Optimization of extraction conditions for the extraction of phenolic compounds from *Moringa oleifera* leaves

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Abstract: The aim of this study was to optimize the extraction conditions for the extraction of phenolic compounds from *Moringa oleifera* leaves using response surface methodology (RSM). A user- defined design was applied to determine the effects of extraction time (min), extraction temperature (^oC) and ethanol concentration (%), on total phenolic content (TPC) from *Moringa oleifera* leaves dried by three methods (oven, sunlight and ambient air). The RSM was used to optimize the extraction conditions for the extraction of TPC of *Moringa oleifera* leaves. The optimum conditions that maximize the extraction of TPC were extraction time, 60 min; extraction temperature, 90^oC and % of methanol, 50 % (v/v). TPC extracted under these conditions were 12.28, 12.65 and 13.14 mg GAE/g DW for samples dried by different methods. Significant difference between drying methods was found ($p<0.001$). Pair wise significant difference was found only between oven and ambient air drying methods ($p<0.001$).

Keywords: *Moringa oleifera*, phenolic compounds, response surface methodology, drying method.

INTRODUCTION

Moringa oleifera is very important plant due its applications in nutritional, agricultural, pharmacological and industrial fields (Kasolo, 2010). The extract of *Moringa oleifera* leaves have antioxidant potential. Sufficient amount of β-carotene, protein, vitamin C, calcium, potassium and natural antioxidants such as ascorbic acid, flavonoids, phenolics and carotenoids is present in *Moringa oleifera* leaves. Due to this reason they are used for inhibiting lipid peroxidation and increases the shelf life of food, which contain fat (Anwar, 2007).

Due to their strong in vitro and in vivo antioxidant activities and their ability to scavenge the free radicals Phenolic compounds have attracted the attention of food and medical scientists (Pinelo *et al.,* 2005; Li *et al.,* 2006; Silva *et al.,* 2007). The research that focused on the recovery of *Moringa oleifera* leaves as a source of phenolic antioxidants is not fully developed.

The availability of phenolic compounds in *Moringa oleifera* leaves as antioxidant source is ensured. High extraction efficiency is necessary for the economical feasibility of an industrial process. There are many factors, which can influence the extraction efficacy, such as extraction methods, particle size, solvent type, solvent concentration, solvent-to-solid ratio, extraction temperature, extraction time and pH (Pinelo *et al.,* 2005; Banik and Pandey, 2007; Silva *et al.,* 2007).

Current study was carried out with the objective primarily to use Response Surface Methodology (RSM) for the optimization of extraction parameters including extraction time, extraction temperature and % of methanol for the extraction of TPC from *Moringa oleifera* leaves and secondly to compare effect of drying methods on TPC extracted from *Moringa oleifera* leaves.

MATERIALS AND METHODS

Plant material

Leaves of *Moringa oleifera* commonly known as sohanjna were collected from a plant in Gujrat. Sunlight, oven and ambient air drying were used for drying the leaves.

Solvent extraction

Leaves dried in sunlight, oven and ambient air were ground into a fine powder in a pestle and mortar. Approximately 5 g of dried sample was weighed and extracted with 50 mL using soxhlet apparatus. Extracts were concentrated reduced pressure at 30-45^oC on rotary evaporator. Concentrated extracts were preserved in labeled sample bottles in a refrigerator (-4^oC) for further analysis.

Determination of phenolic content

Parejo's method was used to determine the total phenolic content by using the Folin-Ciocalteu reagent (Naczk and Shahidi, 2004). Solvent extract (0.5 ml), 0.5 ml of Folin-Ciocalteu reagent, 10 ml of 75g L^{-1} sodium carbonate and deionized water were added to a final volume of 25 ml. After 1 h, the absorbance of the sample was measured at 725 nm against a blank by a spectrophotometer. Calibration curve was prepared using Gallic acid as the standard.

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Experimental design

The effects of three factors, extraction time (tim), extraction temperature (Temp.) and % of methanol on the TPC from *Moringa oleifera* leaves were studied using response surface methodology.

