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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(6): 560-566

www.thepharmajournal.com
Received: 27-04-2019
Accepted: 30-05-2019

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Augmenting the yield of polyphenols and its antioxidant activity from fresh tea leaves of Assam by response surface approach

Sahijul Islam, Manashi Das Purkayastha, Sewali Saikia and Shantanu Tamuly

Abstract
Central composite design (CCD) under response surface methodology (RSM) was used to optimize the process conditions for enhancing the yield of total polyphenols (TP) from fresh tea leaves and its antioxidant activity (AA). The independent variables were leaves-to-solvent ratio (LTSR) (1:20-40 w/v), extraction time (1-3 h), and number of extractions (1-3 times). The solvent used for extraction was a mixture of methanol and acetone (1:1 v/v), as it gave the maximum TP and AA among other tested solvents (water, methanol, ethanol and acetone). The optimum condition of process variables was found to be LTSR 30.4 mL, extraction time 1.5 h, and number of extraction as 1. Under the predicted condition, the TP and AA were obtained as 4.93 g catechol equivalent (CE) per 100 g of tea leaves and 87.74%, respectively. The predicted and experimental values were found to be in congruent with each other, which showed the validity of the developed models.

Keywords: Tea leaves, extraction, total polyphenols, antioxidant activity, response surface methodology

1. Introduction
Tea (*Camelia sinensi*) is mainly valued for its leaves and India ranks second in its production of 1209 million kg by the year 2015 (Peiris, 2016) [1]. The variety which is commonly grown in Assam is *Camelia sinensis* var. *Assamica* (Barua, 1989; Balentine *et al*., 1997; Bezbaruah, 1999) [2, 3, 4]. Fine quality tender tea leaves are collected on the first two flushes; the first flush (spring flush) starts in late march and the second flush starts from end of the May to June. Tea is considered as super food as it is a very good source of antioxidative polyphenols and flavonoids, which make up to 30% of their dry weight (Jayasekera *et al*., 2014) [5]. Unfortunately processing of fresh leaves by oxidation and drying greatly mars the antioxidative properties of these compounds. Thus, extraction of these compounds, without compromising their bioactivity, has become a paramount issue, and researchers are therefore focussing on deriving these benevolent compounds from fresh leaves. Although several authors have reported the optimum conditions for extracting tea leaves polyphenols, most of the research papers mentioned the use of either sophisticated extraction devices such as ultrasound-assisted extraction, supercritical fluid extraction, microwave-assisted extractoion, etc. (Both *et al*., 2014; Prakash Maran *et al*., 2014; Jha *et al*., 2017) [6, 7, 8] or few yield-determining factors (such as time, temperature, and leaves-to-solvent ratio) affecting the extraction process (Turkmen *et al*., 2006; Alothman *et al*., 2009; Do *et al*., 2014; Venkataramanamma *et al*., 2016; Mohammadi *et al*., 2017) [9, 10, 11, 12, 13]. Moreover, the optimum conditions/results derived in these papers are often not consistent with each other. Hence, tea phenolics extraction procedure need to be precisely designed employing low cost extraction techniques. As such, this study was undertaken to optimize the processing parameters for improving the yield of total polyphenols (TP) from fresh tea leaves along with the retention of high antioxidant activity (AA), using simple extraction methodology. In addition, the study was preceded by standardization of a suitable solvent system using commonly available organic solvents.

Response surface methodology (RSM) has been widely employed to generate mathematical models and optimize the process operations in biochemical, biotechnology, food technology and engineering (Sin *et al*., 2006; Gan *et al*., 2007; Singh *et al*., 2010; Yaakob *et al*., 2012; Oliveira *et al*., 2015) [14, 15, 16, 17, 18]; such as, the extraction of phenolic compounds from wheat (Liyana-Pathirana and Shahidi, 2005) [19], antioxidant capacities from *Mangifera pajang*

kosterm peels (Prasad *et al*., 2011) [20], *Parkia speciosa* pod (Gan and Latiff, 2011) [21], total flavonoid content and antioxidant activity of *Limnophila aromatic* (Do *et al*., 2014) [11], etc. Nonetheless, the influence of extraction variables such as leaves-to-solvent ratio (LTSR), extraction time and number of extractions needed for recovery of TP with high antioxidant activity (AA) from tea leaves of Assam, has not been reported yet. Therefore, the preset work was carried out to optimize the conditions for extraction of total polyphenols (TP) and its antioxidant activity (AA) from fresh tea leaves of Assam using response surface methodology.

