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Research Article

Total Phenolics from an Endophytic Fungus *Penicillium sp* Isolated from *Nothapodytes foetida* and its Optimization

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ABSTRACT

Microwave Assisted Extraction (MAE) of total phenolic content (TPC) from an endophytic fungus *Penicillium* sp. was carried out and the conditions for extraction was optimized by Response Surface Methodology (RSM). The effects of operating conditions (extraction time, extraction temperature and ratio of solvent to sample) on the extraction of TPC were studied using Central Composite Design (CCD). TPC was determined by Folin's Ciocalteau method and the results were expressed in (mg GAE/g Biomass). The optimal processing parameters were found to have significant effect on the extraction of TPC from *Penicillium* sp. A mathematical model with high determination coefficient R2 of 0.947 was obtained with optimal conditions of extraction of 5.88 minutes, 51.87 °C, and ratio of solvent to sample of 18.66:1. Under these conditions the experimental yield of TPC was 59.42 mg GAE/g Biomass which was close to the predicted value of 59.88mg GAE/g Biomass. This study indicated the potential use of MAE for extraction of antioxidants from the biomass of an endophytic fungus.

Keywords: Antioxidants, Endophytic fungi, Microwave assisted extraction, *Penicillium* sp., Response Surface Methodology, Total phenolic content

INTRODUCTION

Nothapodytes foetida, a medicinal plant of family Icacinaceae, commonly known as Amrutha or kalagur [1] has been used widely in Indian traditional medicine for various types of cancers, HIV, malaria and for few bacterial infections. It is a small tree distributed in the Western Ghats, a global biodiversity hot spot. Since *N. foetida* has been classified as a 'vulnerable' species, considerable efforts are in progress to map its populations in India and to isolate novel endophytes.[2]

Endophytes are fungi or bacteria which reside inside the healthy plant tissues without causing any infectious symptoms.[3] They are recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities[4]. It has been reported that metabolites from endophytes can be a potential source of novel natural antioxidant compounds possessing anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities as reported by various researchers.[5-10] According to Halliwell and Gutteridge[11] an antioxidant is "any substance that delays, prevents or removes oxidative damage to a target molecule". The antioxidant capacities of endophytic fungal cultures suggested that the phenolic content was the major antioxidant constituents of the endophytes[12]. In order to enhance the extraction efficiency of this bioactive compound it is necessary to understand the behaviour of factors influencing the process conditions. Extraction of phenols or polyphenols including flavanoids from endophytic fungi using solvents constitutes an important step in the manufacture of antioxidant-rich products. Many factors, such as solvent composition, time of extraction, temperature, pH, solid-to-liquid ratio, polarity of the solvent and particle size, may significantly influence the extraction.[13-16]

Interest in Microwave-assisted extraction (MAE) has increased significantly over the past few years as a result of its inherent advantages over conventional extraction techniques such as improved efficiency, reduced extraction time and low solvent consumption[17]. Studies on the extraction of phenolic compounds from endophytic fungus are minimal: indeed there is no report on the microwave assisted extraction of antioxidants from an endophytic fungus. Hence an attempt has been made to optimize microwave assisted extraction of antioxidants

Figure 1. *Fruit of Nothapodytes foetida*

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Table 1: Independent variables and their coded and actual values used in the central composite design

Factors	Levels				
	-α	-1	0	1	+ α
X_1 extraction time/min	2.64	4	6	8	9.63
X_2 extraction temperature/°C	33.18	40	50	60	66.82
X_3 ratio of solvent to sample	3.18	10	20	30	36.82