By using this design, the three variables were tested at 3 different levels: extraction time at 30, 60 and 90 minutes, extraction temperature at 30, 60 and 90ºC, % of methanol at 50, 75 and 100%. Experimental matrix design, with the experimental levels of the independent variables (factors), along with the results obtained for the response analyzed variables for each drying method are shown in table 1.

STATISTICAL ANALYSIS

The experimental results were analyzed using SPSS software (SPSS Version 16). One-way analysis of variance (ANOVA) was used to determine the significant differences (p<0.05) between the means.

The Design Expert (Version 8.0.5, Stat-Ease Inc., Minneapolis) statistical software was employed to analyze the experimental data in RSM.

RESULTS

In this research work, three drying methods were used. Phenolic contents obtained from oven dried, sunlight dried and ambient air dried samples of *Moringa oleifera* leaves are given in table 1. Analysis of variance (ANOVA) was used to study the effect of independent variables on the response variable (phenolic contents). ANOVA for selected 2FI model is given in table 2. Table 3 shows the summary statistics of selected 2FI model. In this study effect of three drying methods was also evaluated. Test of significant difference between average TPC on the basis of three drying methods is given in table 4. Table 5 demonstrates the pair wise significance difference between average TPC obtained from oven dried, sunlight dried and ambient air dried samples of *Moringa oleifera* leaves.

3D response surfaces are visual prediction of future responses, and used for determining factor values that optimize the response function. 3D response surfaces for oven dried, sunlight dried and ambient air dried samples of *Moringa oleifera* leaves are shown in fig. 1, 2 and 3 respectively.

Table 1: Comparison of total phenolic contents from oven dried samples, sunlight dried samples and ambient air dried samples as 27 experimental runs

Run No.	Extraction time	Extraction Temp.	% of Methanol	Oven dried Sample	Sunlight dried Sample	Ambient air dried Sample
1	90	30	75	10	10.43	10.9
2	60	90	75	11.78	12.15	12.64
3	60	60	50	11.25	11.65	12.16
4	30	60	50	11	11.43	11.93
5	30	60	75	10.5	10.96	11.39
6	60	90	50	12.28	12.65	13.14
7	60	60	75	10.75	11.15	11.66
8	60	60	100	10.25	10.55	11.15
9	90	90	50	*11.5*	*11.8*	*12.2*
10	30	90	50	12.03	12.4	12.9
11	30	60	100	10	10.43	10.9
12	60	30	75	9.7	10.13	10.62
13	60	30	50	10.23	10.65	11.1
14	30	90	75	11.53	11.9	12.4
15	90	60	100	10.5	10.93	11.4
16	60	30	100	9.2	9.63	10.12
17	90	30	50	10.5	10.93	11.4
18	90	60	50	11.5	11.93	12.43
19	30	30	50	10	10.46	10.9
20	90	90	100	10.5	10.8	11.4
21	90	30	100	9.5	9.93	10.4
22	30	30	75	9.5	9.95	10.41
23	60	90	100	11.28	11.65	12.14
24	90	90	75	11	11.3	11.7
25	30	90	100	11.03	11.4	11.89
26	90	60	75	11	11.43	11.9
27	30	30	100	9	9.43	9.9

DISCUSSION

In this study phenolic contents of oven dried, sunlight dried and ambient air dried samples of *Moringa oleifera* leaves were determined. Values of TPC are shown in table 1.