2. Materials and Methods

2.1. Materials

Fresh tea leaves (*Assamica sinensis*) were collected on September month from tea garden of Assam Agricultural University, Jorhat, Assam. The chemicals and reagents were purchased from Hi Media®, Mumbai, India.

2.2. Selection of appropriate conditions for extraction

Preliminary experiments were conducted prior to the optimization trails to identify the appropriate amount of solvent (mL), extraction time (h), and number of extraction for the selection of high and low level values of the independent variables in RSM. The primary step of the preliminary experiment was to select an appropriate solvent(s) for the extraction of tea polyphenols. So, neat solvents (water, methanol, ethanol and acetone) and their mixtures at different ratios were tested for the yield of TP and AA (Table 1). For the selection of the appropriate number of extraction steps, the procedure proposed by Zlotek *et al*. (2016) [22] was adopted; wherein the residue left after each extract was re-suspended in fresh solvent, either for 1, 2 or 3 times. The extracts from each experiment were pooled, dried under vacuum at 40 ^{O}C and re-dispersed in a same volume of distilled water. The temperature of extraction was kept at constant 4 ^{O}C, as it is a well-known fact that polyphenols maintains their biological activity and structural integrity at low temperature Zlotek *et al*. (2016) [22].

2.3. Experimental design

A central composite design (CCD) of RSM with three process variables at three levels each was designed to optimize the parameters affecting the extraction of tea leaves (Gan and Latiff, 2011) [21]. The selected independent variables were leaves-to-solvent ratio (X_1), extraction time (X_2) and number of extraction (X_3). The dependent variables were TP (Y_1) and AA (Y_2). The coded and un-coded levels of process variables each at 5 levels with 20 runs including 3 replications are given in Table 2. The design was generated by commercial statistical package, Design Expert® Version 11.0.1.0 (Stat-Ease Inc., Minneapolis, USA).

2.4. Sample preparation and extraction

Fresh tea leaves of second flush were sorted out, washed, cut into small pieces (1 mm approx.) and then dispersed in a dilute volume of solvent. In this study, leaves-to-solvent (LTSR) was varied from 1:20 w/v to 1:40 w/v, extraction time 1 to 3 h and number of extraction 1 to 3 times (derived from preliminary experiments). Extraction was performed in an orbital shaker at a constant speed of 160 rpm, using equimolar mixture of methanol and acetone (1:1 v/v) (Das Purkayatha *et al*., 2013) [23] for different combination of LTSR, extraction time and number of extraction (Table 3). All the extraction was carried out at 4 ^{O}C; extracts were collected after centrifugation at 5,800×g for 10 min and then stored at 4 ^{O}C till analysis.

2.5. Total polyphenols (TP)

TP was determined spectrophotometrically according to procedure mentioned by Singleton *et al*. (1999) [24] using Folin-Ciocalteau reagent (FCR) with some modifications. A 10 g of 20% Na_2CO_3 was mixed in 50 mL of distilled water and FCR of 15 mL added into distilled water having same volume. Catechol was used as working standard; taking 0.05 g into 10 mL distilled water and volume make up to 100 mL and 0.5 ml of FCR was added to each different test tube. After 3 min, 2 mL of Na_2CO_3 solution was added into the test tubes and heated for 1 min in boiling water. During the oxidation of phenolic compounds, a blue colour was developed. After two hours, the absorbance of blue colouration was measured using UV-VIS spectrophotometer (Varian-50 Scan) at 650 nm against a blank. The result was expressed as g catechol equivalent (CE) per 100 g of tea leaves.

2.6. Antioxidant activity (AA)

AA of the tea extract was determined by the method of Leong and Shui (2002) [25] with slight modifications using DPPH (1,1,-diphenyl-2-picrylhydrazyl) radical. The tea leaves extract solution of 100 µL was diluted with 1.9 mL methanol solvent and added to 2 mL methanolic DPPH (0.1mM in methanol) solution. The mixture was incubated at room temperature in dark for 30 minutes and the absorbance was read at 517 nm, taking methanol as blank. A mixture of equal volume of methanol and DPPH reagent served as control. AA was expressed as percentage (%) of scavenging effect.

