Antioxidant Potential of *Penicillium citrinum* and its Optimization through Different Statistical Approaches

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ABSTRACT

Three-step optimization strategies were applied to optimize the antioxidant potential of *Penicillium citrinum*. Primarily, different carbon and nitrogen sources were screened by classical methods, which revealed sucrose and $NaNO_3$ to be the most suitable. Significance of the components of Czapek dox's medium with respect to antioxidant activity was evaluated with Plackett-Burman design, which supported sucrose and $NaNO_3$ to be the most significant. In second step, sucrose and $NaNO_3$ along with temperature were further taken as three variables for response surface methodology to study their interaction. Response surface analysis showed 4% sucrose, 0.05% $NaNO_3$ and incubation temperature of 35° C to be the optimal conditions. Under these conditions, the antioxidant potential assayed through different procedures was 90.1%, 70.9% and 70.1% scavenging effect for 1,1-diphenyl-2-picryl hydrazyl radical, ferrous ion and nitric oxide ion, respectively. The reducing power showed an absorbance of 1.35 with 65.5% activity for ferric reducing antioxidant power assay.

Keywords Antioxidant activity, *Penicillium citrinum*, Plackett-Burman design, Response surface methodology (RSM), Total phenolic content

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INTRODUCTION

Fungi are recognized as producers of various secondary metabolites and have provided several bioactive compounds currently used as pharmaceuticals. Fungi of the genus *Penicillium* are promising candidates for the discovery of novel biologically active compounds. This genus comprises more than 200 described species and many are common soil inhabitants [1]. *Penicillium* species produce a much diversified array of active secondary metabolites, including antibacterial, antifungal substances, immunosuppressants, and cholesterol-lowering agents [2]. Thousands of *Penicillium* isolates have probably been screened for bioprospecting since the discovery of penicillin, and various new bioactive metabolites have been discovered indicating their importance as sources of novel bioactive molecules to be used by pharmaceutical industry [3].

Various *Penicillium* isolates are also known to produce secondary metabolites that possess antioxidant potential. Antioxidants prevent oxidative damage caused by free radicals; it can interfere with the oxidation process by reacting with free radicals, chelating metals and also by acting as oxygen scavengers. The free radicals attributed as reactive oxygen species (ROS), are continuously generated inside the human body which are detoxified by the antioxidants present in the body. However, overproduction of ROS and/or inadequate antioxidant defense can easily affect and cause oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA. This oxidative damage is a critical etiological factor implicated in several chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis and neurodegenerative diseases and also in the ageing process [4].

Hence, the present study was planned to assess the antioxidant potential of soil fungal isolate *Penicillium citrinum* by various assay procedures [1,1-diphenyl-2-picryl hydrazyl (DPPH) assay, reducing power, ferrous ion and nitric oxide ion scavenging activity, ferric reducing antioxidant power (FRAP) assay] and to optimize the conditions for enhancement of antioxidant activity using one-factor-at-a-time classical approach, Plackett-Burman design and response surface methodology.

MATERIALS AND METHODS

Experimental

Penicillium citrinum was isolated from soil of Amritsar, Punjab, India (31° 37' 59" North, 74° 51' 56" East) and identified on the basis of standard protocols and the identity was confirmed by MTCC, IMTECH, Chandigarh, India. To study the antioxidant potential, the fungus was grown on 50 ml Czapek dox's broth (sucrose 3%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, KCl 0.05%, $FeSO_4$ 0.001%). The medium was inoculated with two discs (8mm) of fungal mycelia obtained from 6-7 days grown culture on Yeast extract glucose agar plates. The growth was carried out under stationary conditions at 25° C. After incubation for 10 days, the culture broth was filtered through Whatmann filter paper no.1 and the filtrate so obtained was analyzed for antioxidant potential by different assay procedures and total phenolic content was estimated with Folin-Ciocalteau (FC) method.

Assay procedures for antioxidant activity and determination of total phenolic content (TPC)

Different assay procedures for antioxidant activity was used as described earlier [5]. The total phenolic content was determined colorimetrically using the Folin-Ciocalteau (FC) method according to Arora & Chandra, 2010 [5].