Fitting of model

Fitting of the response function and experimental data, the linearity and quadratic effect of the independent variables, their interactions and regression coefficients on the response variables were evaluated by analysis of variance (ANOVA) (table 2). Models were highly significant due to a very low probability value ($p<0.0001^a$, $p<0.0001^b$ and $p<0.0001^c$) as indicated by the ANOVA of the regression models. Coefficient of determination (R^2) and the significance of lack-of-fit were used to check the fitness and adequacy of the models. R^2 which was defined as the ratio of the explained variation to the total variation was a measure of the degree of fit (Wang *et al.,* 2008). The empirical model fits to the actual data in a better way when the R^2 value closer to unity (Fan *et al.,* 2007). R^2 values for the regression models of TPC were 0.9505^a, 0.9386^b and 0.9340^c which were closed to 1 as shown in table 3. This suggested that the predicted second order polynomial models defined well the real behaviour of the system. In addition, the values of adjusted R^2 (0.9357^a, 0.9201^b and 0.9143^c) were also very high which indicate a high significance of the models. The adjusted R^2 value was obtained from R^2 after the elimination of the unnecessary model terms. In this study, the adjusted R^2 values were very close to the R^2 values. The reliability of the models also strengthened by the absence of any lack of fit ($p>0.05$). Experimental results were precise and reliable as indicated by a small coefficient of variation (2.06^a, 2.16^b and 2.15^c). Significance of each coefficient, which in turn might indicate the interaction patterns between the variables, was checked by P-values (Hou and Chen, 2008). The smaller the P-value, the more significant was the corresponding coefficient. It could be observed from table 2 that both the linear and 2FI terms of all parameters (extraction time, extraction temperature

Table 2: ANOVA for response surface 2FI model

Source	df	SS (MS)[a]	SS (MS)[b]	SS (MS)[c]	F Value (p-value) [a]	F Value (p-value) [b]	F Value (p-value) [c]	
Model	6	18.44 (3.07)	17.30 (2.88)	17.33 (2.89)	64.07 (0.0001)	50.93 (0.0001)	47.21 (0.0001)	Signi-ficant
A-Extraction Time	1	0.31 (0.31)	0.31 (0.31)	0.29 (0.29)	6.44 (0.0196)	5.52 (0.0292)	4.70 (0.0423)	
B-Extraction Temp.	1	1.62 (1.62)	1.57 (1.57)	1.57 (1.57)	33.73 (0.0001)	27.66 (0.0001)	25.59 (0.0001)	
C-% of Methanol	1	0.36 (0.36)	0.38 (0.38)	0.43 (0.43)	7.41 (0.0131)	6.66 (0.0179)	6.99 (0.0156)	
AB	1	0.80 (0.80)	0.88 (0.88)	0.95 (0.95)	16.59 (0.0006)	15.55 (0.0008)	15.56 (0.0008)	
AC	1	0.000 (0.000)	0.00007 (0.0007)	0.0036 (0.0036)	0.000 (1.0000)	0.0013 (0.9713)	0.060 (0.8089)	
BC	1	0.00007 (0.00007)	0.0002 (0.0002)	0.0002 (0.0002)	0.0015 (0.9689)	0.0036 (0.9522)	0.039 (0.8447)	
Residual	20	0.96 (0.048)	1.13 (0.057)	1.22 (0.061)				
Lack of Fit	10	10.69 (1.07)	17.45 (1.75)	23.40 (2.34)	1.69 (0.2923)	4.27 (0.0612)	3.16 (0.1082)	not signi-ficant
Pure Error	20	3.16 (0.63)	2.04 (0.41)	3.71 (0.74)				
Cor Total	26	19.40	18.43	18.55				

Table 3: Summary statistics of selected 2FI model

Quadratic Model	C.V. %	PRESS	R-Squared	Adj R-Squared	Pred R-Squared	Adeq Precision
Model[a]	2.06	1.97	0.9505	0.9357	0.8984	28.859
Model[b]	2.16	2.32	0.9386	0.9201	0.8739	26.213
Model[c]	2.15	2.46	0.9340	0.9143	0.8677	25.502

Model[a,b&c] represent 2FI models used for optimization conditions for the exractions of phenolic contents from oven dried, sunlight dried and ambient air dried samples.

and % of methanol) had significant (at least at $p<0.05$) effect on TPC. TPC was also significantly influenced by the interactions between extraction time and extraction temp. ($p<0.0006^a$, $p<0.0008^b$ and $p<0.0008^c$). Among all the three extraction parameters studied, extraction temperature had the most critical role in the extraction of phenolic compounds from *Moringa oleifera* leaves followed by extraction time and % of methanol. By applying multiple regression analysis, relationship between the tested independent variables and the response was explained in Equation (1):

