0	135.6	44.8
C. papyrus: Hexane	2.51	n.d.	>1000
Trolox	n/a	119.9	4.5
Quercetin	n/a	51.9	3.8

Note: FE: Ferrous equivalent (concentration equivalent to the OD (595nm) of 100 µM $FeSO_4$); EC_{50}; Concentration that produces 50% scavenging of the DPPH; n.d.: Not determined. n/a: not applicable.

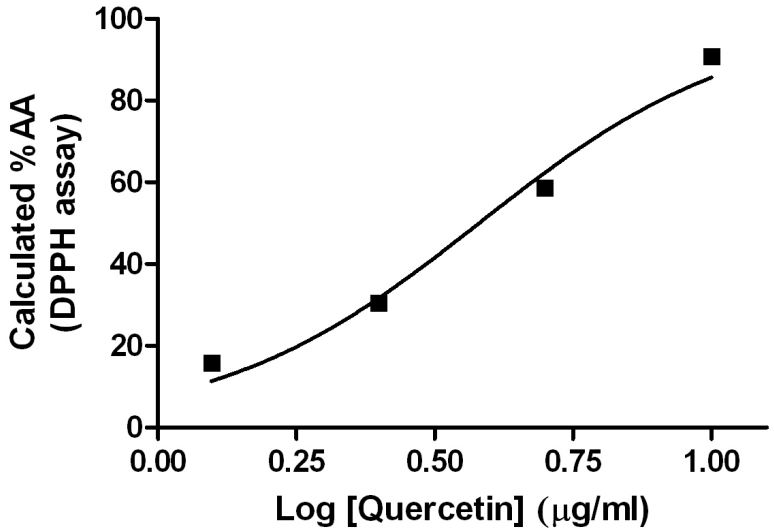

Fig. (1). Representative plots showing non-linear regression of the concentration-response effect of quercetin against calculated antioxidant activity (%AA) (R^2=0.914). Data for the %AA were calculated from the equation stated in the Methods section.

The three extracts from *C. esculentus* and the hexane extracts from both *C. papyrus* and *C. rotundus* showed weak or no antiradical activities (EC_{50} > 1000 µg/ml).

FRAP Assay

In the FRAP assay, $FeSO_4$ (0-250 µM) gave highly reproducible data. As demonstrated in Fig. (**2**), the linear regression analysis revealed a significant linear relationship (P < 0.0001). From this analysis, the OD (595 nm) corresponding to 100 µM $FeSO_4$ (FE) was found to be 1.20 as obtained with interpolation. Similarly, the linear regression analyses of plant extracts/compounds demonstrated significant linear relationships (P < 0.01) of Fe^{3+} reduction with extract/compound concentration. The concentration of the extract/compound that gives an OD (595 nm) value of 1.20 was obtained by interpolation from its linear regression fit. This constructed the FRAP equivalent values (FE) expressed as µg/ml, as shown in Table **1**. The plant extracts of *Cyperus* gave ferrous equivalent values at the order of 80% EtOH extract of *C. papyrus* (FE= 48.7 µg/ml) > $MeCl_2$/MeOH extract of *C. papyrus* (FE = 135.6 µg/ml) > 80% EtOH extract of *C. rotundus* (FE= 160.8 µg/ml).

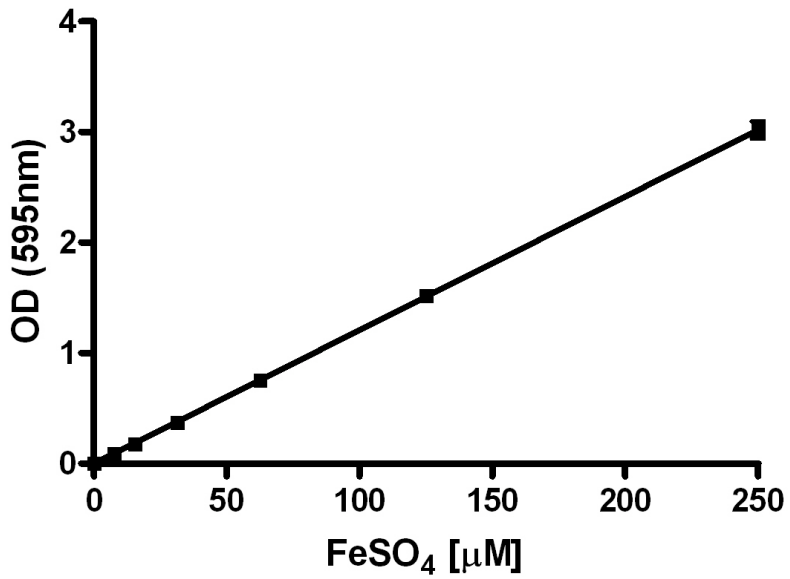

Fig. (2). Linear regression fit of FRAP standard curve for $FeSO_4$ ($P<0.0001$). Data shown are obtained from 6 triplicate absorbance determinations (n=6).

