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Effective extraction of total phenolic compounds bearing anti-obesity activity from *Eucalyptus globulus*

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ABSTRACT

The extraction of total phenolic compounds (TPC) from *Eucalyptus globulus* leaf was carried out using different solvents such as methanol, ethanol, and chloroform. The TPC in the extracts were determined by the Folin-Ciocalteu method. Further, the effective TPC extraction were done using response surface methodology (RSM); which aims to obtain the optimal conditions of different parameters *viz.*, solvent percentage, extraction time and solid-liquid ratio. The TPC in the extract was detected in gas chromatography and mass spectrometry (GC-MS) analysis. The pancreatic lipase inhibition activity of the extract was measured by monitoring the hydrolysis of *p*-nitrophenyl butyrate. Thus, as a result the effective TPC extracts of *Eucalyptus globulus* showed about 90% of lipase-inhibitory activity.

KEY WORDS: Anti-obesity activity, *Eucalyptus globulus*, lipase inhibition, polyphenols.

1. INTRODUCTION

The polyphenols are the secondary metabolites widely distributed in plants and are known to possess many functional properties in plants like involvement in structural roles, defence strategies, and signalling properties (Boudet, 2007). The polyphenols which is present in the bark of *Eucalyptus globulus* contains considerable amount of polyphenolics (Vazquez, 2008; Conde, 1996). The extraction of these TPC from plant materials would give a great result for applications in different industries like food additives and pharmaceuticals.

The biological systems comprising of microorganisms, enzymes, plants and their derivatives finds a wide application in all areas of engineering and biotechnology such as bioremediation, bio-energy (Selvanaveen, 2015; Vinodhini, 2015; Kumar, 2014; Balaji, 2014; Seenuvasan, 2014; 2013).

The design of experiments (DOE) finds wide applications in area of optimizing process parameters (Kumar, 2015; Karthikeyan, 2014; Kumar, 2012). Among these, RSM is a statistical and mathematical technique which facilitates the development and improvement of the process conditions by simple optimization (Vidhyadevi, 2014; Seenuvasan, 2014). RS methodology consumes less time, effort and provides quantitative measurements of possible interactions between factors (Oraon, 2007).

The present investigation focuses on the extraction of TPC from *Eucalyptus globulus* and optimizing the process conditions for effective extraction of TPC using suitable solvents. To analyse the extracted TPC using GC-MS analysis and elucidate the different compounds present in it and to evaluate the efficacy of the TPC in exerting the pancreatic lipase inhibition activity.

2. MATERIALS AND METHODS

2.1. Collection of plant material: The leaves of *Eucalyptus globulus* plant were collected off the road sides of Cuddalore district in Tamil Nadu. The detached plant leaves were washed with distilled water and allowed to air dry for two weeks and further crushed into very small pieces and placed in containers.

2.2. Preparation of TPC extracts: TPC were extracted using methanol, ethanol, and chloroform (100% each) and 10.0 g of grounded plant material was taken in 100 mL of each solvent and placed in a reciprocal shaking water bath incubated at 150 rpm for 2d at room temperature. The crude extracts were centrifuged at 5000×g for 10 to 15 min. The supernatant was collected and the excess solvent was separated by vacuum evaporation at 42°C and the extract was stored at – 20°C.

2.3. Determination of TPC: TPC was determined by the Folin-Ciocalteu method, about 250 µL of the extract was diluted with distilled water to 10 mL. Aliquot of samples were mixed with 5 mL of 10 fold-diluted Folin-Ciocalteu's reagent. After 3 min, 4 mL of 7.5% sodium carbonate was added(Singleton et al., 1999).The mixture were allowed to stand for 30 min at 40°C prior measuring the absorbance at 734 nm. TPC in the extract was calculated and expressed as tannic acid equivalent (TAE: g/100 g dry mass) using tannic acid (0–120 mg/L) as standard. The TPC in the extracts was calculated according to the following formula:

$$TPC\ (mg/g\ plant\ extract) = \frac{(C \times V)}{m} \quad (1)$$

Where c is the concentration of tannic acid from the calibration curve (mg/mL); V is the volume of extract (mL) and m is the weight of extract (g).

2.4. GC-MS analysis of TPC: GC-MS analysis was used to find the composition of the TPC. The HPLC grade methanol dissolved TPC were used for GC-MS analysis. The column temperature was maintained at 70˚C for 5 min and then 150˚C with an ionization voltage of 70 eV. The compounds were identified on the basis of mass spectra using the available databases from National Institute of Standards and Technology (NIST) library.