$TPC^a = 9.274 + 0.019\ t + 0.045\ T - 0.020\ \%\ MeOH + 0.00028\ t.T + 0.0000\ t\ .\ \%\ MeOH + 0.000003\ T.\ \%\ MeOH$

$TPC^b = 9.78 + 0.019\ t + 0.044\ T - 0.021\ \%\ MeOH + 0.0003\ t\ .T + 0.000003\ t\ .\ \%\ MeOH + 0.000005\ T.\ \%\ MeOH$

$TPC^c = 10.3 + 0.019\ t + 0.044\ T - 0.022\ \%\ MeOH + 0.0003\ t.T + 0.00002\ t\ .\ \%\ MeOH + 0.00001\ T.\ \%\ MeOH$

Equation 1(a, b, c)

Analysis of response surfaces

3D response surfaces are used for visually predicting future responses, and for determining factor values that optimize the response function.

Effect of extraction time on extraction of total phenolic compounds

Figs. 1, 2, 3 present effect of extraction time on the TPC extracted from *Moringa oleifera* leaves. Figs. show that the time factor has a very low influence in the final response. The highest value of TPC for extraction time in extraction of phenolic compound was at 60 minutes. Extraction time has no significant effect on the extraction of phenolic compounds ($p>0.05$). The range of time was selected based on the practical and economical aspects because longer time will increase cost. There was small increase in TPC with the increase of time. It was clear that a shorter time would extract the same amount of phenolic extracts as longer time while saving cost. Too much extraction time is not useful to extract more phenolic antioxidants (Silva *et al.,* 2007). Small increase of total phenolic contents at a longer extraction time may be due Polymers and wall-bound phenolics retained in cells that were extracted out as well as the polymerization reaction that occurs and new components produced as reported by Spigno and De Faveri (2007).

Effect of extraction temperature on extraction of total phenolic compounds

Figs. 1, 2, 3 show that temperature has a highly significant ($p<0.0001$) effect on extraction of phenolic compounds from *Moringa oleifera* leaves. The extraction of phenolic compounds as shown in figs. 1, 2, 3 was at its peak at 90°C with the value of total phenolic content (TPC) 12.28, 12.65 and 13.14 mg GAE/ g DW. The value then decreases to 9, 9.43 and 9.9 at 30°C. Wang and Zheng (2001) reported that temperature strongly changes antioxidant properties in strawberries. Extraction of TPC increases with the increase of temperature due increasing both solubility of solute and diffusion coefficient but after a certain temperature, phenolic compounds can be denatured (Spigno *et al*., 2007). Dutra *et al.* (2008) reported that extraction made under reflux using ethanol/water (70:30, v/v) provided the highest polyphenol levels in Vogel seeds. This might be due to release of some bound phenolics extracted under reflux conditions (Antolovich *et al.,* 2007).

Effect of % of methanol on extraction of total phenolic compounds

It can be seen from figs. 1, 2 and 3 that total phenolic content (TPC) decreases with the increase of % of methanol. Mixture of alcohols and water is more efficient to extract phenolic constituents as compared to mono-component solvent system (Spigno *et al.,* 2007). Addition of small quantity of water to organic solvent usually creates a more polar medium which facilitate the extraction of polyphenols (Spigno *et al.,* 2007). Polarity of the solvent increases by increasing the proportion of

Table 4: Test of Significant Difference between Average TPC on The Basis of Three drying methods

TPC (mg/g)	Sum of Squares	df	Mean Square	F	p-value
Between drying methods	10.496	2	5.248	7.260	.001
Within in drying methods	56.382	78	.723		
Total	66.877	80			

Table 5: Pair wise Test of Significance Difference between Average TPC (%) based upon three Drying methods

Average TPC (mg/g)		Mean Difference (1-2)	Std. Error	p-value
1	2			
Oven dried sample	Sunlight dried sample	-.39778	.2314	.205
Oven dried sample	Ambient air dried sample	-.88037*	.2314	.001
Sunlight dried sample	Ambient air dried sample	-.48259	.2314	.099

*The mean difference is significant at the 0.05 level.