Table 2: Central Composite Experimental Design and Response for TPC

Run	X_1 Extraction Time (min)	X_2 Extraction Temperature (°C)	X_3 Ratio of Solvent to Sample (ratio)	Y TPC Actual	TPC Predicted	TPC Residual
1	6(0)	66.82(1.682)	20(0)	52.5	50.38	2.116
2	6(0)	50(0)	36.82(1.682)	41.2	35.26	0.760
3	6(0)	50(0)	20(0)	58.2	58.61	-0.411
4	6(0)	50(0)	20(0)	58.4	58.61	-0.211
5	6(0)	33.18(-1.682)	20(0)	26.9	26.30	-0.311
6	6(0)	50(0)	20(0)	58.9	58.61	0.289
7	6(0)	50(0)	20(0)	58.7	58.61	0.089
8	8(1)	60(1)	10(-1)	49.5	52.17	-2.675
9	4(-1)	60(1)	30(1)	22.9	29.41	5.936
10	6(0)	50(0)	20(0)	58.3	58.61	-2.021
11	8(1)	40(-1)	30(1)	25.6	31.48	-3.228
12	8(1)	40(-1)	10(-1)	31.4	26.81	4.590
13	4(-1)	40(-1)	30(1)	26.9	26.14	0.760
14	4(-1)	40(-1)	10(-1)	29.0	31.01	-5.888
15	6(0)	50(0)	3.18(-1.68)	42.5	45.728	-6.506
16	8(1)	60(1)	30(1)	44.7	44.6	0.097
17	6(0)	50(0)	20(0)	58.7	58.61	0.089
18	2.64(-1.682)	50(0)	20(0)	29.5	28.47	1.327
19	4(-1)	60(1)	10(-1)	50.5	46.53	3.972
20	9.36(1.682)	50(0)	20(0)	39.1	37.72	1.381

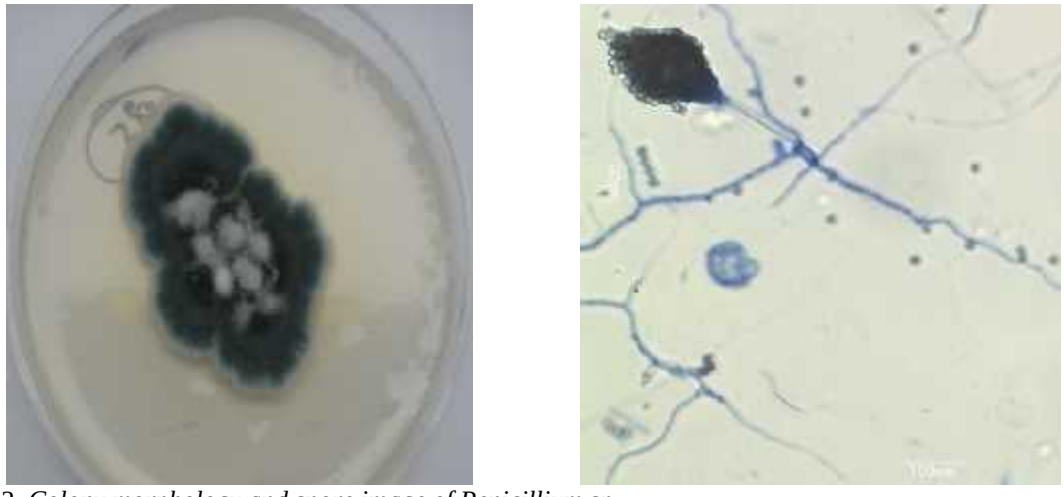

Figure 2. *Colony morphology and spore image of Penicillium sp*

from an endophytic fungus *Penicillum* sp. isolated from N. foetida using RSM.

MATERIALS AND METHOD

Collection of Plant Materials: The fruit samples were collected from Agumbe forest of Western Ghats, Dakshina Karnataka, India (Fig.1). The sample was identified and authenticated by an experienced botanist. Fresh and healthy parts of fruits were cut with a sterile scalpel and stored at 4ºC in a sterile polythene bag prior to use.

