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Optimization Studies and Chemical Investigations of *Aspergillus terreus*-18 Showing Antioxidant Activity

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Aspergillus terreus-18 ethyl acetate extract was chemically analyzed using High performance liquid chromatography (HPLC). This led to the isolation of the butenolide butyrolactone I (BL-1) which was identified using nuclear magnetic resonance (NMR) and mass spectra. As a major compound, BL-1 was tested for its antioxidant activity through its ability to scavenge the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). It showed high scavenging activity with IC_{50} 439.09 (µg/ml). *A. terreus*-18 local isolate was chosen among 18 other isolates screened for their DPPH scavenging activities. *A.terreus*-18 showed highest scavenging activity (% inhibition), 80.4± 1.35. Malt peptone broth was then used as production media where *A.terreus*-18 was grown under static conditions, hence DPPH scavenging activity reached 85%. Optimization of different physicochemical parameters to enhance bioactivity and increase the total phenolic content (TPC) of the promising isolate was done through response surface methodology (RSM). Statistical approaches led to 1.17 fold increase of DPPH scavenging activity, 99.5% inhibition. This was obtained upon using (g/L) malt extract, 25; peptone, 6 as fermentation media with initial pH, 7 and volume 50% media /flask (50% aeration) and using inoculum size 15% v/v of 60 h aged culture incubated statically for 18 days at temperature 27±2 °C. Extracellular TPC and antioxidant activity positively correlated where TPC increased from 122.475 to 290.51 mg gallic acid/g of tested extract.

Keywords: *Aspergillus terreus*, Chemical investigations, Antioxidant activity, DPPH assay, Response surface methodology, Butyrolactone I

Introduction

Oxidation is crucial to produce energy to fuel biological processes for many living organisms [1, 2]. Cellular damage is caused during normal metabolic processes of aerobic cells by free radicals such as reactive oxygen species (ROS) or reactive nitrogen species (RNS). The most important class of radical species generated in living organisms are free radicals derived from molecular oxygen [3]

Oxidative stress is either the loss of natural antioxidant defenses or the imbalance in free radical production such as overproduction of ROS. This may lead to the oxidation of lipids, DNA or protein and is a major contributor to aging [4, 2], degenerative diseases such as cancer, compromised immune function, inflammation, renal failure [5, 6] and cardiovascular disease [7].

Antioxidants are essential for keeping normal cell function and health. They are compounds that prevent the initiation or propagation of oxidizing chain reactions and thus inhibit or delay oxidative damage related to aging and disease. Although animals have developed natural mechanisms to guard cells against free radical damage by neutralizing them, the amount of antioxidant produced under normal conditions is not always enough.

Consumption of synthetic antioxidants, for example butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have negative impact on health [2]. With increasing consumer awareness of food preparation, contention has surrounded the use of artificial antioxidants as preservatives, which has led to increasing interest in finding effective and safe preservatives from natural sources. Natural compounds obtained from

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fungi, plants, citrus fruits and leafy vegetables such as ascorbic acid, vitamin E, carotenoids, flavonols and phenolic acids, have the capacity for free radicals scavenging in living organisms [8].

Fungi are a well-known producer of many bioactive products, such as antibiotics, immunosuppressants, anticancers and antioxidants. Fungi produce diverse bioactive antioxidant secondary metabolites including phenolic compounds, polyketides and terpenes [9, 10]. Pharmacological studies of fungi have shown that the Ascomycete and Basidiomycete divisions are enormous sources of biologically active compounds, however less than ten percent of all species have been described and fewer have been tested for therapeutic importance [11, 12]. Being a natural source of antioxidants, joined with enormous biological activities, fungi are considered excellent health promoters.

The traditional method of optimization of parameters encompasses the optimization of one parameter at a time. This is not only time consuming, but also leads to an incomplete understanding of interaction between the factors [13]. In the optimization of media compounds, Plackett- Burman [14] designs are used to select the constituents that influence a system. However, they do not give an interactive effect for each constituent [15, 16] and further optimization is needed. Response surface methodology [17] is commonly used to understand and assess the interactions between different physiological and nutritional parameters [18].

In the present study, different fungal isolates were screened for their antioxidant activities through DPPH free radical scavenging ability. Results are presented as scavenging activity (% inhibition) of DPPH free radical. The fungal isolate with the highest activity was then subjected to statistical studies to optimize its antioxidant activity together with its total phenolic content (TPC). After optimization, the ethyl acetate extract was subjected to chromatographic and spectroscopic analysis for the isolation and purification of the major antioxidant compound.

Materials and Methods

Microorganisms

Fungal isolates used for screening were donated by the Center of Cultures of Chemistry of Natural and Microbial Products Department, National Research Center, Cairo, Egypt. The fungal strains were routinely grown on potato-dextrose agar (PDA) medium at 27± 2°C for 7 days until well sporulated and preserved at -80ºC in 50% (v/v) glycerol and subcultured every 3 months for maintenance.

Preparation of inoculum and fermentation conditions

Five ml distilled water were added to a 7 days old slant and a spore suspension was prepared to inoculate 50 mL modified glucose peptone yeast broth [2], composed of (g/L of distilled water): glucose, 20; peptone, 5; yeast extract, 5; NaCl, 5; K_2HPO_4, 5, in 250 mL Erlenmeyer flask. Final pH was adjusted to 7 at 25°C before autoclaving. Inoculum flask was then incubated in a rotatory shaker (Sanyo Gallenkamp PLC Refrigerated orbital incubator shaker model 220/240) at 150 rpm and temperature 27± 2°C for 2 days. Five ml inoculum were then transferred to either 250 mL Erlenmeyer flasks containing 50 mL fermentation broth, or spread on the surface of the petri dishes (100 mm x 15mm) containing solid media.

Extraction

At the end of the incubation period, the whole culture broth or the solid media, were mixed with or soaked in equal volume of ethyl acetate overnight then filtered using Whatman filter paper No.1. The extract was evaporated *in vacuo* under reduced pressure using rotary evaporator at a temperature not exceeding 50°C. The obtained residue was then assayed for its bio-activity.

Screening experiment and production media

Fifty mL of the production media, glucose peptone yeast broth, were inoculated with 5 mL inoculum of each of the 18 tested fungi then incubated in a rotary shaker at 150 rpm at 27± 2°C for 7 days before extraction.

Fungal isolate(s) that showed best antioxidant activity was used to inoculate malt peptone broth [2] (malt extract, 20 g; peptone, 5 g; distilled water to 1L; pH, 7) as production media, and incubated under static conditions at 27± 2°C for 14 days.

Scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical

The scavenging activity for DPPH free radical was measured according to Zhao *et al.* [19] with some modifications. Samples (100µLfungal extract) from stock solution of concentration 1 mg/ml in ethyl acetate were mixed with 900µl of 0.1mM DPPH solution in methanol. The mixture

was shaken vigorously and allowed to reach a steady state for 30min in dark at temperature 37°C. Decolourization of DPPH was determined by measuring the absorbance at 517nm, and the DPPH radical scavenging was calculated according to the following equation:

Scavenging activity (%) = $[(A_1-A_2)/A_1]$ x100

Where A_1 was the absorbance of the DPPH solution without adding extract and A_2 was the absorbance of DPPH with the test sample. Ascorbic acid was used as the standard, all the tests were performed in triplicate and the results are the averages of products percentage.

To estimate the IC_{50}, different concentrations (100, 250, 500 and 1000 µg/mL) of the total extract were tested for their scavenging activity and a linear equation was established.

Determination of total phenolic content (TPC)

The total phenolic content was determined colorimetrically according to the Folin-Ciocalteu procedure as described by Singleton *et al*. [20]. Total extract (100 µL) was transferred into a test tube and the volume was adjusted to 3.5mL with distilled water and was oxidized through the addition of 250µL of Folin-Ciocalteau reagent. After 5 min, the mixture was neutralized with 1.25mL of 20% aqueous sodium carbonate (Na_2CO_3) solution. After 40min, the absorbance was measured at 725nm against the solvent blank. The total phenolic content was determined by means of a calibration curve prepared with gallic acid, and expressed as mg of gallic acid equivalent (mg GAE) per gm of sample.

Statistics

All experiments were performed in triplicates, the data shown in the corresponding tables and figures were the mean values of the experiments and the relative standard deviations were shown (mean ± SE).

Experimental design and optimization

Experimental design as two steps sequential optimization is used to screen many variables together in one experiment and to optimize them for the desired response in a much faster way than examining one variable at a time.

Plackett-Burman design

Plackett-Burman design was used to screen the most significant parameters affecting antioxidant potential, represented by percent of scavenging activity of DPPH free radical, as well as the total phenolic content of the crude extract of the active isolate. Seven independent variables were screened in nine combinations, organized according to the Plackett-Burman design matrix. For each variable, a high level (+) and low level (-) was tested. All trials were performed in triplets and the averages of products percentage were treated as the responses. The main effect of each variable was determined by the following equation:

$$E_{xi} = (\Sigma_{Mi+} - \Sigma_{Mi-}) / N$$

Where E_{xi} is the variable main effect, Mi+ and Mi- are either the product scavenging activity or the total phenolic content of the extract in trials where the independent variable (xi) was present in high and in low settings, respectively, and N is the number of trials divided by 2.

Box-Behnken design

In the second phase of medium formulation for maximum antioxidant potential, the Box-Behnken experimental design was applied where the most significant independent variables, named (X_1), (X_2) and (X_3) were included and each factor was examined at three different levels, low (-), high (+) and central or basal (0). Thirteen combinations and their observations were fitted to the following second order polynomial mode:

$$Y_{(1,2)} = b_o+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{27}X_2^2+b_{33}X_3^2$$

Where, Y is the dependent variable (Y_1, scavenging activity (% inhibition) and Y_2 total phenolic content); X_1, X_2 and X_3 are the independent variables; b_o is the regression coefficient at center point; b_1, b_2 and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are second-order interaction coefficients; and b_{11}, $b_{22}$and b_{33} are quadratic coefficients. The values of the coefficients were calculated and the optimum concentrations were predicted using JMP software. The quality of the fit of the polynomial model equation was expressed by R^2 (regression coefficient). If the proposed model is adequate, as revealed by the diagnostic tests provided by an analysis of variance (ANOVA) and residual plots, contour plots can be usefully employed to study the response surface and locate the optimum operational conditions [21, 22]. The 3D graphs were generated to understand the effect of selected variables individually and in combination to determine their optimum level for maximal scavenging activity. The F-test was performed to determine factors having a significant effect ($P < 0.1$).

Chemical investigations and Purification of fungal major secondary metabolite(s)

High performance liquid chromatography (HPLC)

Semi preparative separations were carried out at Nawah scientific laboratory, Egypt (www.nawah-scientific.com), with a PuriFlash 4100 system (Interchim; Montluçon, France) consisting of a mixing HPLC quaternary pump, a PDA-UV-Vis detector 190-840 nm, a fraction collector, and a sample loading module. For system controlling and process monitoring, Interchim Software 5.0 was used. Crude extract (2 g) was dissolved in 50 ml of ethyl acetate then introduced into the column (80 gm - flash/prep - NP/RP column) via dry load using 12 gm silica/celite.

Preparative HPLC was done for isolation of pure compounds from fractions obtained from the PuriFlash Column, using LC10AD VP SHIMADZU HPLC (Kyoto, Japan) with Waters pump and UV detector at λ = 254 or 210 nm. The used column was reversed phase (Cosmosil 20 x 250 nm) column, with typical flow rate 4.3 mL/min. About 40 mg of the sub-fraction was dissolved in appropriate amount of column mobile phase, and then was injected to HPLC manually using a micro syringe. The solvent systems were 70% methanol, in isocratic manner. The fractions were collected separately in small vials using UV detector adjusted to 254 and 210 nm.

Structure Elucidation

Structural elucidation of the isolated pure compound(s) were carried out by combination of spectroscopic techniques, mainly using 1D-NMR (^{1}H and ^{13}C NMR) and MS spectroscopy methods. The comparison of NMR data of pure compound(s) with other known published compounds was performed using the Reaxys databases and Scifinder database (Chemical Abstracts), which are available at websites (https://www.reaxys.com), and (http://www.cas.org/products/scifindr/index.html) respectively.

The NMR measurements (^{1}H, ^{13}C) of the major compound was done using a Varian 500 MHz. Spectra of NMR instrument were calibrated using solvent reference signals (^{13}C: CD_3OD 49.0 ppm) or a signal of the portion of the partially or not deuterated solvent (CH_3OD in CD_3OD δ 3.30 ppm). Structural assignments were based on spectra resulting from one or more of the following NMR experiments: ^{1}H, ^{13}C. The observed chemical shift *(δ)* values were given in ppm and coupling constants *(J)* in Hz.

The apparatus used for mass spectroscopy (MS) was Electrospray ionization mass spectrometry (ESI): Bruker Esquire 3000 pulse.

Results and Discussion

Screening experiment

The importance of fungi had been established for long time ago as potential sources of pharmaceutical leads, as many of fungi were reported to produce unique bioactive metabolites as anticancer, antiviral and antimicrobial agents. DPPH assay is one of the most popular and frequently employed method among antioxidant assays. The method is efficient, simple, quick and relatively inexpensive [23].