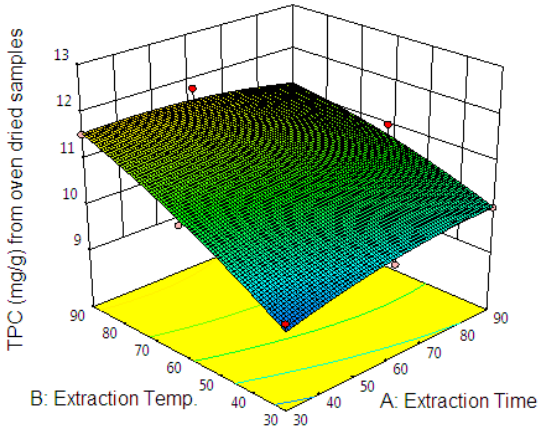
water to methanol. This type solvent system can extract the phenolic substances of all the types (highest polarity substances, low polarity substances and moderate polarity substances) (Zhang *et al.*, 2007).

Optimum conditions

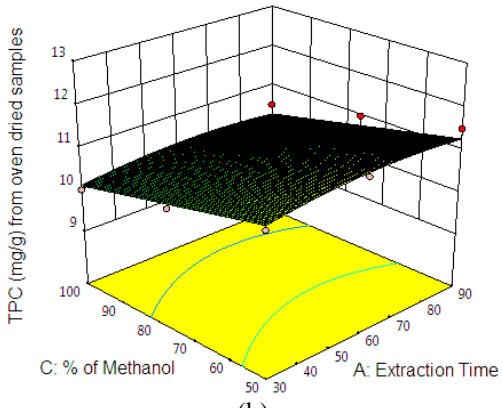
Optimum conditions are bolded in table 1. The optimal extraction conditions of TPC from *Moringa oleifera* leaves extracts acquired using the model were as follows: extraction time 60 minutes, extraction temperature 90°C and % of methanol, 50% (v/v). Under these optimal conditions, the model predicted a maximum response of 12.28, 12.65 and 13.14 mg GAE/g DW of *Moringa oleifera* leaves extracts dried by oven, sunlight and ambient air respectively.

Comparison of drying methods

The effect of drying methods on the TPC from *Moringa oleifera* leaves was determined. Three drying methods (oven, sunlight and ambient air) were used. It was found that ambient air drying method gives best results among the three drying methods. The maximum TPC observed from ambient air dried samples was 13.14 mg GAE/g DW while for sunlight and oven dried samples the maximum TPC was 12.65 and 12.28 mg GAE/g DW respectively.



(a)



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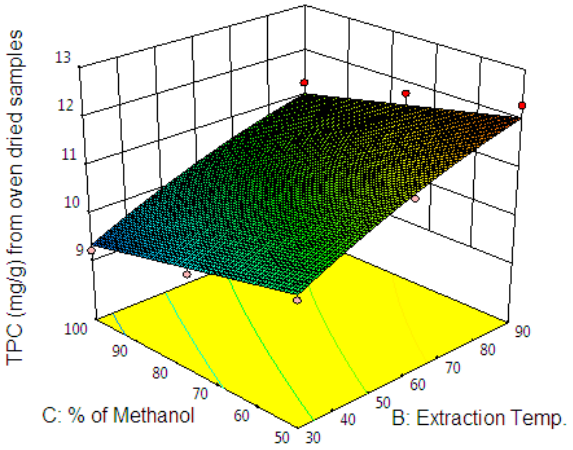
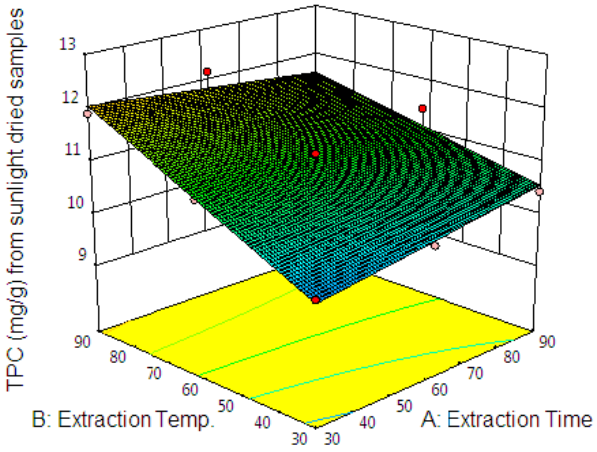