2.5. RSM modelling of process parameters using central composite design (CCD) matrix: CCD matrix under the RSM was employed to demonstrate the nature of the response and evaluate the optimal conditions (Box and Wilson, 1992). Three major independent variables namely solvent percentage, extraction time (h) and solid-liquid ratio (mL/g) were included in this model. Each parameter was studied at three different levels (-1, 0, +1). A matrix of 20 experiments with 3 factors was generated using MINITAB 14 as shown in Table 1. The average maximum TPC was taken as the dependent variable or response. An empirical model was constructed based on the variables at the 95 % confidence level.

Table.1. Experimental range and variable levels of CCD experiment

Parameters	Symbol	Low (-1)	Middle (0)	High (+1)
Solvent %	X_1	60	80	100
Solid-liquid ratio (mL/g)	X_2	40	50	60
Extraction time (h)	X_3	20	24	28

Each parameter was studied at three different levels (-1, 0, +1). All the parameter were taken at central coded value considered as 0. The minimum and maximum ranges of parameter were investigated and full experimental plan with respect to their values.

2.6. Evaluation of anti-obesity activity: The porcine pancreatic lipase (PPL, type II) activity was measured using *p*-nitrophenyl *p*-NPB as substrate (Zheng, 2010; Changhyun and Uhee, 2012). The PPL stock solutions (1 mg/mL) were prepared using 0.1 mM potassium phosphate buffer (pH 6.0) and were stored at -20°C.The plant extract (final concentration 10-100 µg/mL) was used as a positive control which were pre-incubated with PPL for 1h (in 0.1mM potassium phosphate buffer, pH 7.2, 0.1% tween 80) at 30°C before assaying the PPL activity. The reaction was initiated by adding 0.1µL p-NPB, all in a final volume of 100µL. After incubation at 30°C for 5min, the amount of p-nitro phenol (p-NPP) released was measured at 405 nm using a UV–vis spectrophotometer. The activity of negative control was also examined with and without an inhibitor. The percentage inhibitory activity (I) was calculated according to the following relation;

$$I(\%) = 100 - \left(\frac{X-x}{Y-y} \times 100\right) \qquad (2)$$

Where Y is the activity without control; y is the negative control without inhibition; X is the activity with inhibitor; and x is the negative control with inhibition. DMSO was used as negative control and its activity was also measured.

3. RESULTS AND DISCUSSION

3.1. Selection of solvent: It was observed from Fig.1 that among the various solvents employed for the extraction of TPC, methanol extraction had produced higher yield than ethanol and chloroform. The highest yield of TPC was obtained in methanol (9.35 g/mL) and the range of solvent (%) was selected on the basis of individual experiment carried out on particular range. The TAE value of the TPC was evaluated by establishing the tannic acid standard graph (Fig.2).

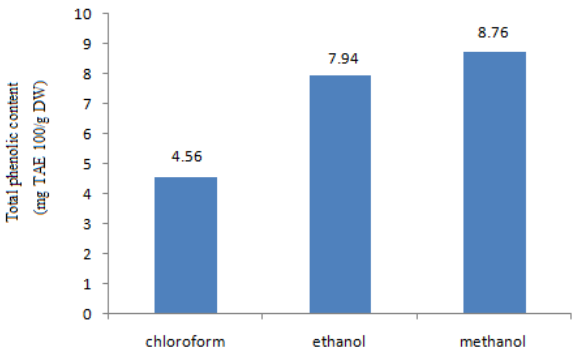

Fig.1. Effect of various solvents on total phenolic content extraction

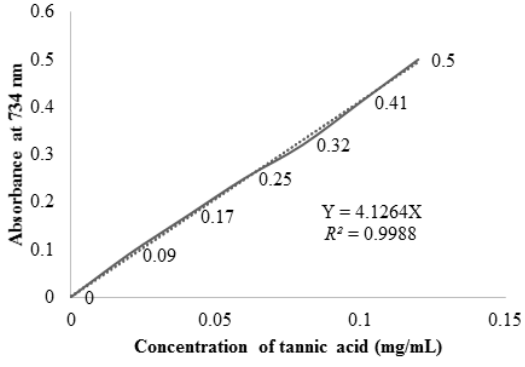

Fig.2. Tannic acid calibration curve

3.2. Optimization of extraction: The observed experimental value given in Table 2 was analyzed for multiple linear regression. The significance of regression coefficients were evaluated by performing Student's t-test. The statistical analyses including the regression coefficient and P-values for linear, quadratic and interaction effect were given in

Table 4 with 95% significance level. The lower P-value indicates more significance of the coefficient and its effect on the TPC (mg TAE/100g DW). The significance of each coefficient and the interactions between the variables were evaluated by the P-value.