Isolation and Identification of Endophytic Fungi: Isolation was carried out as described by Wang et al.[18] with little modifications. Plant samples were surface sterilized with 75% ethanol (1 minute) and 2.5% sodium hypochlorite (3 minute) followed by washing with sterile distilled water (2 minute). Each plant sample was cut aseptically into 0.5 cm long segment and placed on petri dishes containing

potato dextrose agar (PDA) supplemented with chloramphenicol (50 µg/mL, Sigma) and streptomycin sulphate (250 µg/mL Sigma). The plates were sealed using Parafilm™ and incubated at 25°C±1°C in a light chamber with 12 hours of light followed by 12 hours of dark cycles[19]. The petri dishes were monitored every day to check the growth of endophytic fungal colonies from the plant segments. Isolated, subcultured and brought to pure culture by serial sub-culturing. Identification of fungal endophyte was done based on their morphological characteristics using lactophenol cotton blue staining method[20] and visualized under microscope (MOTIC BA 400).

Fermentation: The fungal fermentation was carried out using potato dextrose broth (PDB) in 250 ml of Erlenmeyer flask at a temperature of 26±1°C and at 120 rpm. Each flask containing 100 ml of medium was inoculated with a spore suspension of 100 µL prepared by dispersing (1cmx1cm) mycelial agar plug taken from an actively growing colony on potato dextrose agar plate in a solution containing Tween 80 (0.1% v/v), filtered using Whatmann No.1 filter paper and counted for number of spores in colony forming unit using Haemocytometer. The biomass was separated from the broth by filtration using sterilized pre-weighed filter cloth after 14 days of fermentation.

Experimental design by Response surface methodology

Selection of Experimental variables: A set of experimental trials were performed to select the relevant factors (independent variables) such as extraction temperature, extraction time and ratio of solvent to sample before the development of the study through RSM on extraction yield of total phenolic content (dependent variable). In general, efficiency of the extraction is influenced by various multiple parameters such as temperature, time solvent concentration and polarity, and their effects may be either independent or interactive[21]. In this study, effect of extraction time ranging from 4 to 8 minutes, effect of extraction temperatures between 40 to 60°C and ratio of solvent to sample 10 to 30 mL/ g Biomass were investigated on the extraction of TPC as given in Table1.

A five level, three factors central composite design (CCD) (Minitab version 14; Minitab Ltd., Coventry CV3 2TE, UK) was utilized to examine the optimum combination of extraction variables for extraction of TPC from biomass of *Penicillium* sp. The CCD design comprised of 20 experimental runs with eight factorial points, six axial points (two axial points on the axis of each design variable at a distance of 1.68179 from the design center) and six replicates at the centre point as shown in Table 2 were used to obtain a quadratic model, consisting of factorial trails and star points, to estimate quadratic effects and central points to estimate the pure process variability with TPC as response. To simplify the calculation, the independent variable coded as Z was used.

$$Z = (X - X_0)/\Delta X \quad (1)$$

Where X Corresponds to natural value X_0 is the natural value in the centre of domain and ΔX is increment of X corresponding to one unit of Z.

Experimental data were fitted to a second-order polynomial model as given below in Eq. (2) and regression coefficients were obtained using multi-variant regression analysis.

$$y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=j}^{2} \sum_{j=i+1}^{3} A_{ij} X_{ij} \quad (2)$$

Where

$y =$ Response Function

$A_0 =$ Intercept

$A_i, A_{ii}, A_{ij} =$ Coefficients of the linear, quadratic and interactive terms respectively

X_i & X_{ij} = Level of Independent Variables

The model evaluated the effect of each independent variable to a response. Three additional confirmation experiments were conducted to verify the validity of the statistical experimental strategies.

Verification of model: Optimal extraction conditions on TPC of *Penicillium* sp. samples crude extract were obtained using the predictive equations generated by RSM. TPC was tested using Folin-Ciocalteu method after solvent extraction under specific optimal conditions. Each set of experiment was conducted in two replicates, and the experimental and predicted values were compared in order to examine the validity of the model.