In this study, screening the DPPH free radical scavenging activities of the ethyl acetate extracts of 18 local endophytic fungal isolates was conducted. The activities of the extracts were compared to that of ascorbic acid. Among the screened fungi, the highest radical scavenging activity was observed for the extract of the fermentation media of *Aspergillus terreus*-18, 80.4%, and *Aspergillus ochraceus,* 75.3%, at concentration 1mg/mL (Fig. 1).This result link with some previous findings of endophytic fungi and their antioxidant activity. Terrestrial or marine endophytes showed promising antioxidant potential in different assays such as *Aspergillus sp* Y16 isolated from mangrove [24]. Yadav *et al.* [25] investigated crude extracts of endophytic fungi isolated from *Eugenia jambolana* for their antioxidant potential. *Chaetomium sp*. and *Aspergillus sp*. showed a high antioxidant capacity value of 80%, *Aspergillus peyronelii* and *Aspergillus niger* strain were having 71% and 72% of reducing potential respectively. 114 out of 347 endophytic fungi isolated from *Rhodiola spp*. showed DPPH radical-scavenging rates >50%, and those of five isolates were >90% [26]. Also *Chaetomium sp.* isolated from medicinal plant *Gynostemma pentaphylla showed* promising antioxidant potential in different assays [27].

Selection of the suitable production media is an essential part in biopharmaceutical industries targeting the highest possible product of interest. Regarding fungal physiology, it is important for growth, building of bioactive metabolites [28]. Malt extract agar or broth medium is frequently used for cultivation of filamentous fungi especially *Aspergillus* species as it provides the fermentation environment rich in nutrients supporting their natural growth and sporulation.

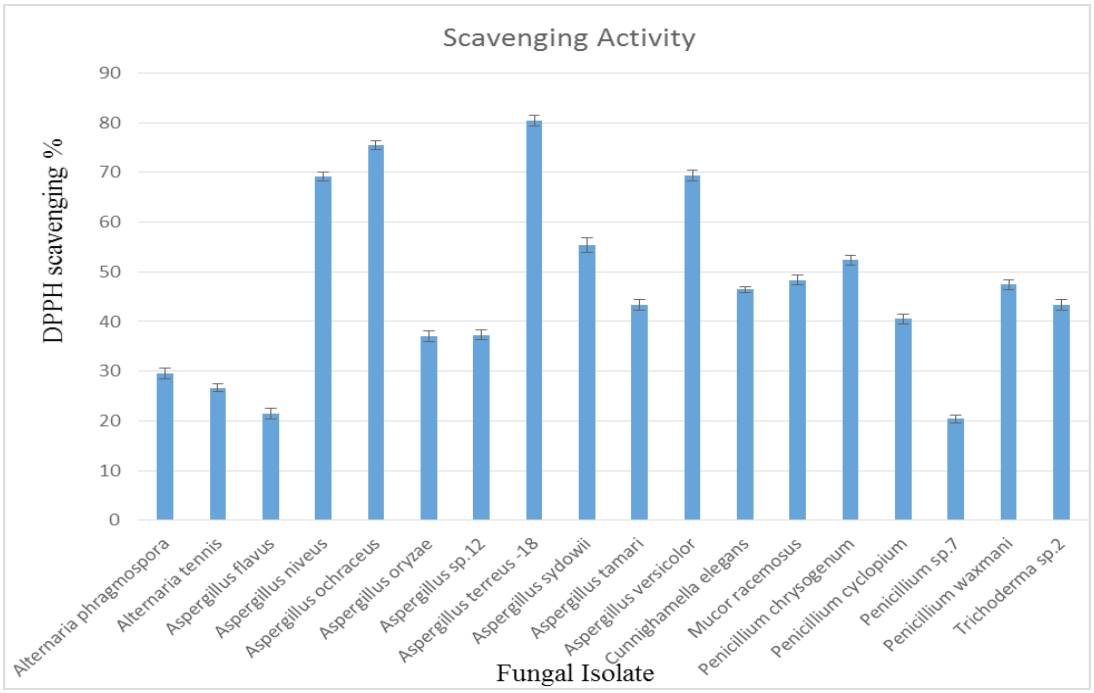


Fig. 1. Graph comparing scavenging activities of different fungal isolates for DPPH free radical.

Static condition or low rpm have been employed by several researchers for producing secondary metabolites showing antioxidant activities [29, 30, 31, 32, 33]. This may be due to the production of low amount of phenolic compounds thought to be responsible for fungal antioxidant activity under shaking conditions [30].

Thus, malt peptone broth was used as antioxidant secondary metabolites production media from *Aspergillus terreus*-18 under static condition for 14 days. Upon using these conditions, DPPH scavenging activity reached 85%.

Quantitative estimation of the total phenolic content in the crude extract of the active fungal Isolate:

The total phenolic content of *Aspergillus terreus*-18 extract has been expressed as gallic acid (GA) equivalents (mg GA per gm of active extract), by using standard curve of GA with R^2 = 0.998. By substitution in the equation

$$Y=0.024X+0.018$$

The total phenolic content of *Aspergillus terreus*-18 extract was equivalent to 122.475 mg gallic acid/gm of tested extract. Chandra and Arora [34] reported that TPC of three fungal isolates, *Aspergillus terreus* 1, *Aspergillus terreus* 2 and *Aspergillus fumigatus*, obtained from fermentation of Czapek Dox's medium was 16.75, 13.05 and 5.68 mg/ml.

Determination of fermentation factors affecting the scavenging activity of fungal extract using Plackett-Burman design

Optimization of fermentation medium and conditions is very important for minimizing the production cost and maximizing the yield of many secondary metabolites. Most of the recent optimization efforts have relied on statistical experimental design and response surface analysis. Statistical design is an efficient approach that can be applied to explain the main as well as interactive effects of fermentation parameters on the process performance. It is an effective way to get useful information with limited experimentation, thus minimize the process development time and cost [35]. Previous studies have shown linear correlation between antioxidant activity and total phenolic content, thus the scavenging activity was assayed and compared to the total phenolic content throughout the optimization experiment.