Statistical optimization of the medium

Plackett-Burman experimental design

The screening of most significant parameters affecting antioxidant potential was studied by the Plackett–Burman design. The 5 factors, which are components of Czapek dox's medium (sucrose, $NaNO_3$, K_2HPO_4, KCl, and $MgSO_4$) were examined. Total 14 tests were designed including 12 combinations and 2 repetitions at central point which contain different concentration of each factor and the effect of each factor was determined by the difference between the average of the + and – responses. The significance level of effect of each factor was determined by student's t test. The most common mean of assessing significant value is the *p* value which was also evaluated for each factor.

Response surface methodology through Box-Behnken designs

On the basis of earlier study, screening of different carbon and nitrogen sources through one-factor-at-a-time classical method (data not shown) and different components by Plackett-Burman design, sucrose and $NaNO_3$ were found to be the best for antioxidant activity. Sucrose as carbon source, $NaNO_3$ as nitrogen source and temperature were taken independent variables for the optimization by RSM using Box-Behnken designs of experiments. Each variable was studied at three levels (-1, 0, +1); for sucrose these were 5%, 3% and1%; $NaNO_3$: 0.05%, 0.2% and 0.35 %; temperature: 15°C, 25°C and 35°C

The experimental design included 17 flasks with five replicates having all the three variables at their central coded values. The DPPH assay, reducing power, ferrous ion and nitric oxide ion scavenging activity, FRAP assay and their total phenolic contents was taken as a responses $G_{(1\text{-}6)}$. The mathematical relationship of response G (for each parameter) and independent variable X (X_1, Sucrose; X_2, $NaNO_3$; and X_3, temperature) was calculated by the following quadratic model equation.

$$G_{(1\text{-}6)} = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X^2_1 + \beta_{22}X^2_2 + \beta_{33}X^2_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \quad (1)$$

Where, G is the predicted response; β_0, intercept; β_1, β_2, and β_3, linear coefficients; β_{11}, β_{22} and β_{33}, squared coefficients and β_{12}, β_{13} and β_{23} interaction coefficients. MINITAB statistical software was used to obtain optimal working conditions and generate response surface graphs. Statistical analysis of experimental data was also performed using this software.

Estimation of half maximal effective concentration (EC_{50}) value

On the basis of above experimentation, *Penicillium citrinum* was grown on Czapek dox's medium and to estimate half maximal effective concentration (EC_{50}) value, the extracellular fungal extract was lyophilized. The lyophilized extract was weighed and stock solution of 2 mg/ml was made for the fungal extract which was further diluted to obtain the different concentrations ranging from 0.02 to 1 mg/ml. EC_{50} represents the amount of sample (mg extract/ml) necessary to scavenge free radicals by 50%. EC_{50} value is also the effective concentration at which the absorbance for reducing power is 0.5. Such EC_{50} value was calculated from the graph plotting inhibition percentage against extract concentration [6].

RESULTS AND DISCUSSION

Natural compounds like secondary metabolites produced from various fungi have the potential to serve as alternatives to many conventional and synthetic bioactive

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compounds. Recent studies demonstrated the effectiveness of such natural compounds from various fungi as bioactive agents including antioxidant activity along with their amenability to easy manipulations. Nutritional and fermentation conditions for the growth of fungi have intense effect on secondary metabolites production. Optimization of fermentation medium and conditions is important step to be carried out for maximum yield of many secondary metabolites. The application of statistical experimental design techniques in fermentation process development can result in improvement of product yield and reduce process variability. Thus, the present study is based on statistical design as it is an efficient way to generate useful information with limited experimentation, thereby cutting the process development time and cost [7].

Initially, to assess the antioxidant potential by various assay procedures all the experimentation was done by growing *Penicillium citrinum* on Czapek dox's broth medium. Effect of different carbon and nitrogen sources on antioxidant potential were studied by various assay procedures (data not shown). Of the various carbon and nitrogen sources tested, sucrose and $NaNO_3$ supported the maximum antioxidant activity by *Penicillium citrinum* which is in consonance with earlier studies carried out on various *Aspergillus* spp. [8].