Statistical analysis for central composite design (CCD): The statistical software package Minitab version 14 (Minitab Ltd., Coventry CV3 2TE, UK) was used to analyze the experimental data. The optimal concentrations of the critical variables were obtained by analyzing surface and contour plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

Table 3: Analysis of variance (ANOVA) of second order polynomial model for optimization of TPC

Source	DF	Seq.SS	Adj SS	Adj MS	F-value	*p*-value
Regression	9	3207.5	3207.5	356.3	19.78	0.00
Linear	3	935.08	935.08	311.6	17.30	0.00
Square	3	2103.08	2103.08	701.1	38.91	0.00
Interaction	3	169.14	169.14	56.38	3.313	0.075
Residual Error	10	180.20	180.20	18.02		
Lack of fit	5	179.83	179.83	35.96	481.68	0.00
Pure Error	5	0.37	0.37	0.075		
Total	19	3387.73				

Table 4: Estimated Regression coefficients of second order polynomial model for optimization of TPC

Terms	Coeff	SE Coeff	T	*p*-value
Constant	58.61	1.731	33.853	0.000*
X_1	2.749	1.149	2.393	0.000*
X_2	7.158	1.149	6.231	0.000*
X_3	-3.111	1.149	-2.708	0.0.022*
$X_1 X_2$	2.463	1.501	1.641	0.132
$X_1 X_3$	2.387	1.501	1.591	0.143
$X_2 X_3$	-3.062	1.501	-2.041	0.069
X_1^2	-9.021	1.118	-8.067	0.000*
X_2^2	-7.165	1.118	-6.407	0.000*
X_3^2	-6.405	1.118	-5.728	0.000*

*SE- Standard Error; T- Student's T test; p- Corresponding level of significance; * Significant*

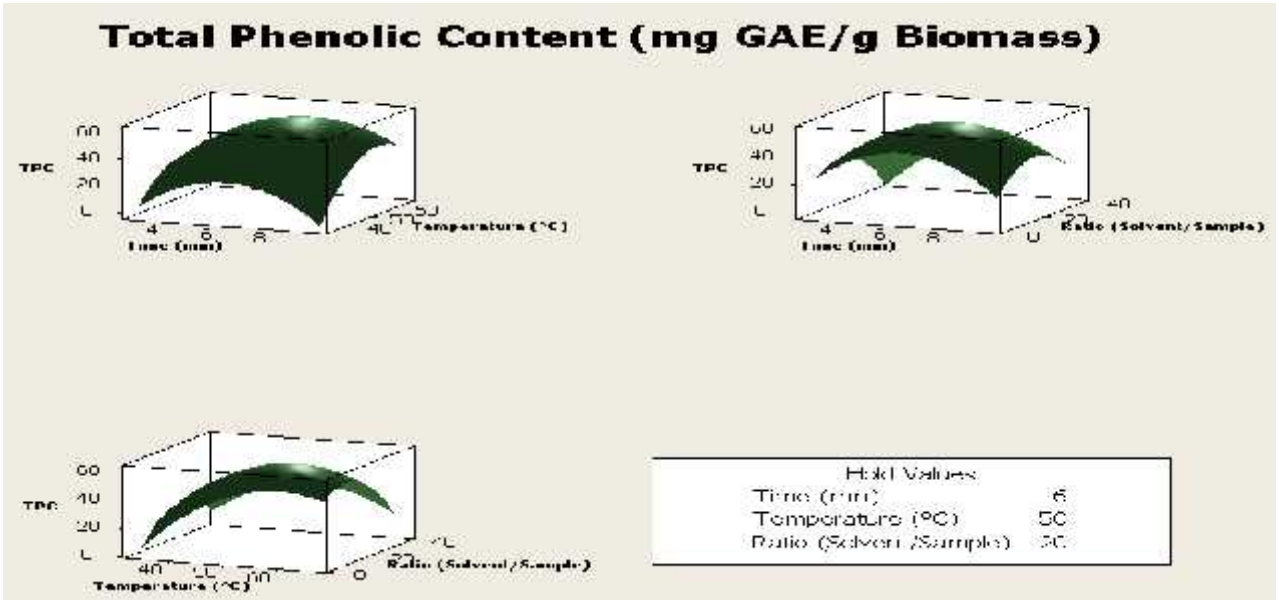

Figure 3. *Response surface plot, (a) Time vs Temperature (b) Time vs Ratio of Solvent to Sample (c) Temperature vs Ratio of Solvent to Sample on TPC*