The screening of most significant parameters affecting antioxidant potential was studied by the Plackett-Burman design. Seven components were selected for the study where each variable was represented at two levels, high concentration

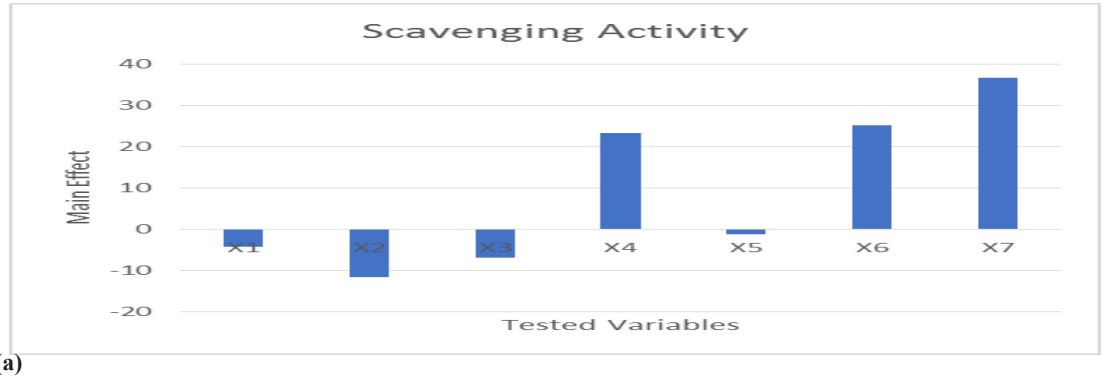
(+) and low concentration (−) in 8 trials. The maximum scavenging activity along with high TPC was observed in run ordered 8 (Table 1) where the scavenging activity increased from 85% to be 97% and the TPC was increased from 122.475 to 239.6 mg gallic acid equivalent per gram total extract.

Analysis of the effect of the physicochemical parameters of the media showed that the scavenging activity as well as TPC are highly affected by incubation time, inoculum size and

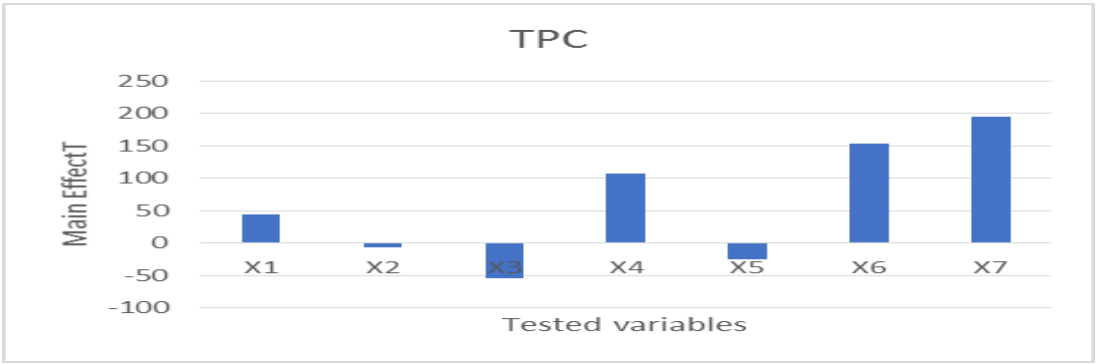
TABLE 1. Factors examined as independent variables affecting scavenging activity and TPC of *Aspergillus terreus*-18 and their levels in the Plackett-Burman experiment.

Trial no.	Independent variables							Scavenging activity (% Inhibition)	TPC (mg*/g)
	X_1 Malt (gm/l)	X_2 Peptone (gm/l)	X_3 PH	X_4 Time (days)	X_5 Inoculum age (h)	X_6 Inoculum Size (%)	X_7 Aeration (% medium/flask volume)		
1	-(15)	-(4)	-(5)	+(16)	+(60)	+(15)	-(10)	90.00	150.300
2	+(25)	-(4)	-(5)	-(12)	-(36)	+(15)	+(30)	95.20	203.223
3	-(15)	+(6)	-(5)	-(12)	+(60)	-(5)	+(30)	78.30	89.650
4	+(25)	+(6)	-(5)	+(16)	-(36)	-(5)	-(10)	70.10	55.330
5	-(15)	-(4)	+(7)	+(16)	-(36)	-(5)	+(30)	92.90	155.055
6	+(25)	-(4)	+(7)	-(12)	+(60)	-(5)	-(10)	60.20	38.657
7	-(15)	+(6)	+(7)	-(12)	-(36)	+(15)	-(10)	69.70	53.704
8	+(25)	+(6)	+(7)	+(16)	+(60)	+(15)	+(30)	97.00	239.600
9	0(20)	0(5)	0(6)	0(14)	0(48)	0(10)	0(20)	85.00	122.475

*Gallic acid equivalent



(a)



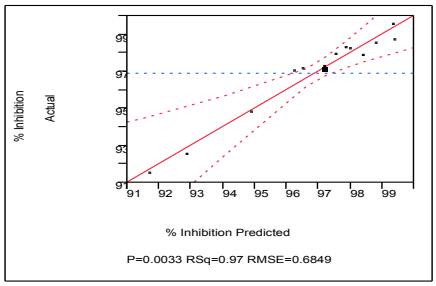
(b)

Fig. 2. Main effects of independent variables according to the results of the Plackett-Burman experiment (a) on DPPH scavenging activity of *Aspergillus terreus*-18 (b) on total extract TPC.

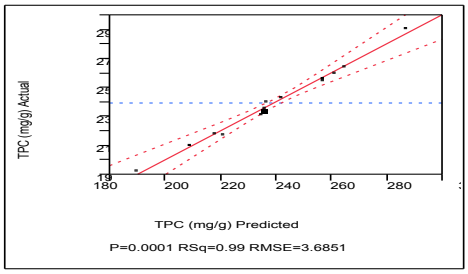
aeration (represented by media/flask volume ratio). On the other hand, concentration of malt and peptone, pH adjustment and inoculum age had relatively negative or lower effect (Fig. 2a and b). Based on the results obtained from the Plackett-Burman experiment a pre-optimized medium of the following composition: malt 25 g/L and peptone 6 g/L with initial pH 7, inoculated with inoculum size 15% of 60 h old culture was incubated statically for 16 days in 30% media/flask volume (aeration 70%), was used in response surface methodology (RSM) for further conditions optimization.

Optimization of the culture conditions using Box – Behnken design

Box – Behnken design of RSM was used to estimate the influence of the individual factors and their interaction effects on bioactive extract production. Response surface analysis showed 50% aeration, 15% v/v inoculum size and an incubation time of 18 days to be the optimum conditions. Other variables were kept in their (zero) initial values. The Actual by Predicted plot provides a visual assessment of model fit that reflects variation due to random effects. Figure 3a and b plots the actual values of either responses, scavenging activity and TPC respectively, against their marginal predicted values. The three-dimensional response surface plots represent the regression equations graphically. They are useful in understanding both the interaction and the main effects of tested factors on the response value. Figures 4a-c show the response surface plots of aeration and inoculum size, aeration and time and inoculum size and time on fungal scavenging activity respectively. While Fig. 5a-c show the



(a)



(b)

Fig. 3. Response Actual by Predicted plot of (a) Scavenging activity (% inhibition) (b) TPC.

TABLE 2. Box-Behnken design of different variables with their responses.