2.7. Statistical analysis

A second order polynomial equation was fitted to the experimental data of each response variable (Y_k) and is expressed as (Singh *et al*., 2010) [16]:

$$Y_k = \beta_{k0} + \sum_{i=1}^{n} \beta_{ki} x_i + \sum_{i=1}^{n} \beta_{kii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{kij} x_i x_j + e_k \quad (1)$$

Where, Y_k = response variable, Y_1 = TP (g CE/100 g of tea leaves), Y_2 = AA (%). Coefficient β_{k0} is the value of fitted response at the center point design, i.e., point (0, 0, 0), and β_{ki}, β_{kii} and β_{kj} are the linear, quadratic, and interaction regression coefficients, x is the independent variable, n is the number of independent parameters $(n = 4)$, and $e_k =$ error. Analysis of variance (ANOVA) was used to evaluate the effect of process variables on dependent variables. The significant and non-significant model terms were found by ANOVA for each response within 95% of confidence interval ($p<0.05$). The effect of the function of two independent variables on dependent variables (while holding the third independent variable at centre point) was visualized in three dimensional (3-D) response surface and two dimensional (2-D) contour plots.

2.8. Optimization

Optimum values of process variables (LTSR, extraction time and number of extraction) were evaluated through numerical optimization and desirability function method. The maximum

desirability (D) for optimization as calculated using the following equation (Chakraborty *et al*., 2014) [26].

$$D = (d_1^{r_1} \times d_2^{r_2} \times d_3^{r_3} \times d_4^{r_4})^{\frac{1}{(r_1+r_2+r_3+r_4)}} \quad (2)$$

Where, d_i = desirability index for i^{th} responses having the relative importance of r_i, set to its target (maximize or minimize) corresponding to each process and response parameters. The D value as well as d_i varied in the range between 0 (least desirable) and 1 (most desirable). The process parameters were targeted to be minimized and response parameters maximized in the industrial point of view.

3. Results and Discussion

3.1. Selection of design variables and their levels

The effect of various solvents (water, methanol, ethanol and acetone) and their mixtures on TP and AA were studied (Table 1). Out of the tested neat solvents, methanol gave the TP yield (5.66±0.08 g CE/100 g) and water gave the lowest yield (2.03±0.01 g CE/100 g), while the AA of methanol was 89.79±0.37% and that of water was 80.55±0.27%. The present result is in partial agreement with Jaime Guerrero *et al*. (2010) [27], where the authors found high antioxidant activity in methanolic extract of cultivated barriers. The TP of methanol, ethanol and acetone were almost comparable ($p>0.05$); however AA of ethanolic extract was lower than those of acetone and methanol ($p<0.05$). Next, following the methodology of Das Purkayastha *et al*. (2013) [23], equimolar mixture of these solvents was tested for TP and AA, wherein methanol and acetone (1:1 v/v) gave the highest yield of TP (5.79±0.16 g CE/100 g). So, this mixture was used as the extracting medium (solvent) for further optimization. Based on preliminary study, the lower, middle and upper levels for the independent variables, namely, leaves-to-solvent ratio (LTSR) were set at 1:20, 1:30 and 1:40 (w/v), and those for extraction time was set at 1, 2 and 3 h (Table 2). Zlotek *et al*. (2016) [22] reported that the number of extraction is a crucial factor for extraction of phenolic compounds of basil leaves (*Ocimum basilicum*) extracts. So, the number of extraction was varied from 1-3 times in the present study, as inclusion of more of number extraction steps might incur cumbersomeness.