Microwave assisted extraction and Determination of Total phenolic content (TPC) Assay: Microwave assisted extraction of Total phenolic content was carried out in a Microwave ashing system (Questron Q Ash 800, Canada). The TPC of *Penicillium* sp. extracts was determined spectrophotometrically using Folin-Ciocalteu's reagent according to the method described by Lim et al.[22] with slight modifications.

Approximately 0.3 ml sample was added into the test tubes followed by 1.5 ml of Folin-Ciocalteau reagent (10% v/v) and 1.2 ml of sodium carbonate (7.5% w/v). The test tubes were covered with aluminium foil, mixed for 10 seconds using vortex and allowed to stand at room temperature for 30 minutes in dark environment. Absorption was measured at 750 nm using spectrophotometer. Blank sample was prepared by adding 0.3 ml solvent without the extract. Gallic acid was used as standard and TPC were expressed in Gallic acid equivalents, mg GAE / g Biomass.

RESULTS AND DISCUSSION

The endophytic fungus isolated from *N.foetida* was identified as *Penicillium* sp. Based on the microscopic and macroscopic characteristics as shown in the Fig.2.

Experimental design Model fitting and Analysis of Response for optimization of TPC extraction: RSM is a sequential procedure for efficient and rapid determination of optimum value of any process parameter [23]. It also determines statistically the importance of individual factors, appropriateness of the functional form and sensitivity of response on each factor [24].

The experimental design of five-level, three-variable CCD and the predicted and experimental results of extraction are shown in Table 3. The maximum content of total phenolics (58.9 mg GAE/ g Biomass) was recorded under 6th trail with experimental parameters of extraction time of 6 minutes, extraction temperature of 50°C and ratio of solvent to sample of 20:1. The lowest TPC (26.9 mg GAE/ g Biomass) was found at 4 minutes of extraction time, 40°C extraction temperature and ratio of solvent to sample of 30 under 13th trail. The larger the magnitude of the t-value and the smaller the *p*-value, indicate more significant of the corresponding coefficient and its effect on extraction of TPC. The *p*-values were used as a tool to check the significance of each of the coefficients and to understand the interactions between the best variables.

Linear effect of X_1, X_2 and interaction effect of X_1 X_2 and X_1X_3 was highly significant ($p < 0.05$), which showed the

existence of the optimal value within the experimental area. This suggested that the change in either factor will influence TPC distinctly as shown in Table 3. The statistical analysis of the model was done by Fisher's statistical test for analysis of variance (ANOVA).The coefficient of determination (R^2) was calculated to be 0.947 this implies that 94.7% of the experimental data of TPC extraction was compatible with the data predicted by the model (Table 4). The value of the adjusted determination coefficient Adj R^2= 89.9% was also high to advocate for a high significance of the model.

According to Joglekar and May[25] for a good fit of model, a regression coefficient R^2 should be at least 80%. Application of RSM yielded the following second order