	Independent variables			Response	
	Aeration (% medium/flask volume)	**Inoculum size (ml)**	**Time (h)**	**Scavenging activity (% Inhibition)**	**TPC (mg*/g)**
Trial	X_1	X_2	X_3		
1	-(30)	-(5)	0(18)	92.50	209.770
2	+(50)	-(5)	0(18)	99.50	290.510
3	-(30)	+(10)	0(18)	94.76	217.400
4	+(50)	+(10)	0(18)	98.26	256.220
5	-(30)	0(7.5)	-(16)	97.00	218.000
6	+(50)	0(7.5)	-(16)	98.20	254.386
7	-(30)	0(7.5)	+(20)	91.50	192.360
8	+(50)	0(7.5)	+(20)	98.70	264.167
9	0(40)	-(5)	-(16)	98.50	259.804
10	0(40)	+(10)	-(16)	97.85	239.583
11	0(40)	-(5)	+(20)	97.13	231.322
12	0(40)	+(10)	+(20)	97.90	243.056
13	0(40)	0(7.5)	0(18)	97.23	235.520

*Gallic acid equivalent

response surface plots of aeration and inoculum size, aeration and time and inoculum size and time on fungal extract TPC respectively. Table 2 represents the design matrix of the coded variables together with the response of the scavenging activity and TPC of the extract.

Under these conditions, the scavenging activity of *Aspergillus terreus*-18 against DPPH was 99.5%. Fungal extract total phenolic content correlated well with the antioxidant activity which is in consonance with earlier studies [30, 36]. Total phenolic content increased upon optimization to reach 290.51 gallic acid equivalent per gm total extract.

Acceptability and fitness of the model were evaluated by ANOVA analysis **(**Tables 3 and 4**)**.

The regression equation obtained was as follows:

$$Y_{1(\%Inhibition)} = 97.23 + 2.3625\ X_1 + 0.1425\ X_2 - 0.79\ X_3 - 0.875\ X_1X_2 + 1.5\ X_1X_3 + 0.355\ X_2X_3 - 1.235X_1^2 + 0.26\ X^2_2 + 0.355\ X^2_3$$

$$Y_{2(TPC)} = 253.52 + 28.469125\ X_1 - 4.393375\ X_2 - 5.1085\ X_3 - 10.48\ X_1X_2 + 8.85525\ X_1X_3 + 7.98875\ X_2X_3 - 1.629\ X_1^2 + 9.584\ X_2^2 - 1.66275\ X_3^2$$

Where Y is the predicted response, scavenging activity or TPC, and X_1, X_2 and X_3 are the coded independent variables for aeration, inoculum size and incubation time respectively. The *F* ratio and the *P* value were used to check the significance of each coefficient, which also indicated the interaction strength between independent variables. The larger the magnitude of the *F* ratio and smaller the *P* value, the more significant is the

TABLE 3. Results for ANOVA analysis for optimization of *Aspergillus terreus*-18 scavenging activity.

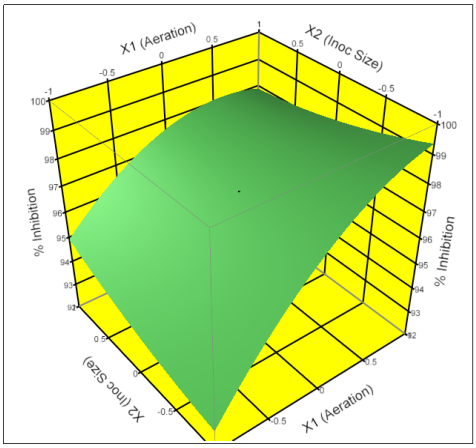
Term	Coefficient estimate	DF	SE	SS	t-value	F-ratio	P-value
Corrected Model		9		69.145993		16.3793	0.0033*
Intercept	97.23	1	0.395415		245.89		<.0001*
X_1 (Aeration)	2.3625	1	0.242141	44.651250	9.76	95.1930	0.0002*
X_2 (Inoc Size)	0.1425	1	0.242141	0.162450	0.59	0.3463	0.5818
X_3 (Time)	-0.79	1	0.242141	4.992800	-3.26	10.6443	0.0224*
X_1 X_2	-0.875	1	0.34244	3.062500	-2.56	6.5290	0.0509
X_1 X_3	1.5	1	0.34244	9.000000	4.38	19.1873	0.0072*
X_2 X_3	0.355	1	0.34244	0.504100	1.04	1.0747	0.3474
X_1^2	-1.235	1	0.356423	5.631600	-3.46	12.0061	0.0179*
X_2^2	0.26	1	0.356423	0.249600	0.73	0.5321	0.4984
X_3^2	0.355	1	0.356423	0.465323	1.00	0.9920	0.3650

DF: Degrees of freedom, SE: Standard error, SS: Sum of squares. R2 =R Squared = 0.967195 (Adjusted R Squared = 0.908145),*Significant at 5% level.

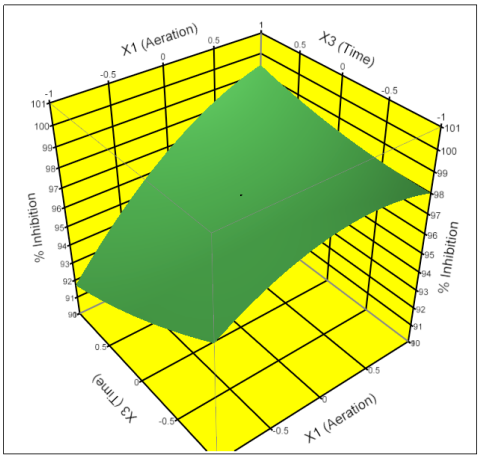
TABLE 4. Results for ANOVA analysis for optimization of TPC of *Aspergillus terreus*-18 total extract.

Term	Coefficient estimate	DF	SE	SS	t-value	F-ratio	P-value
Corrected Model		9		8233.9084		67.3709	0.0001*
Intercept	235.52	1	2.127575		110.70		<.0001*
X_1 (Aeration)	28.469125	1	1.302868	6483.9286	21.85	477.4712	<.0001*
X_2 (Inoc Size)	-4.393375	1	1.302868	154.4140	-3.37	11.3709	0.0198*
X_3 (Time)	-5.1085	1	1.302868	208.7742	-3.92	15.3740	0.0112*
X_1 X_2	-10.48	1	1.842534	439.3216	-5.69	32.3513	0.0023*
X_1 X_3	8.85525	1	1.842534	313.6618	4.81	23.0978	0.0049*
X_2 X_3	7.98875	1	1.842534	255.2805	4.34	18.7986	0.0075*
X_1^2	-1.629	1	1.91777	9.7981	-0.85	0.7215	0.4344
X_2^2	9.584	1	1.91777	339.1497	5.00	24.9747	0.0041*
X_3^2	-1.66275	1	1.91777	10.2083	-0.87	0.7517	0.4256

DF: Degrees of freedom, SE: Standard error, SS: Sum of squares, R^2 =R Squared = 0.991821 (Adjusted R Squared = 0.977099)*Significant at 5% level.