The total phenolic content (TPC) of *Penicillium citrinum* extracts have been expressed as gallic acid equivalent (GAE) i.e. mg gallic acid /100 ml culture. TPC are known to be responsible for antioxidant activity and the *Penicillium citrinum* possessed high TPC, which is positively correlated with its antioxidant potential. The highest TPC yield was 17.2 mg/ml in the presence of sucrose and $NaNO_3$ in the medium. It is known that the phenolic compounds is responsible for the antioxidant properties which can play an important role in absorbing and neutralizing the free radicals by acting as reducing agents and hydrogen donor or quenching singlet and triplet oxygen or decomposing peroxides. The importance of phenolic contents has been endorsed by their high content in *Penicillium citrinum* and their antioxidant activity is quite comparable to that of various mushrooms as well as medicinal plants [9].

Plackett-Burman design for selection of significant components

A Plackett-Burman design experiment was employed to evaluate the influence of five factors (sucrose, $NaNO_3$, K_2HPO_4, KCl, and $MgSO_4$) and their importance in culture medium to obtain better antioxidant activity. Antioxidant potential of *Penicillium citrinum* assayed by different procedures and extra-cellularly produced total phenolic content varied significantly with the 14 run of different combinations of the media components (Table 1). The maximum antioxidant potential along with high TPC was observed in run order 13 and run order 14 which was followed by run order 5. The results were subjected to regression analysis and the analysis of variance (ANOVA) which revealed sucrose and $NaNO_3$ to have statistically significant effect on antioxidant potential with p value ≤ 0.05 and ≤ 0.5, respectively which showed that of the five variables only sucrose and $NaNO_3$ played a critical role for antioxidant activity. Based on these results, sucrose and $NaNO_3$ were selected as two variables and applied to optimize the medium composition by RSM. To know the optimum temperature and its interaction with other variables (sucrose and $NaNO_3$), it was chosen as a third variable as it is the important physical parameter that affects the activity as well as fungal growth.

Box-Behnken design

Fitting the model

The data obtained from quadratic model equation was found to be significant. It was verified by F value and the analysis of variance (ANOVA) by fitting the data of all independent observations in response surface quadratic model (Table 2). R^2 value for all the responses ranged between 91-76% which showed suitable fitting of the model in the designed experiments. The final predictive equations for each response: DPPH assay (G_1), reducing power (G_2), ferrous ion scavenging activity (G_3), FRAP assay (G_4) and nitric oxide ion scavenging activity (G_5) and total phenolic contents (G_6) obtained are as follow:

$$G_{(1)} = -13.85 + 25.54X_1 + 127.24X_2 + 2.51\,X_3 - 2.74X^2_1 + 26.49\,X^2_2 - 0.03\,X^2_3 - 44.50\,X_1X_2 - 0.01\,X_1X_3 - 0.62\,X_2X_3 \quad (2)$$

$$G_{(2)} = -1.813 + 0.553\,X_1 + 4.551X_2 + 0.099\,X_3 - 0.054X^2_1 - 0.560X22 - -0.001\,X23 - 1.283X1X2 + 0.002\,X1X3 - 0.029X2X3 \quad (3)$$

$$G\,(3) = -28.88 + 23.89X1 + 115.26X2 + 2.75X3 - 1.99X21 - 11.89X22 - 0.03X^2_3 - 36.25X_1X_2 - 0.18\,X_1X_3 - 0.63\,X_2X_3 \quad (4)$$

$$G_{(4)} = -27.53 + 25.41X_1 + 125.32X_2 + 2.26X_3 - 2.03\,X^2_1 - 24.22X^2_2 - 0.02X^2_3 - 36.56\,X_1X_2 - 0.22\,X_1X_3 - 0.75\,X_2X_3 \quad (5)$$

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Table 1. Plackett-Burman design variables with different antioxidant potential as response