3.2. Model fittings

The mathematical models for TP and AA were generated individually using 20 experiments of CCD (Table 2). The observed data were fitted to second-order polynomial equation and model was tested to describe the variability in the responses, by evaluating the coefficients of determination. Regression summary of ANOVA conducted for the second order polynomial models of TP and AA are presented in Table 3 and 4. The high values of coefficient of determination ($R^2>0.90$) indicate the good fit between the observed and predicted values. Non-significant lack of fit suggests a good model for prediction. The adequate precision measures the signal to noise ratio, which was found to be 17.92 for TP and 20.57 for AA. If this ratio is >4, it is considered desirable for the responses, which indicates the best fitness of the developed models (Canettieri *et al*., 2013) [28]. Moreover, the small values of coefficient of variation (CV) gave the better reproducibility of process conditions for the responses (Liyana-Pathirana and Shahidi, 2005) [19]. The p-values indicated that all linear and quadratic model terms of X_1, X_2, and X_3 are statistically significant at 95% confidence levels (Table 3 and 4). The regression equations obtained from the second order polynomial model (in the coded forms of process variables) for TP (Y_1) and AA (Y_2) are as follows:

$$Y_1 = +5.63 + 1.17X_1 + 0.5168X_2 + 0.2066X_3 - 0.1325X_1X_2 - 0.0550X_1X_3 + 0.1925X_2X_3 - 0.3602X_1^2 - 0.4168X_2^2 - 0.3001X_3^2 \quad (3)$$

$$Y_2 = +89.91 + 3.27X_1 + 1.25X_2 + 0.9323X_3 - 0.6213X_1X_2 - 0.4188X_1X_3 + 0.0287X_2X_3 - 1.38X_1^2 - 1.21X_2^2 - 0.5278X_3^2 \quad (4)$$

Where, X_1, X_2, and X_3, are the coded values of independent parameters (LTSR (w/v), extraction time (h), and number of experiment, respectively).

3.4. Total polyphenols (TP)

Although the interaction terms showed non-significant effect on TP (Table 3); LTSR contributed a major effect, followed by extraction time. Both extraction time and LTSR had significant quadratic effects on TP (Table 3). Therefore, increase in LTSR and extraction time tends to increase TP yield, which is clearly depicted in Fig. 1. Nevertheless, prolonged extraction of bioactive compounds, especially polyphenols, is usually not recommended because these compounds get oxidized, polymerized or degraded (Chew *et al*., 2011; Maheshu *et al*., 2013) [29, 30].

3.5. Antioxidant activity (AA)

Likewise, in AA, the linear and quadratic terms of X_1, X_2, and X_3 were found to be significant ($p<0.05$) (Table 4). Thus, LTSR followed by extraction time were the main determining terms of AA amongst the process variables. Moreover, the interaction term X_1X_2 produced a significant effect ($p<0.05$), indicating that AA increases with increasing these two factors (Fig. 2). This can be related to the higher extractability of antioxidative polyphenols with increasing solvent volume and time. Similar observation was reported by McDonald *et al*. (2001) [31] wherein the solvent volume played a critical role on antioxidant activity of olive leaves extracts.

3.6. Optimization and validation of the models

In order to obtain the optimum value of process conditions for obtaining high TP and AA of fresh tea leaves, the second order polynomial equation was utilized for each response. Using Desirability function, the optimum values predicted for the independent variables were 30.4 mL of LTSR, 1.5 h of extraction time and number of extraction as 1, while the predicted optimum values for the responses were 4.93 g CE/100 g of tea leaves of TP and 87.74% AA (Table 5). The optimized conditions obtained by RSM were performed to validate the predicted models. The data obtained from the trials conducted under the predicted optimum condition, were reasonably close to the predicted values, which confirmed the validity of the derived models. Thus, the verification of the experiments at optimized conditions proved that the observed value of TP being 4.56±0.12 g CE/100 g of sample and AA of 88.14±0.26 % could be achieved satisfactorily within 95% confidence interval levels (Table 6).

Table 1: Values of total polyphenols (TP) and antioxidant activity (AA) at different extraction conditions

Solvent (v/v)	Total Polyphenols (g CE/100 g of tea leaves)	Antioxidant activity (%)
Methanol	5.66±0.08[b]	89.79±0.37[b]
Acetone	5.45±0.12[b]	89.46±0.06[b]
Ethanol	5.39±0.24[b]	85.10±0.36[c]
Water	2.03±0.01[a]	80.55±0.27[a]
Methanol: Acetone (1:1 v/v)	5.79±0.16[c]	89.40±0.16[a]
Acetone: Ethanol (1:1 v/v)	5.15±0.03[c]	84.45±0.38[c]
Methanol: Ethanol (1:1 v/v)	5.58±0.03[b]	87.39±0.44[c]