(a)



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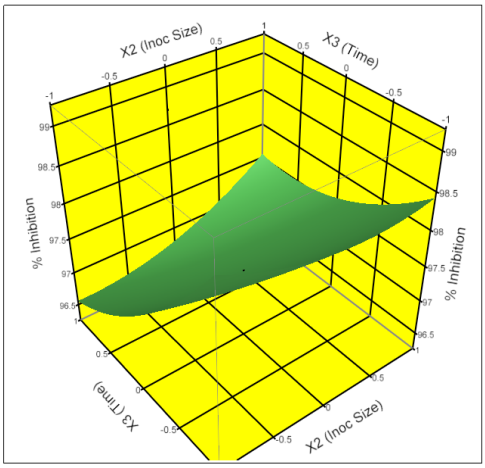

(c)

Fig. 4. Response surface plot of scavenging activity by interaction between (a) aeration and inoculum size (b) aeration and time (c) inoculum size and time.

corresponding coefficient.

Regarding the scavenging activity, ANOVA analysis showed a significant *F* ratio (95.1930) for the effect of aeration, which suggested the significance of the model. The probability value of the model is less than 0.05 (P =0.0002) which is considered highly significant. The determination of the coefficient (R^2) was calculated as (0.9672) for scavenging activity (a value of R^2>0.75 indicated the aptness of the model) which indicates the statistical model can explain 96.72% of variability of the response.

As for TPC of the extract, ANOVA analysis showed a significant *F* ratio (477.4712) also for the effect of aeration, which suggested the significance of the model. The probability value of the model is less than 0.05 (P <0.0001) which is considered highly significant. The determination of the coefficient (R^2) was calculated as (0.991821) for TPC which shows the statistical model can explain 99.2% of variability of the response.

This shows that the medium most suitable for growth may or may not be equally effective for secondary metabolites and thus enhancement of secondary metabolites can only be achieved through systematic manipulation of different parameters.

Verification of the optimization methods

A confirmatory experiment was performed under the predicted optimal conditions for optimum scavenging activity and higher TPC the basal medium was used as control. Under

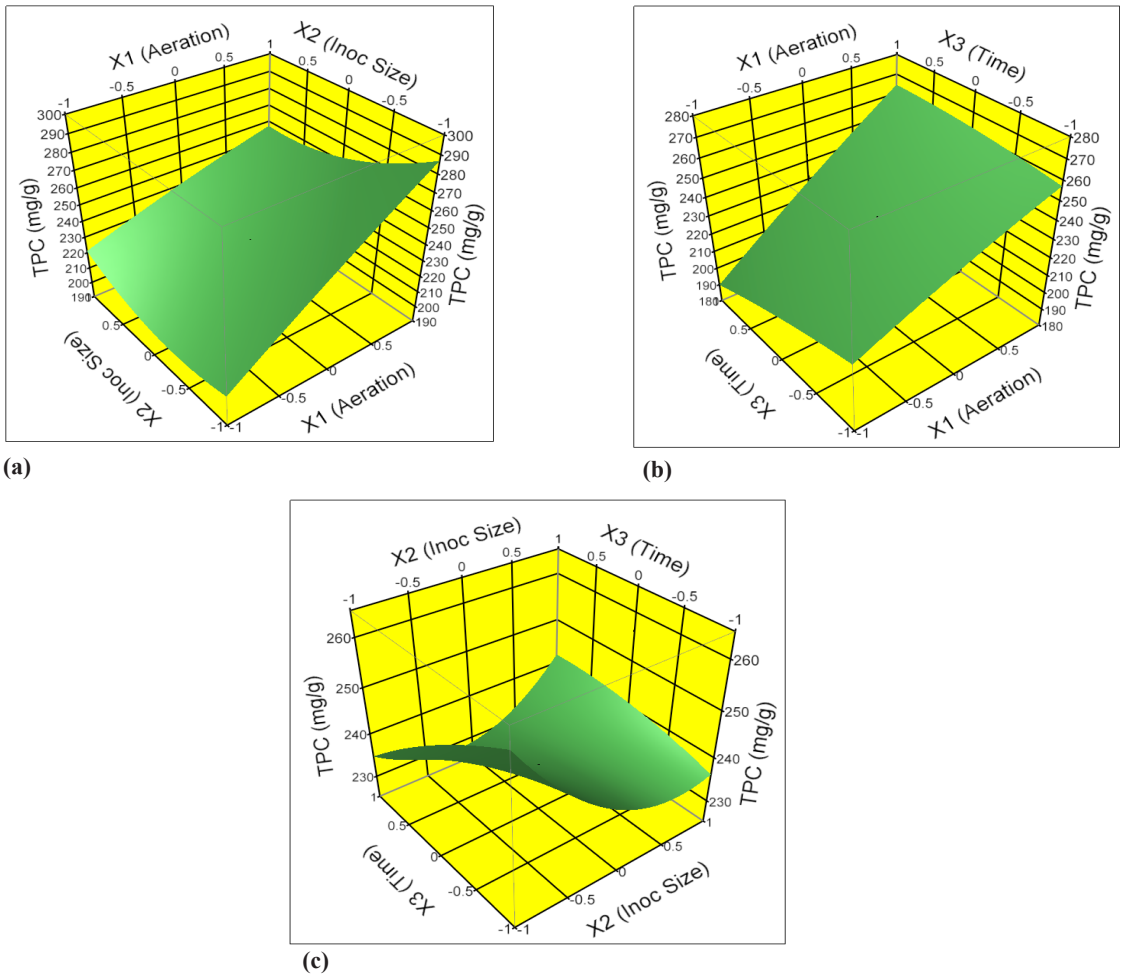

Fig. 5. Response surface plot of TPC by interaction between (a) aeration and inoculum size (b) aeration and time (c) inoculum size and time.

optimized conditions scavenging activity reached 99.5 % inhibition. The result indicated that the optimization condition increased activity by 1.17 fold times (99.5%) of the basal medium (85%). The two steps of optimization resulted in a formula of the following condition and composition (g/l): malt extract, 25; peptone, 6; with initial pH 7, inoculated with 15% v/v inoculum of age 60 h old and incubation time 18 days in media with 50% aeration under static condition.