Variables (%)						Antioxidant activity (% activity)					
Run	Sucrose	$NaNO_3$	K_2HPO_4	$MgSO_4$	KCl	DPPH Assay	Reducing power	Fe^{2+} scavenging activity	FRAP assay	NO scavenging activity	TPC (mg/ml)
1	5.0	0.000	0.18	0.000	0.000	65.0	0.60	50.2	48.9	54.4	4.2
2	5.0	0.350	0.00	0.090	0.000	72.0	0.92	54.6	52.4	56.8	12.4
3	0.0	0.350	0.18	0.000	0.090	29.0	0.19	20.4	23.6	34.0	1.3
4	5.0	0.000	0.18	0.090	0.000	45.0	0.32	30.4	27.9	35.6	3.8
5	5.0	0.350	0.00	0.090	0.090	78.0	1.03	61.8	58.7	67.4	18.8
6	5.0	0.350	0.18	0.000	0.090	70.0	0.83	52.4	53.0	55.2	10.8
7	0.0	0.350	0.18	0.090	0.000	24.0	0.12	18.2	17.4	25.7	1.2
8	0.0	0.000	0.18	0.090	0.090	45.0	0.34	30.9	30.3	32.4	3.2
9	0.0	0.000	0.00	0.090	0.090	20.0	0.08	16.3	18.7	22.9	0.8
10	5.0	0.000	0.00	0.000	0.090	68.0	0.64	52.4	50.8	53.2	7.8
11	0.0	0.350	0.00	0.000	0.000	52.0	0.54	48.8	45.4	50.3	6.4
12	0.0	0.000	0.00	0.000	0.000	0.0	0.00	0.0	0.0	0.0	0.0
13	2.5	0.175	0.09	0.045	0.045	77.5	1.03	60.6	62.4	65.4	17.6
14	2.5	0.175	0.09	0.045	0.045	78.0	1.01	61.2	60.3	65.9	19.2

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Table 2. Box-Behnken designs of different variables with their responses

Variables (%)				Antioxidant activity (% activity)					
Run	Sucrose	$NaNO_3$	Temperature	DPPH Assay	Reducing power	Fe^{2+} scavenging activity	FRAP assay	NO scavenging activity	TPC (mg/ml)
1	1	0.05	25	60.40	0.520	50.8	49.33	50.8	10.9
2	5	0.05	25	80.90	1.200	62.8	62.40	67.3	20.8
3	1	0.35	25	81.20	1.200	63.8	60.40	68.9	20.9
4	5	0.35	25	48.30	0.340	32.3	29.60	38.3	7.8
5	1	0.2	15	46.90	0.240	30.4	29.40	35.8	5.2
6	5	0.2	15	52.60	0.430	48.9	50.80	62.8	9.3
7	1	0.2	35	76.20	0.846	58.2	56.70	62.8	15.4
8	5	0.2	35	80.80	1.200	62.3	60.60	67.4	20.8
9	3	0.05	15	72.30	0.700	52.6	50.30	56.3	10.4
10	3	0.35	15	73.40	0.732	54.8	55.80	57.8	12.2
11	3	0.05	35	79.80	1.120	62.4	60.30	65.8	18.2
12	3	0.35	35	73.44	1.180	65.5	62.5	70.90	16.3
13	3	0.2	25	72.70	0.980	64.2	61.8	68.90	19.2
14	3	0.2	25	73.80	1.020	64.3	61.8	68.90	18.8
15	3	0.2	25	72.80	0.970	64.7	61.6	68.90	17.6
16	3	0.2	25	73.90	1.100	65.7	62.6	69.99	18.6
17	3	0.2	25	72.06	1.100	65.8	64.7	70.10	19.8

$$G_{(5)} = -28.4 + 25.0\,X_1 + 174.6X_2 + 2.5X_3 - 1.5X^2_1 - 117.8X^2_2 - 0.0X^2_3 - 39.3\,X_1X_2 - 0.3\,X_1X_3 - 0.8\,X_2X_3 \quad (6)$$

$$G_{(6)} = -35.28 + 7.80X_1 + 89.00X_2 + 2.23X_3 - 0.66\,X^2_1 - 46.67X^2_2 - 0.03\,X^2_3 - 19.17\,X_1X_2 - 0.02\,X_1X_3 - 0.62\,X_2X_3 \quad (7)$$

The optimized values of factors were validated by repeating the experiment in triplicate flasks.