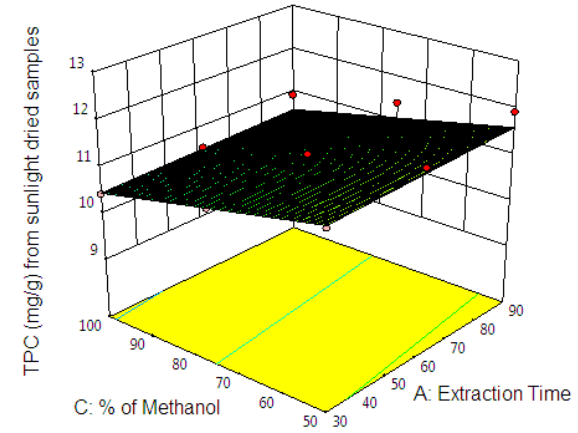
(c)

Fig. 1: Response surface plot corresponding to total phenolic content (TPC) of Moringa oleifera *leavesdried in oven* as a function of (a) extraction temperature and extraction time (b) % of methanol and extraction time (c) % of methanol and extraction temperature

Analysis of variannce ANOVA (table 4) was carried out to assess the equality of TPC from *Moringa oleifera* leaves by three different drying methods. The p-value (0.01 <0.05) showed that there is significant difference between the average TPC from *Moringa oleifera* leaves dried by three different methods. Further Post Hoc test (Tukey's test) was applied to check the pairwise significant difference among the average TPC (table 5). The p-value (0.01) for oven and ambient air dried samples depicted clearly that significant difference exist between the TPC from oven and ambient dried samples. The p-values (0.205 and 0.099) for oven dried and sunlight dried samples, sunlight dried and ambient dried samples indicated non-significance difference between these samples.



(a)



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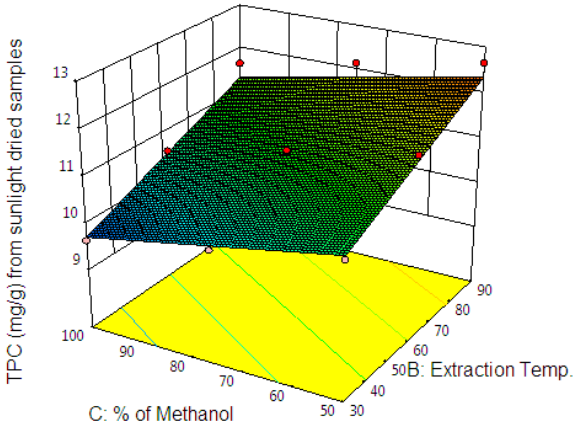
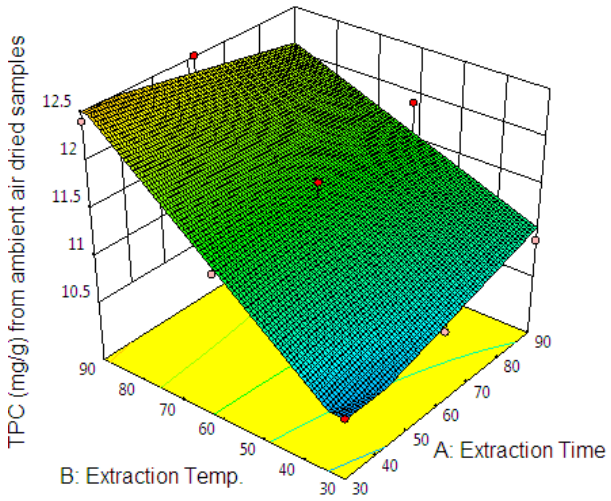
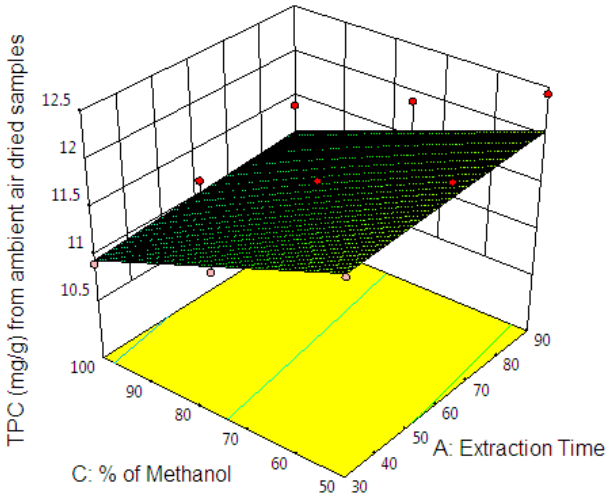