Table 2. Integration of Anti-DPPH and Cell-Based Anti-TBHP Assays

Treatment	Anti-DPPH		Anti-TBHP	
	Max. Scavenging (%)*	Conc. of Max Scavenging (µg/ml)	Cytoprotection (% Viability) †	Corresponding Conc. (µg/ml)
C. papyrus: 80% EtOH	86	62.5	63.1 ± 5.9 98.3 ± 2.0	50 100
C. rotundus: 80% EtOH	90	125	46.3 ± 7.8 70.1 ± 8.0	50 100
C. papyrus: $MeCl_2$/MeOH	86	250	44.5 ± 4.6 53.5 ± 7.0	50 100
C. papyrus: Hexane	10	1000	54.9 ± 7.4 57.9 ± 1.7	50 100
Quercetin	90	10	98.1 ± 1.9	12.5‡
Sulforaphane	0	n. d.	79.4 ± 3.2	4.4‡
β-naphthoflavone	0	n. d.	72.8 ± 3.4	0.85‡
Non-TBHP control	n/a	n/a	100	n/a

Note: EC_{50}: concentration of the extract/compound producing 50% scavenging of the DPPH; Max.: Maximum; *: % was calculated relative to DPPH control; †: % was calculated relative to the non-TBHP control. ‡: concentrations were selected based on a preliminary experiment (data not shown); n. d.: not determined due to the scavenging activity was lower than 50%; n/a: not applicable. The TBHP control has showed only 32.6 % ± 2.6 viability (i.e caused 67.4 % loss of viability relative to the non-TBHP control.

Cytoprotective Potential of *Cyperus* Extracts against TBHP Toxicity in Hepa1c1c7 Cells

The most active extracts that showed scavenging activity against DPPH ($EC_{50} \leq 44.8$ µg/ml) were the 80% EtOH extracts of both *C. papyrus* and *C. rotundus* followed by the $MeCl_2$/MeOH extract of *C. papyrus*. These extracts were tested in parallel with the active standard individual phytochemicals (quercetin, sulforaphane) in the development of the intracellular assay. The hexane extract of *C. papyrus* and β-naphthoflavone were also examined as they gave weak and no radical scavenging activity respectively, in order to test

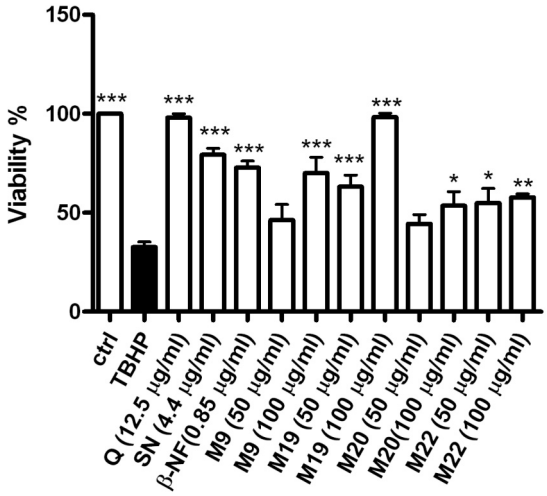

Fig. (3). Results of quercetin, sulforaphane, β-NF and some selected plant extracts in the anti-TBHP cytotoxicity assay. Ctrl: control; TBHP: tertiary-butyl hydroperoxide; Q: quercetin; SN: sulforaphane; β-NF: β-naphthoflavone; other ligands refer to the plant name, extract name and concentrations used in the assay (M9: 80% EtOH extract of *C. rotundus*; M19: 80% EtOH extract of *C. papyrus*; M20: $MeCl_2$/MeOH of *C. papyrus*; M22: hexane extract of *C. papyrus*). Data shown are means ± SEM (n=5 experiments). Data were analyzed using One-way ANOVA followed by Dunnett's test to compare all groups including the control to the TBHP group (colored in black). Asterisks *, ** and *** refer to significantly different values where $P< 0.05$, $P<0.01$, and $P<0.001$, respectively.

the possibility of indirect anti-TBHP effect. Table **2** demonstrates the integration between the direct cell free and cell based assays where Fig. (**3**) represents the results of the anti-TBHP bioassay. The TBHP used at 200 µM has caused 67% loss of hepa1c1c7 cell viability as compared to the non-TBHP treated control. Quercetin, used at final concentration of 12.5 µg/ml has showed a significant full cytoprotection against TBHP cytotoxicity. Sulforaphane (4.4 µg/ml) and β-NF (0.85 µg/ml) produced a significant 79 and 73% viability respectively.