Table.2. CCD matrix with observed and predicted response

Run	Solvent (%) X_1	Solid liquid ratio (mL/g) X_2	Extraction time (h) X_3	TPC (mg TAE 100/g DW)	
				Observed	Predicted
1	60	60	28	8.55	8.65
2	80	50	24	12.51	12.08
3	100	40	28	10.97	10.99
4	100	60	28	10.85	10.92
5	100	60	20	10.75	10.52
6	100	40	20	10.67	10.29
7	80	40	24	9.99	10.74
8	80	50	24	12.49	12.08
9	80	50	24	12.5	12.08
10	60	50	24	9.01	9.61
11	80	50	24	12.31	12.08
12	100	50	24	11.43	11.95
13	80	60	24	10.82	11.18
14	60	40	28	8.05	8.00
15	60	40	20	8.23	7.88
16	80	50	28	12.22	12.07
17	80	50	24	12.45	12.08
18	80	50	24	12.47	12.08
19	60	60	20	9.13	8.83
20	80	50	20	10.54	11.81

Table.3. Estimated regression coefficients for TPC

Term	Constant	Coefficient	SE Coefficient	t-value	P-value	Significance
Constant	β_0	12.0834	0.2237	54.016	0.000	Significant
X_1	β_1	1.1700	0.2058	5.686	0.000	Significant
X_2	β_2	0.2190	0.2058	1.064	0.003	Significant
X_3	β_3	0.1320	0.2058	0.641	0.536	Insignificant
X_1^2	β_{11}	-1.3059	0.3924	-3.328	0.008	Significant
X_2^2	β_{22}	-1.1209	0.3924	-2.857	0.002	Significant
X_3^2	β_{33}	-0.1459	0.3924	-0.372	0.718	Insignificant
$X_1 X_2$	β_{12}	-0.1800	0.2301	-0.782	0.452	Insignificant
$X_1 X_3$	β_{13}	0.1450	0.2301	0.630	0.543	Insignificant
X_2X_3	β_{23}	-0.0750	0.2301	-0.326	0.751	Insignificant

It was observed that the coefficients for the linear effect of solvent %, solid-liquid ratio (mL/g) and combined effect of solvent % with solvent % were found to be more significant ($p < 0.005$) than the other linear and combined effect of medium variables. The second order polynomial for the response, TPC (mg TAE 100/g DW) was constructed using the regression coefficients (in coded units) from Table 2 is given by,

$$\text{TPC } (mg\ TAE\ 100/g\ DW) = 12.0834 + 1.17X_1 + 0.219X_2 + 0.312X_3 - 1.3059X_1^2 - 1.1209X_2^2 - 0.145X_3^2 - 0.18X_1X_2 + 0.145X_2X_3 - 0.075X_1X_3$$

Table.4. ANOVA for polynomial model for TPC (mg TAE 100/g DW)

Source	Degree of freedom	Sum of square	Meansquare	F-value	P-value
Regression	9	40.6383	4.51536	10.66	0.000
Linear	3	14.3428	4.78095	11.29	0.001
Square	3	25.8230	8.60768	20.33	0.000
Interaction	3	0.4724	0.15747	0.37	0.775
Residual Error	10	4.2343	0.42343		
Lack-of-Fit	5	4.2068	0.84136	152.70	0.617
Pure Error	5	0.0275	0.00551		
Total	19	44.8729			

The statistical significance of the equation was evaluated by the F-test of ANOVA and the residuals analysis was performed to validate the model at 95% confidence level as shown in Table 4. ANOVA indicates linear and quadratic effects between the variables in the second order polynomial model were highly significant and no significance was found in the lack-of-fit. This indicated the accuracy of the model was adequate to represent the relationship between TPC and solvent (%), solid-liquid ratio (mL/g) and extraction time (h).

To determine the optimum conditions for the extraction of TPC, the three-dimensional plots were constructed (Fig.3). The influence of solvent (%) and extraction time on TPC at a fixed solid-liquid ratio (mL/g) as shown in Fig. 3(a).The TPC increased with solvent (%) and reaches maximum at middle value of the solvent (%). The effect of solid-liquid ratio (mL/g) and extraction time (h) on TPC as shown Fig. 3(b). The third variable was kept constant at their middle level and it was observed that the TPC was high at middle value of solid-liquid ratio and at middle level of extraction time. The effect of solvent (%) and solid-liquid ratio (mL/g) on TPC (Fig. 3(c)). The third variable was kept constant at their middle level. It was observed that theTPC was high at middle value of both solvent (%) and solid-liquid ratio (mL/g). Based on the model the maximum predicted yield of TPC of 12.59 mg TAE 100/g DW.