Value=Mean ± Std. Dev. (N=3). Solvent volume=40 ml, Extraction time=2 h, No. of Extraction=2. Same superscript letters with a column are not statistically different at $p \leq 0.05$

Table 2: Central Composite Design (CCD) with experimental and predicted values of response variables for total polyphenols (TP) and antioxidant activity (AA) of tea leaves

Exp. Run	Actual and coded values			Response values			
	LTSR (w/v) (X_1)	Extraction time (h) (X_2)	No. of Extraction (X_3)	Total Polyphenols (g CE/100 g of tea leaves) (Y_1)		Antioxidant activity (%) (Y_2)	
				Experimental	Predicted	Experimental	Predicted
1	30 (0)	2 (0)	2 (0)	5.60±0.05	5.63	90.04±0.45	89.91
2	20 (-1)	3 (1)	3 (1)	4.21±0.04	4.49	86.38±0.29	86.76
3	40 (1)	1 (-1)	1 (-1)	5.68±0.10	5.39	89.22±0.54	88.94
4	46.82 (1.68)	2 (0)	2 (0)	6.64±0.06	6.59	92.08±0.67	91.50
5	30 (0)	2 (0)	2 (0)	5.88±0.18	5.63	90.30±0.21	89.91
6	13.18 (-1.68)	2 (0)	2 (0)	2.56±0.03	2.64	80.06±0.40	80.49
7	20 (-1)	1 (-1)	3 (1)	3.05±0.07	2.80	83.98±0.34	82.96
8	30 (0)	2 (0)	2 (0)	5.66±0.08	5.63	89.92±0.21	89.91
9	30 (0)	2 (0)	4 (1.68)	5.24±0.11	5.13	89.68±0.48	89.98
10	30 (0)	4.08 (1.68)	2 (0)	5.70±0.05	5.32	89.33±0.18	88.58
11	30 (0)	2 (0)	2 (0)	5.25±0.09	5.63	89.04±0.44	89.91
12	40 (1)	3 (1)	3 (1)	6.32±0.04	6.46	91.26±0.28	91.23
13	40 (1)	1 (-1)	3 (1)	5.30±0.10	5.30	89.67±0.57	89.91
14	30 (0)	2 (0)	1(-1.68)	4.30±0.07	4.44	87.30±0.70	86.85
15	20 (-1)	1 (-1)	1 (-1)	2.82±0.02	2.66	80.18±0.23	80.32
16	30 (0)	2 (0)	2 (0)	5.91±0.08	5.63	90.60±0.30	89.91
17	40 (1)	3 (1)	1 (-1)	5.54±0.13	5.77	89.02±0.62	90.15
18	30 (0)	2 (0)	2 (0)	5.50±0.05	5.63	89.52±0.50	89.91
19	30 (0)	0.32 (-1.68)	2 (0)	3.18±0.09	3.58	83.78±0.59	84.38
20	20 (-1)	3 (1)	1 (-1)	3.60±0.03	3.58	84.14±0.25	84.00

Value=Mean ± Std. Dev. (N=3)

Table 3: ANOVA showing the variables as linear, interaction, and quadratic terms on total polyphenols (TP)

Source	df	β	SS	F-value	p-value
Model	9		28.25	32.36	<0.0001
X_1-LTSR	1	1.17	18.80	193.78	<0.0001
X_2-Extraction time	1	0.5168	3.65	37.61	<0.0001
X_3-No. of Extraction	1	0.2066	0.5827	6.01	0.0342
X_1 X_2	1	-0.1325	0.1404	1.45	0.2566*
X_1X_3	1	-0.0550	0.0242	0.2495	0.6282*
X_2 X_3	1	0.1925	0.2965	3.06	0.1110*
X_1^2	1	-0.3602	1.87	19.28	0.0014
X_2^2	1	-0.4168	2.50	25.81	0.0005
X_3^2	1	-0.3001	1.30	13.38	0.0044
Residual	10		0.9700		
Lack of Fit	5		0.6660	2.19	0.2047*
Pure Error	5		0.3039		
Corrected Total	19		29.22		
R^2		0.9668			
Adjusted R^2		0.9369			
Adeq. Precision		17.9182			
C.V. %		6.36			

*Non-significant at 5% level, df: degree of freedom, β: coefficients, SS: sum of squares