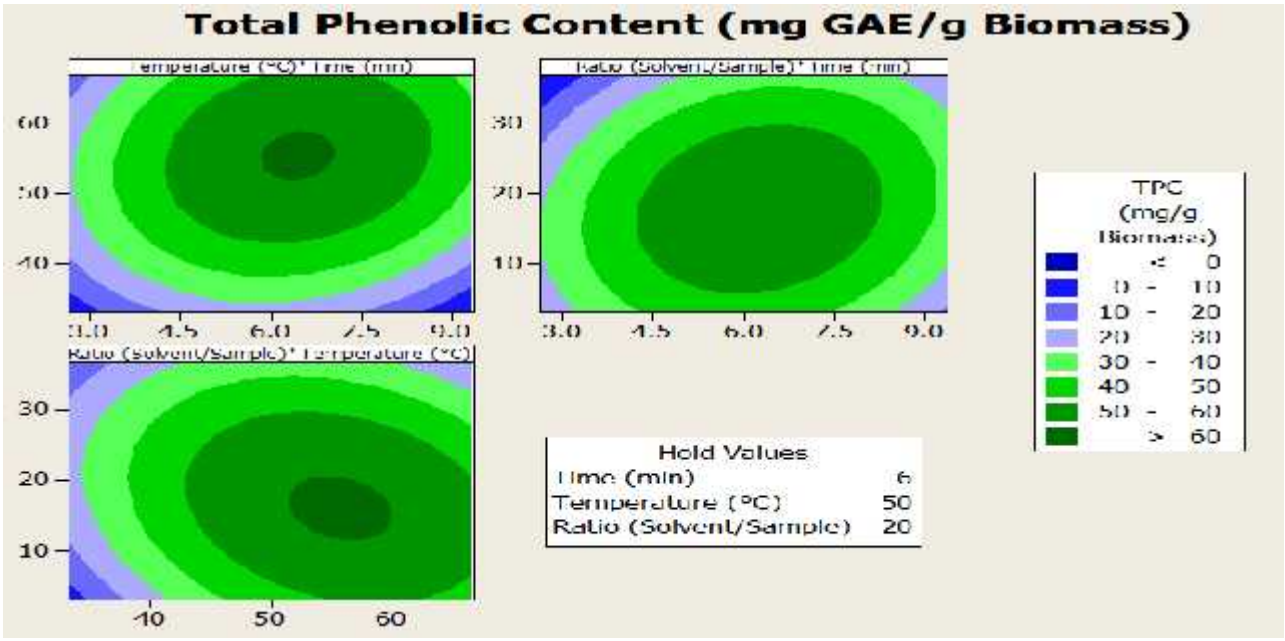

Figure 4. *Contour plots showing the interaction effect of Time, Temperature and ratio of Solvent to Sample on the yield of TPC*

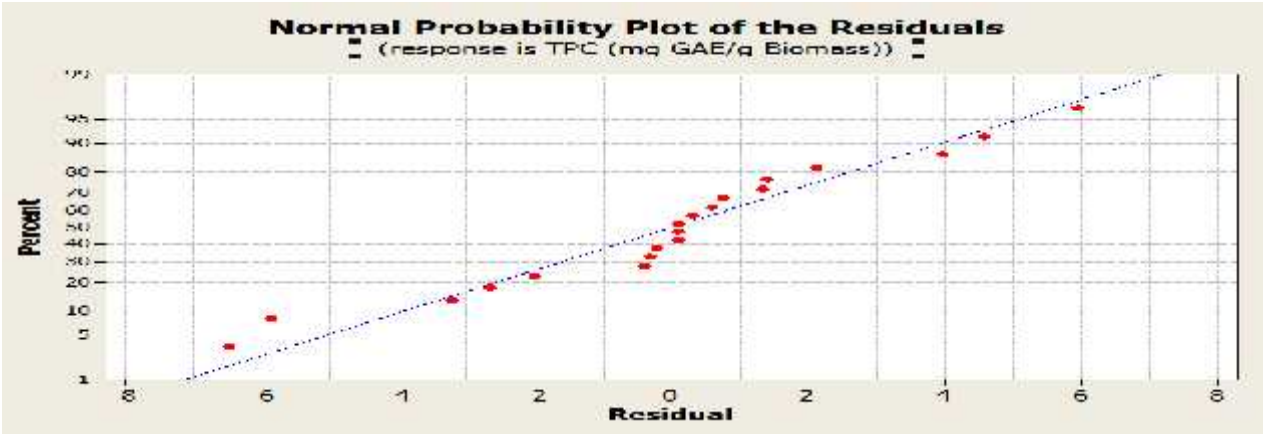

Figure 5. *Normal probability plot of residuals*

Table 5: Predicted and experimental values of the response at optimum conditions

Conditions	Extraction Time (min)	Extraction Temperature (°C)	Ratio of Solvent to Sample	TPC (mg/g of Biomass)
Predicted	5.99	53.42	21.42	59.88
Experimental	5.99	53.42	21.42	59.42