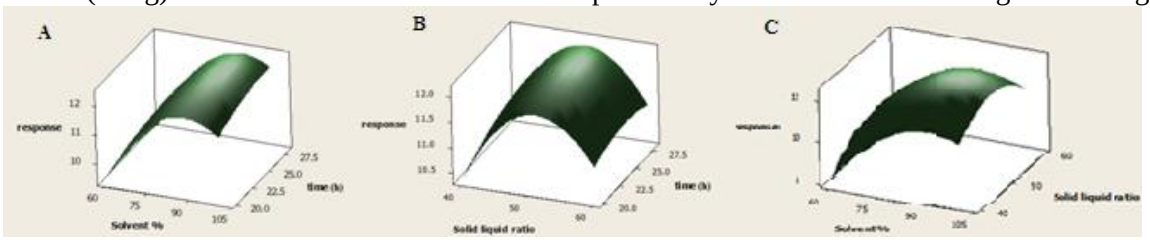

Fig.3. Response surface plot of TPC extract of *Eucalyptus globules*

3.3. GC-MS analysis of TPC: The compounds in the methanol extract of *Eucalyptus globulus*were identified using GC-MS analysis. Our suggested GC-MS conditions enabled rapid baseline separation starts at R_t =5.98 min.The presence of TPCwas confirmed by comparing with data library and the eluted TPC were tabulated as in Table 5.

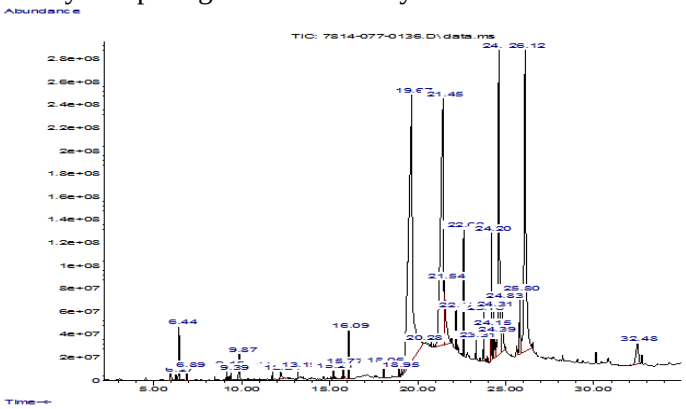

Fig.4. TPC obtained by GC-MS analysis from methanol extract of *Eucalyptus globulus*

Table.5. Mass spectra of TPC in methanol extract

***R_t* min)**	**Molecular formula**	**Name of products**
5.968	$C_{10}H_{16}$	alpha-Phellandrene Cyclopentene
6.273	$C_{10}H_{14}$	p-Cymene
6.443	$C_{10}H_{18}O$	Eucalyptol
6.897	$C_{10}H_{16}$	gamma.-Terpinene, 3-Carene
9.147	$C_{10}H_{18}O$	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)- (R)
9.393	$C_{10}H_{18}O$	L-.alpha.-Terpineol
9.868	$C_6H_6O_3$	5-Hydroxymethylfurfural, 4-Mercaptophenol
11.755	$C_6H_9CH_2OH$	3-Cyclohexene-1-methanol
15.217	$C_{15}H_{26}O$	Globulol
15.766	$C_{10}H_7CH_2OH$	2-Naphthalenemethanol
18.946	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
19.674	$C_{16}H_{32}O_2$	n-Hexadecanoic acid
21.449	$C_{18}H_{34}O_2$	oleic acid
24.309	$C_{16}H_{31}ClO$	Palmitoyl chloride
32.481	$C_{18}H_{27}NO_4$	4-Nitrophenyl laurate

3.4. Inhibitory effect of TPC on pancreatic lipase: The inhibitory activity of TPC against pancreatic lipase was determined using different concentrations (10-100 µg/mL) of TPC and the extract from *Eucalyptus globulus* had 90% of lipase-inhibitory activity.

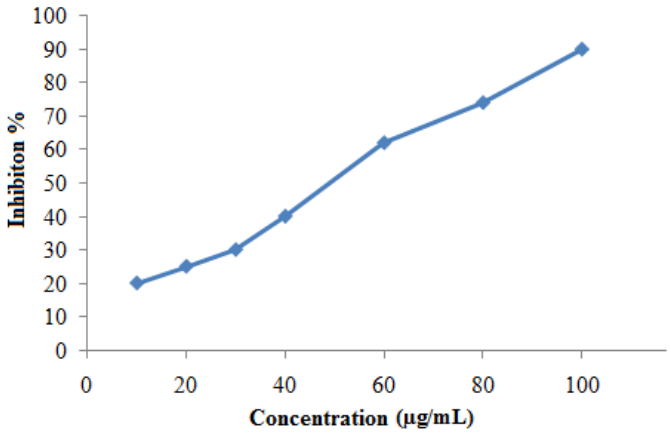

Fig.5. Inhibitory effect of TPC on pancreatic lipase

4. CONCLUSION

The effective extraction of TPC from *E. globulus* leaf was successfully carried out using methanol and the TPC extraction was optimized using RSM. The TPC of the extract had a good pancreatic lipase inhibition activity of about 90%. Thus, the TPC extracts obtained from *E. globulus* plants exhibited inhibitory effects on fat digestion.

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