Purification and structure elucidation of the major antioxidant compound

The interest of finding safe and effective natural compounds possessing cytoprotective activity has increased in order to develop lead compounds for chemopreventive agents. In an attempt to isolate and characterize the active compound(s) produced by the fungal isolate *Aspergillus terreus*-18, its ethyl acetate extract obtained from fermentation of malt peptone broth was dried to yield 2g of oily dark brown crude extract. It was then fractionated into nine fractions using PuriFlash Column. Based on the abundance of fraction, fraction 1, 4 and 8 were selected for further investigation and purification of its pure compounds. The three major fractions were spotted on TLC, developed using hexane/ethyl acetate (1:1) observed in a UV chamber at 254 nm and 365 nm for the fraction with the best separated spots. Upon staining developed TLC plates with DPPH solution, fraction (4) showed scavenging activity represented by pale yellow spots against purple background. Hence, Fraction (4) was selected and separated into its components using HPLC using solvent system methanol (70%) on reversed phase (Cosmosil 20 x 250 nm) column, with flow rate 4.3 mL/min at UV 254 and 210 nm to yield pure compound **1** (6 mg) at R_t 33 min.

Compound **1** was obtained as colorless crystals. On TLC, it showed dark spot under short UV light and changed to dark brown by spraying with anisaldehyde reagent and heating. The low resolution ESI mass spectrum, positive mode (Fig. 6) revealed a molecular ion peak [M+Na] at *m/z* 447 which was in agreement with the molecular formula $C_{24}H_{24}O_7$. Careful comparison of NMR data (Table 5) of compound **1** with compounds isolated from the same fungus, *Aspergillus terreus*, showed that compound **1** is Butyrolactone I (Fig.7) [37].

BL-1 is a butenolide commonly produced by *Aspergillus terreus.* It was first isolated in 1977 from *Aspergillus terreus* var. *africanus*

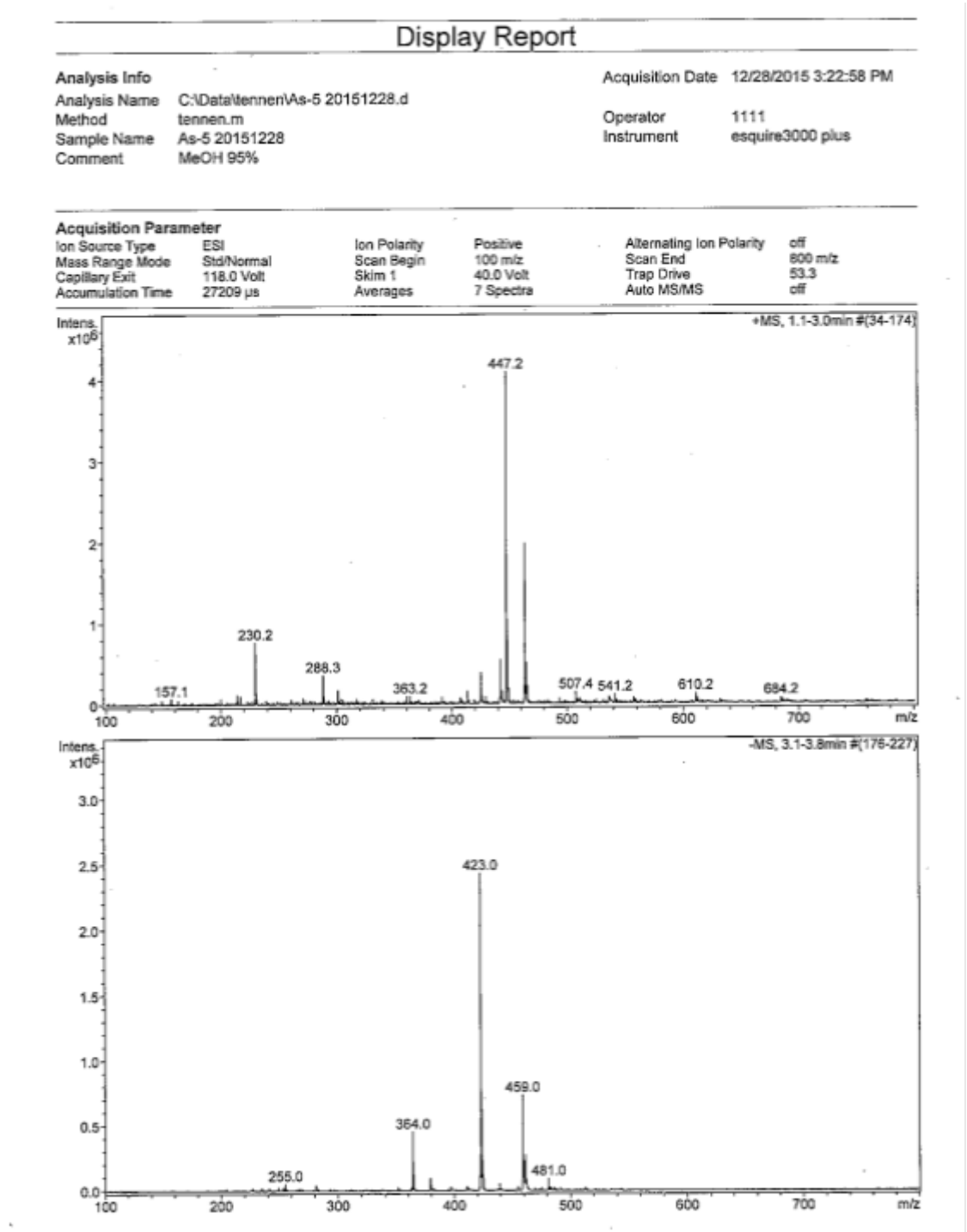

Fig. 6. ESI MS of Butyrolactone I.

TABLE 5. ^{13}C and ^{1}H NMR data of Butyrolactone I .

Atom	δ_H (*J* in Hz)	δ_C
1	-	170.4
2	-	139.8
3	-	129.1
4	-	86.8
5	3.42 (d, *J*=8.0)	39.6
6	-	171.7
7	3.77	53.8
1’	-	123.2
2’	7.58 (d, *J*= 8.8)	130.4
3’	6.85 (d, *J*= 8.8)	116.6
4’	-	159.3
5’	6.85 (d, *J*= 8.8)	116.6
6’	7.58 (d, *J*= 8.8)	130.4
1’’	-	125.0
2’’	6.39 (d, *J*= 2.0)	132.4
3’’	-	128.4
4’’	-	155.1
5’’	6.52 (dd, *J*= 8.0, 2.0)	115.0
6’’	6.47 (d, *J*= 8.0)	129.8
7’’	3.06 (d, *J*= 7.2)	28.7
8’’	5.05 (t, *J*= 1.2)	123.6
9’’	-	133.0
10’’	1.66	26.0
11’’	1.56	17.8

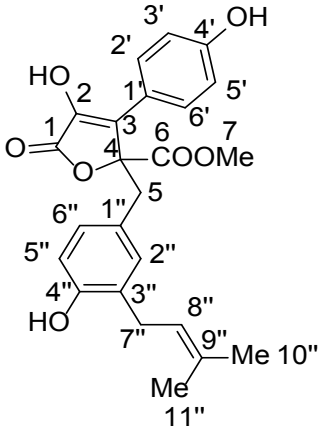

Fig. 7. Chemical structure of Butyrolactone I (BL-1)

[38]. BL-1 has been described in *Aspergillus terreus* endophyte living in *Camellia sinensis* var. *assamica* [39] and in *Laurencia ceylanica* [40], and also in *Microsphaeropsis olivacea* isolated from *Pilgerodendron uviferum* [41].