polynomial regression equation, Eq. (3) in terms of the coded values

$TPC = 58.611 + 2.749\ X_1 + 7.158X_2 - 3.111X_3 + 2.463\ X_1X_2 + 2.387\ X_1X_3 - 3.062X_2X_3 - 9.021X_1^2 - 7.165X_2^2 - 6.405X_3^2$ (3)

The graphical representation of the regression equation in terms of response surface is shown from Fig.3a- Fig.3c and contour plots in Fig.4. The 3-dimensional response surface and 2- dimensional contour plots together were used to determine the interaction effects of the factors on the response. These response surfaces display the variation of two factors while the third was kept at the optimum level. From the figures it was observed that the curves are convex in nature suggesting that the conditions were well optimized.

The ranges of variables chosen are quite appropriate and thus the optimum lies in the designed space. As reflected in Fig.3a, the maximum yield of TPC obtained was found to fall in the region of extraction time between 5.8 to 6.4 minute and its corresponding temperature between 48°C to 52°C. Further increase in the time and temperature of extraction decreased the yield of TPC in the extract. Fig.3b shows the effect of extraction time and ratio of solvent to sample on the extraction of TPC at temperature of zero level. The amount of TPC found to increase with time ranging from 5.8 to 6.4 minute and ratio of solvent to sample from 16:1 to 22:1 beyond which there was a decrease in the TPC. Similarly Fig.3c shows the relation between extraction temperature and ratio of solvent to sample on the extraction yield of TPC at time of zero level. The extraction of TPC increased gradually with the increase in temperature.

The comparison of the residuals with the residual variance indicates that none of the individual residual exceeds twice the square root of its residual variance. Normal probability plot of the residuals shown Fig.5 follows a straight line indicating a uniform distribution of residuals with its corresponding residual variance.

Validation of the model was performed at 95% confidence level. The optimal values from the model was 5.88 minutes of extraction, 51.87ºC of extraction temperature and ratio of solvent to sample of about 18.66 with TPC yield of 59.88 mg GAE/g Biomass. The model fitted well and experimental yield at the optimized condition was 59.42 mg GAE/g Biomass.

Liu et al. [26] reported total phenolic content in the range of 54.51 mg/g from *Xylaria* sp. isolated from *Ginkgo biloba*. 18.33 ± 0.68 mg /g of total phenols from an endophytic fungus *Phyllosticta* sp. of *Guazuma tomentosa* have also been reported by Srinivasan et al.[27]. Total phenolic content of 5.68 mg GAE/mL culture was reported from *Aspergillus fumigates* 28 and *Penicillium granulatum* by optimization of various carbon and nitrogen sources using response surface methodology.[29]

CONCLUSION

Most of the literature available reveals that the antioxidant activity of metabolites from plants where as very limited work reports on mushrooms and fungi. To the best of our knowledge, apparently this is the first systematic report on antioxidant activity in terms of Total phenolic content of an endophytic *Penicillium* sp. demonstrated by TPC assay and optimization by statistical methods for enhancing the extraction efficiency. The results obtained are comparable with the antioxidant activity of various other fungi, *Aspergillus candidus*, *Chaetomium* sp., *Cladosporium* sp., *Colletotrichum gloeosporioides*.[30] This may be due to the difference in extraction process used. Hence these findings will facilitate the use of microwave assisted extraction of antioxidants to enhance the extraction efficiency even from the biomass of an organism.

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