(c)

Fig. 2: Response surface plot corresponding to total phenolic content (TPC) of Moringa oleifera *leavesdried in sunlight* as a function of (a) extraction temperature and extraction time (b) % of methanol and extraction time (c) % of methanol and extraction temperature



(a)



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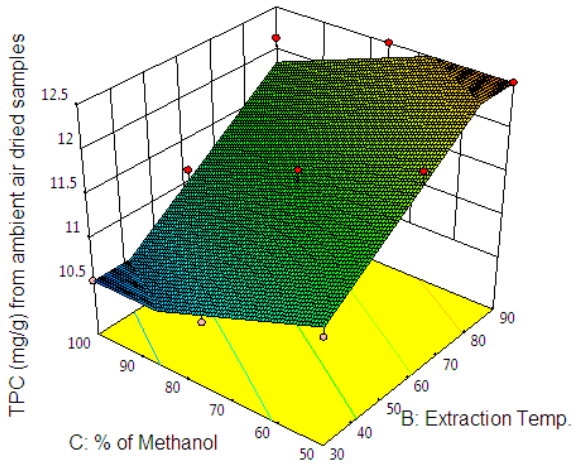

(c)

Fig. 3: Response surface plot corresponding to total phenolic content (TPC) of Moringa oleifera *leavesdried in ambient air* as a function of (a) extraction temperature and extraction time (b) % of methanol and extraction time (c) % of methanol and extraction temperature

Ambient air drying provided best results. Sunlight drying could lead to an uneven loss of antioxidants. During sunlight drying climate factors play important role as evidenced by Mueller-Harvey (2001). During oven drying intensive heat is generated which could inactivate the antioxidant. Antioxidants decompose rapidly in direct sunlight or elevated temperature.

Drying process would generally result in a reduction of naturally occurring antioxidants due intense and/or prolonged drying (Tomaino *et al.*, 2001). Drying process significantly destroy the natural antioxidants, as most of these compounds are comparatively unstable (Nicoli *et al.*, 1999).

CONCLUSION

The present study indicates the advantages of RSM over classical method in optimizing the extraction conditions for the extraction of phenolic antioxidants from *Moringa oleifera* leaves. The results from RSM showed that TPC of *Moringa oleifera* leaves were most affected by extraction temperature followed by % of methanol and extraction time. The optimum conditions that maximize the extraction of TPC were extraction time, 60 min; extraction temperature, 90^{o}C and % of methanol, 50 % (v/v). TPC extracted from samples dried by different methods under these conditions were 12.28, 12.65 and 13.14 mg GAE/g DW. Ambient air drying method provides best results. There was pairwise significance difference between TPC from *Moringa oleifera* leaves dried by oven and ambient air drying methods.

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