Scavenging activity of Butyrolactone I

BL-1 has been reported to have various biological activities. It displays antidiabetic, antioxidant [42,43], anti-inflammatory [39], antimicrobial [44] and antitumor [45, 37] potentials, and it inhibits the enzyme CDK (cyclin dependent kinase), preventing apoptosis [46]. Therefore the scavenging activity (% inhibition) of the purified BL-1 using the DPPH radical was assessed and compared to that of ascorbic acid (Table 6, Fig. 8). They showed scavenging ability in a concentration-dependent manner. The IC_{50} was obtained by interpolation with linear regression analysis. Lower IC_{50} indicates higher antioxidant activity. BL-1 showed high scavenging activity with IC_{50} 439.09µg/mL (1.035µM) this result indicates that BL-1 could be a very promising antioxidant compound.

Conclusion

Aspergillus terreus -18 total extract after optimization contained high amount of phenolic compounds (TPC 290.51 mg GA equivalent/gm extract) which positively correlates with its DPPH scavenging activity. Scavenging activity increased 1.17 fold times (99.5%) of the basal medium (85%) after optimization. Phenolics have more ability to quench free radicals such as DPPH, their effectiveness depends on the number of aromatic rings, the molecular weight and substitution of hydroxyl groups than the specific functional groups [47, 48]. In an attempt to isolate and purify the major compound responsible for the antioxidant activity of the fungal extract using spectroscopic techniques, BL-1 was isolated, which was tested for its scavenging potential. BL-1 showed relatively high DPPH radical scavenging activity. Compounds showing antioxidant activity may also possess other bioactivities of pharmaceutical interest, thus BL-1 isolated from the local isolate *Aspergillus terreus* -18 could be further investigated for other health promoting bioactivities.

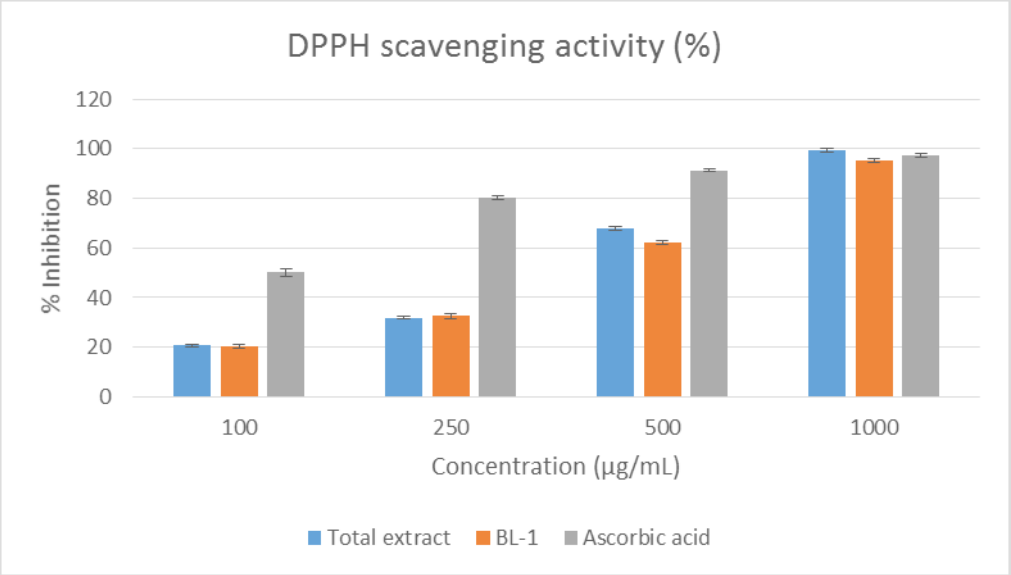

Fig. 8. DPPH scavenging activity of total extract of and BL-1 compared to that of ascorbic acid.

Acknowledgments

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فحوصات كيميائية و دراسات تحسينية للعزلة المحلية *Aspergillus terreus*-18 ذات النشاط المضاد للأكسدة

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تم تحليل مستخلص *Aspergillus terreus*-18 كيميائياً باستخدام تحليل كروماتوجرافي (HPLC). وقد أدى ذلك إلى عزل بوتنولايد البوتيرولاكتون الأول (BL-1) الذي تم تحديده باستخدام الرنين المغناطيسي النووي (NMR) والأطياف الكتلية. تم اختبار نشاط BL-1 المضاد للأكسدة من خلال قدرته علي ازالة الراديكال الحر DPPH . أظهر مركب BL-1 نشاطًا عاليًا في الازالة (439.09 IC_{50} ميكروجرام / مل). كانت العزلة المحلية *A.terreus*-18من بين 18 عزلة أخرى تم فحصها لنشاط كسح DPPH. أظهر *A.terreus*-18 أعلى نشاط (تثبيط ٪) ، 80.4 ± 1.35.عندما تم تنمية العزلة الفطرية على مستخلص الشعير المخمرمع البيبتون كوسط انتاجي تحت ظروف ثابتة لمدة 14 يوم أظهرت مستخلصات *A.terreus*-18 نشاط مضاد للأكسدة قيمته 85٪. تم إجراء تحسين لمعاملات فيسولوجية مختلفة لتعزيز النشاط الحيوي وزيادة المحتوى الكلي الفينولي (TPC) للعزلة الواعدة من خلال منهجية النمذجه الاحصائية (RSM). أدت النهج الإحصائية إلى زيادة نشاط ازالة الراديكال الحر DPPH 1.17 ضعف (تثبيط 99.5 ٪). تم الحصول على هذا باستخدام الوسط الغذائي المكون من: مستخلص الشعير 25 جم\لتر؛ بيبتون 6 جم\لتر, وأس هيدروجيني 7 و نسبة تهويه 50 ٪ واستخدام زريعة عمرها60 ساعة و نسبتها 15٪ من حجم بيئة الانتاج , علي ان يتم التخمربشكل ساكن لمدة 18 يوما في درجة حرارة 27 ± 2 درجة مئوية. أثبتت التجارب ان هناك ارتباط بين المحتوى الكلي الفينولي TPC في مستخلص الفطرو النشاط المضاد للأكسدة بشكل إيجابي حيث زاد TPC من 122.475 إلى 290.51 ميللي جرام من حمض الجاللیك لكل جرام من مستخلص الفطر.