Effect of different variables on DPPH assay

Sucrose significantly affected the DPPH activity. The Linear effect (X_1), squared effect (X_1^2) as well as interactive effect (X_1X_2) was highly significant (p value < 0.05). Linear effect (X_2; X_3) was significant with p value <0.5. The response surface graphs showed the highest activity at concentration of sucrose in between 4-5% but with least amount of $NaNO_3$. Maximum predicted value for DPPH scavenging effect (90 %) was obtained at 4.5 % of sucrose, 0.05 % of $NaNO_3$ and at 35° C (Fig.1a).

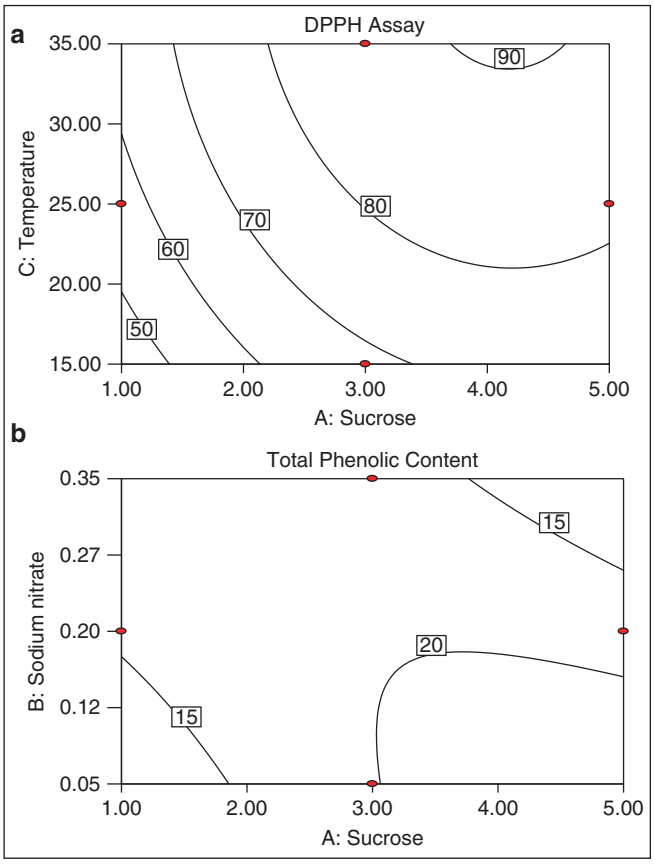

Figure 1. Contour plot showing effect of different variables on **(a)** DPPH assay (% activity) (hold value: 0.05% of sodium nitrate);**(b)** Total phenolic content (mg/ml) (hold value: 35°C Temperature).

Effect of variables on reducing power

Linear effects (X_1, X_2, X_3) and squared effects (X_1^2) were significant with p value <0.5. Squared effect (X_1^3) was most significant at p value ≤ 0.005. The response surface graphs showed the highest reducing potential with an absorbance of 1.4, when the concentration of sucrose is 4.0 % with 0.05 % of $NaNO_3$ and at a temperature of 35° C.

Effect of variables on FRAP assay, ferrous ion and nitric oxide ion scavenging activity

The linear (X_1, X_2, X_3), squared effect of sucrose (X_1^2) and interactive effect between sucrose-sodium nitrate (X_1X_2) are significant with p value ≤ 0.05 for all the scavenging activities. Ferric reducing antioxidant power was maximum (65 %) at 35° C, with medium composition of 4.5 % of sucrose and 0.05 % of $NaNO_3$. Similarly, highest scavenging effect of 70 % for both nitric oxide and ferrous ion was observed at 35° C with 4.5 % and 0.05 % of sucrose and $NaNO_3$ respectively. Antioxidant potential as assayed by different procedures demonstrated decrease in activity with the further increase of $NaNO_3$ concentration.