Table 4: ANOVA showing the variables as linear, interaction, and quadratic terms on antioxidant activity (AA)

Source	df	β	SS	F-value	p-value
Model	9		229.73	43.20	<0.0001
X_1-LTSR	1	3.27	146.34	247.66	<0.0001
X_2- Extraction time	1	1.25	21.37	36.17	<0.0001
X_3-No. of Extraction	1	0.9323	11.87	20.09	0.0012
X_1 X_2	1	-0.6213	3.09	5.23	0.0453
X_1X_3	1	-0.4188	1.40	2.37	0.1544*
X_2 X_3	1	0.0287	0.0006	0.0112	0.9178*
X_1^2	1	-1.38	27.58	27.58	<0.0001
X_2^2	1	-1.21	21.17	21.17	<0.0001
X_3^2	1	-0.5278	4.01	4.01	0.0262
Residual	10				
Lack of Fit	5			2.80	0.1413*
Pure Error	5				
Corrected Total	19				
R^2		0.9749			
Adjusted R^2		0.9524			
Adeq. Precision		20.5748			
C.V. %		0.87			

*Non-significant at 5% level, df = degree of freedom, β = coefficients, SS = sum of squares

Table 5: Optimum solutions of process and response variables at desired goal and criteria

Parameters	Desired goal	Criteria		Importance	Predicted
		Upper limit	Lower limit		
LTSR (w/v)	Minimize	20	40	3	30.384
Extraction time (h)	Minimize	1	3	3	1.46613
No. of Extraction	Minimize	1	3	3	1.07577
Total Polyphenols (g CE/100 g of tea leaves)	Maximize	2.56	6.64	5	4.93472
Antioxidant activity (%)	Maximize	80.06	92.08	5	87.7473

Table 6: Validation at optimum conditions of extraction parameters for total polyphenols (TP) and antioxidant activity (AA) of tea leaves

Optimum conditions			Responses		
LTSR (w/v)	Extraction time (h)	No. of Extraction	Parameters	Observed	Predicted
30.4	1.5	1	Total Polyphenols (g CE/100 g of tea leaves)	4.56±0.12	4.93
			Antioxidant activity (%)	89.39±0.26	87.74

Value=Mean ± Std. Dev. (N=3)