The pretreatment of hepa1c1c7 with 50 µg/ml of the 80% EtOH extract of *C. papyrus* tubers produced a 63% viability while the toxicity of TBHP was completely inhibited at the extract concentration of 100 µg/ml. Lower anti-TBHP activities were produced by the $MeCl_2$/MeOH extract of the same plant producing a significant 54% viability at the extract concentration of 100 µg/ml. In addition, the pretreatment of hepa1c1c7 cells with the hexane extract of *C. papyrus* tubers (50 and 100 µg/ml) produced a significant increase in the obtained cell viability recording about 55 and 58% viability, respectively. The 80% EtOH extract of *C. rotundus* tubers produced 70% viability at the highest concentration tested (100 µg/ml).

DISCUSSION AND CONCLUSION

Nine extracts from three Egyptian Cyperus tubers were prepared and subjected to antioxidant investigation. The study included two chemical assays; FRAP and DPPH and one cell-based bioassay using hepa1c1c7 cells where TBHP was the inducer of cytotoxicity. The results are part of a Ph.D. thesis [17] that suggested one chemical assay may be enough for representing the direct antioxidant effect where the DPPH assay was more recommended than the FRAP assay.

Three extracts namely; the 80% EtOH extract of *C. papyrus* and *C. rotundus* and the $MeCl_2$/MeOH extract of *C. papyrus* were the most active as radical scavengers (EC_{50} < 44.8 µg/ml) whereas the hexane extract of *C. papyrus* showed weak activity (10% scavenging at maximum concentration of 1000 µg/ml). These extracts simultaneously with three phytochemical standards were used in the development of the cytoprotective bioassay to investigate the integration of the chemical and cell-based assays as indicated in Table **2**. Interestingly, the 80% EtOH extract of *C. papyrus* produced a significant nearly full cytoprotection against TBHP cytotoxicity which was similar to that produced by the reference antioxidant quercetin. Moreover, the recorded concentration values of the concerned extract (nearly full gain of control viability level at 100 µg/ml; EC_{50}=5.1 µg/ml; FE of 48.7 µg/ml) are comparable to quercetin as single compound (nearly full gain of control viability level at 12.5 µg/ml; EC_{50}=3.8 µg/ml; FE of 51.9 µg/ml) suggesting that the activity could be attributed to more than one active principle. The $MeCl_2$/MeOH extract was less potent than the 80% EtOH extract and the activity obtained at higher concentrations (54% viability at 100 µg/ml; EC_{50} =44.8 µg/ml; FE of 135.6 µg/ml).

The chemical structures of the reference compounds sulforaphane and ß-NF are devoid of directly acting antioxidant chemical groups, therefore their produced cytoprotective activities are mainly through induction of the cellular antioxidant capacity. Similarily, the hexane extract of *C. papyrus* produced a significant cytoprotection (58% viability) and weak cell-free activity in the chemical antioxidant assays. Therefore, it might be suggested that hexane extract is acting indirectly to enhance the cellular antioxidant capacity of hepa1c1c7 cells. It should be mentioned here that the use of integrated cell-free and cell-based set of assays are very important to conclude the potential of antioxidants.

Concerning *C. rotundus*, the 80% EtOH extract was less potent than the corresponding extract of *C. papyrus*. It possessed the direct and indirect antioxidant activity as quercetin (70% viability at 100 µg/ml; EC_{50} =44 µg/ml; FE=160.8 µg/ml). $MeCl_2$/MeOH of the same species showed EC_{50} at relatively higher concentration (222.2 µg/ml).

The increasing order of the activity shown by both *C. papyrus* and *C. rotundus* extracts are in the order of the increase in their polarity; 80% EtOH> $MeCl_2$/MeOH> hexane, which emphasizes the presence of polar active substances. Previous reports showed the presence of polar classes of secondary metabolites in these two species such as polyphenols, alkaloids and essential oils which are known to possess antioxidant activities [18-23].

The cytoprotective activities shown by the studied extracts from Cyperus species are in agreement with some reports (ref. [24] by Soltan on isolated rat hepatocytes where these extracts showed a good protection against CCl_4 toxicity model, the study performed by Natarajan and co-authors [25] who reported that *C. rotundus* has showed a significant scav-

enging activity and the finding by Nagulendran and co-authors [26] who reported the direct and indirect antioxidant potency of *C. rotundus*).

The results obtained in the present study can provide, at least in part, an additional experimental justification for some reported folk medicinal uses of these plants. The traditional herbal reports mentioned that the selected species were edible [27, 28] and used to cure various chronic disorders such as cancer and to reduce many of the symptoms implicated in the free radical-related diseases.

CONFLICT OF INTEREST

Declared none.

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ABBREVIATIONS

DPBS	=	Dulbecco's modified phosphate buffered saline
DPPH	=	2,2-Diphenyl-1-picryl hydrazyl radical
FBS	=	Foetal bovine serum
FE	=	Ferrous equivalents
FRAP	=	Ferric reducing antioxidant power
NRU	=	Neutral red uptake
TBHP	=	Tertiary-butyl hydroperoxide
β-NF	=	β-naphthoflavone

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