Effect of variables on total phenolic content

Interactive effect (X_1X_2) was significant with p value ≤ 0.005 while linear effect (X_1, X_2, X_3) showed significance at p ≤ 0.05 for total phenolic content. The response surface graphs showed the highest TPC yield at concentration of sucrose in between 3-5% but with range of 0.05-0.2% of $NaNO_3$. The highest amount of TPC (20 mg/ml) was obtained at 4 % of sucrose and with 0.05 % of $NaNO_3$ concentration at 35° C (Fig. 1b)

Validation of results

Thus, from the overall assessment, the optimized conditions for different assay procedures may be concluded as, 4.5% sucrose, 0.05 % $NaNO_3$ and incubation temperature of 35° C while other media components were retained as standard concentration in Czapek Dox's medium. The F value and R^2 value showed that the model correlated well with measured data and was statistically significant. To confirm the model adequacy for predicting maximum scavenging activity, the verification experiment using the optimum medium composition was performed. Under optimal condition, *Penicillium citrinum* showed 90.1%, 70.9% and 70.1% scavenging effect for DPPH radical, ferrous ion and nitric oxide ion, respectively. The yield for TPC

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was 22.3 mg/ml and reducing power showed absorbance of 1.35 with 65.5% activity for FRAP Assay. The results showed the scavenging effect for DPPH radical, ferrous ion and nitric oxide ion was enhanced by 1.14, 1.1 and 1.12 folds, respectively while reducing potential and ferric reduction rate was enhanced by 1.3 and 1.1 folds. The production of TPC was enhanced by 1.4 folds.

A good agreement between the predicted and experimental results verified the validity of the model and the improvement of antioxidant activity indicated that RSM is a powerful tool for determining the exact optimal values of the individual factors and the maximum response value.

EC50 value of lyophilized extracellular fungal extract against different free radicals

In order to calculate the EC_{50} value against different free radicals, antioxidant activity was estimated at different concentrations. The results indicate the increase in activity with the increase in concentration of the extract irrespective of the assay procedure used (Fig. 2). The EC_{50} values of extract of *P. citrinum* (0.05 mg/ml) were similar for DPPH ion scavenging activity, reducing potential, ferric ion reduction capacity, ferrous ion scavenging potential and nitric oxide ion scavenging activity.

Correlation between antioxidant activity and TPC

The results obtained indicate *Penicillium citrinum* to be a potent antioxidant producer having broad spectrum against various free radicals. To measure the total antioxidant potential using a single assay procedure seems to be unrealistic, yet there are numerous published methods claiming to measure total antioxidant activity *in vitro* [4].Thus, to validate the reliability of different methods, the correlation between the different assay procedures is necessary. Previous studies have shown the linear correlation between total phenolic content and antioxidant activity, in this study too, total phenolic content of *Penicillium citrinum* correlated well with the antioxidant activity.

The results of *Penicillium citrinum* are comparable with the antioxidant activity of various other fungi, *Aspergillus candidus*, *Chaetomium* sp., *Cladosporium* sp, *Colletotrichum gloeosporioides*, [10] many mushrooms and medicinal plants[11].

The study thus suggests that not only mushrooms and plants but some other fungi may also be a good source of antioxidant compounds and *Penicillium citrinum* is one such potential candidate offering a better scope for production and easier down streaming of such bioactive compounds. These findings will facilitate the further studies to gain better understanding of production of bioactive metabolites in fungi, which will be helpful in their biotechnological mass production in near future.

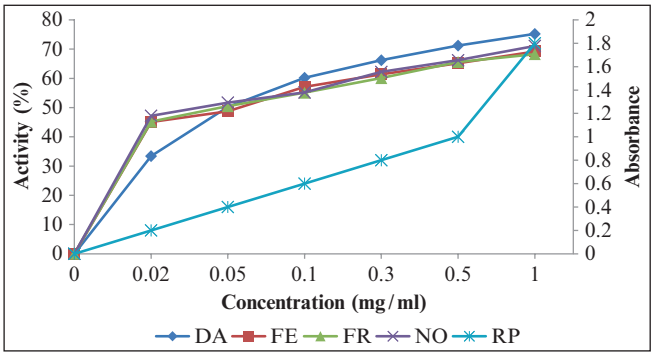

Figure 2. Antioxidant potential assayed by various procedures shown by lyophilized extract of *Penicillium citrinum* (DA: DPPH scavenging activity; RP: reducing power; FE: Ferrous ion scavenging activity; FR: FRAP assay; NO: Nitric oxide scavenging activity).

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