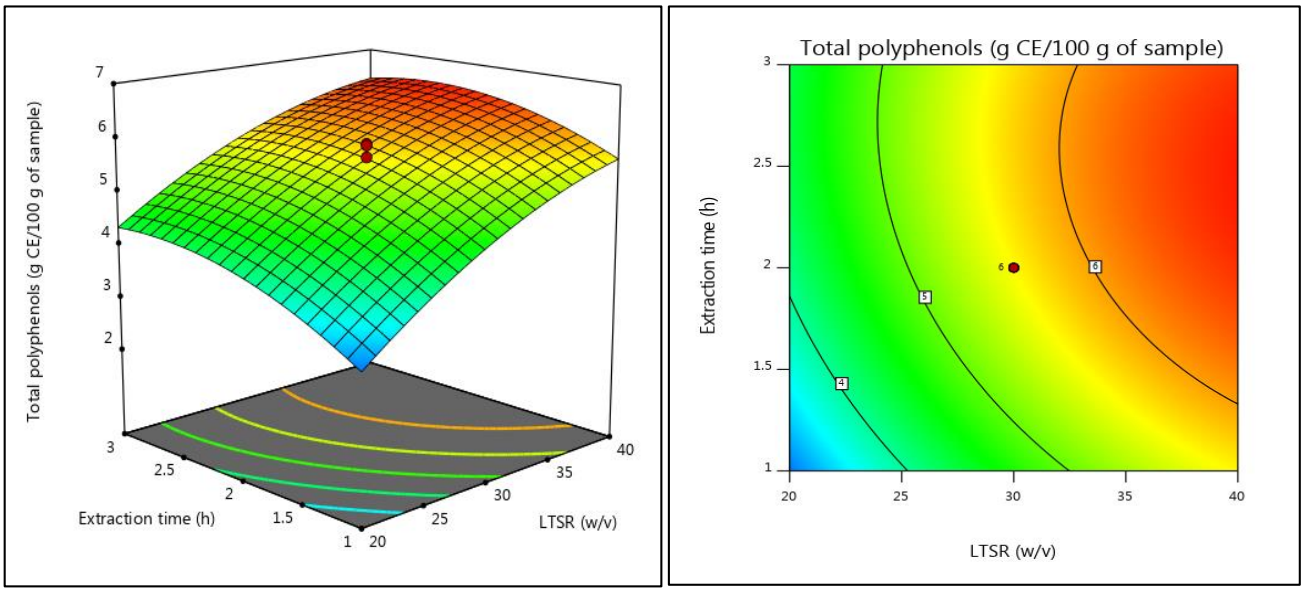

Fig 1: Response surface and contour plots for the effects of LTSR and extraction time on total polyphenols (TP)

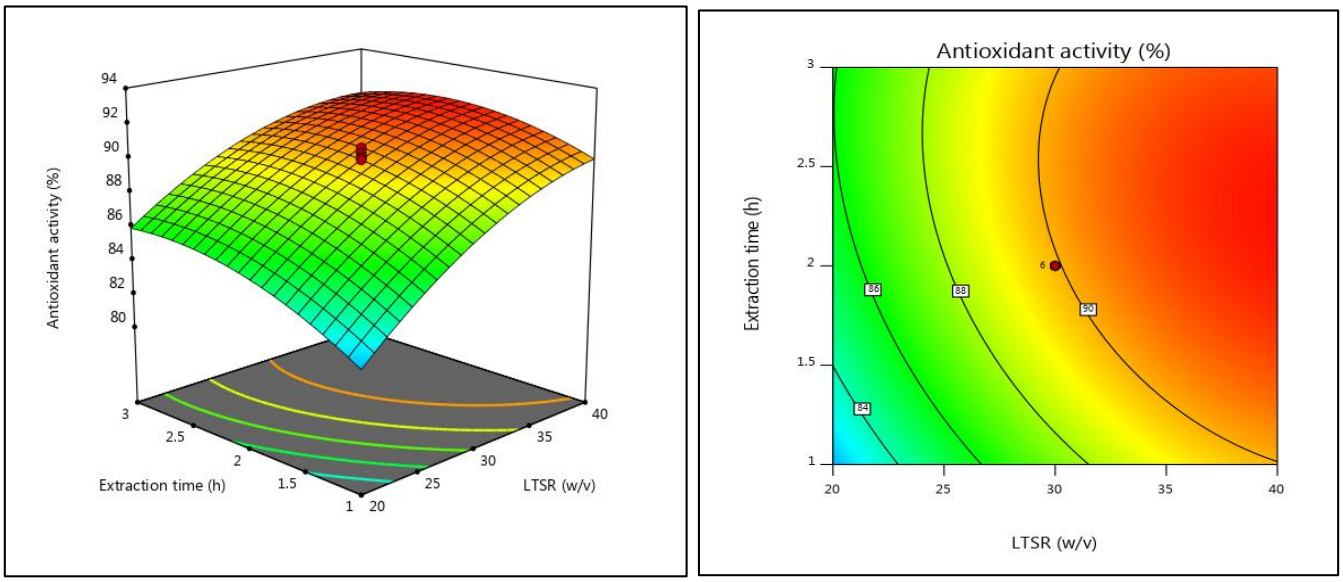

Fig 2: Response surface and contour plots for the effects of LTSR and extraction time on antioxidant activity (AA)

4. Conclusions

Among the tested solvents, combination of methanol and acetone (1:1 v/v) was found to be the most effective extractant in obtaining maximum yield of TP and AA from tea leaves. RSM was successfully employed in optimizing the extraction process and response parameters. Desirability function was applied to locate the optimum operating conditions of the responses, which was experimentally verified and found to be adequately reproducible within the predicted 95% confidence interval levels. Under the suggested optimum conditions, the experimental values were in congruent with the predicted values, which vouched the validity of the predicted model. The extracted antioxidative phenolics can be used in a myriad of food models.

5. Acknowledgements

We would like to acknowledge the grant received from DBT U-Excel, DBT, Govt. of India and Assam Agricultural University (AAU) for financial support.

6. Conflict of Interests

The author(s) declare that there is no conflict of interest with respect to the research, authorship, and/or publication of this article to disclose.

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