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Effect of Extraction Conditions on the Total Phenolic Yield of *Madhuca longifolia* Leaves and Evaluation of Its Physico-chemical and Antioxidant Properties

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Abstract

Madhuca longifolia is a traditional medicinal plant used in the treatment of several diseases including cancer in Indian system of medicine. Even though the chemical composition, bioactive principles and medicinal value of *M. longifolia* leaf have investigated earlier, the extraction of phytochemical compounds is not yet optimized, because preparation of extract will be of great importance as it could be used in several herbal formulations to treat variety of diseases. In the present study, we have employed different solvents (Hexane, ethyl acetate, methanol, ethanol and water) and different extraction conditions and also applied to optimize the extraction of phytochemicals from *M. longifolia* leaves. Effect of different solvent concentrations (50, 75 & 100%), extraction time (30, 75 and 120 min) and extraction temperature (30, 50 & 70^{o}C) on the phenolic yield was evaluated using Response Surface Methodology (RSM). Methanol was the suitable solvent to yield maximum phenolic content (6744.24 mg GAE/100 g). Maximum yield of total phenols (7755 mg GAE/100 g) was achieved under the optimal conditions such as extraction temperature (50^{o}C), extraction time (88 min) and solvent concentration (88%). Analysis of physico-chemical properties indicated that the methanolic extract of *M. longifolia* possesses acceptable sensory qualities with low water solubility (36%). Analysis of antioxidant activity in terms of DPPH radical scavenging potential indicated that the methanolic extract of *M. longifolia* (62.45 mg/L) was stronger when compared to other medicinal plants. Extraction of phenolic compounds with strong antioxidant power from *M. longifolia* was optimized in the present work, which could be useful to incorporate it in the development of novel herbal formulations. In this direction, the presently optimized conditions will be helpful for their large-scale extraction for industrial applications.

Key words: *Madhuca longifolia*; Extraction conditions; RSM; Antioxidant power; Physico-chemical properties.

INTRODUCTION

Madhuca longifolia (J. Konig) J.F. Macbr. belongs to the family Sapotaceae and commonly known as Mahua / Butter nut tree. It is a medium to large sized deciduous tree, can grow up to 17 m height and distributed in Nepal, India and Sri Lanka [1, 2]. *M. longifolia* is a multipurpose forest tree species that provide food, fodder and fuel [3]. Large numbers of Mahua trees are found in India and estimated production of its flowers is more than one million tonne in the country [4].

Leaves are coriaceous, elliptic, shortly acuminate and base cuneate and are clustered at end of the branches; young branches, leaves and petiole have pubescent. Flowers are numerous and drooping on pedicels near the ends of branches [5]. Calyx coriaceous, densely clothed with rusty tomentum whereas corolla is yellowish with fleshy tube. Stamens are 20-30 in number, anthers hispid at the back with stiff hairs. Fruit berries are ovoid, fleshy and green and seeds are 1-4 in number. Flowering takes place during March-April and fruit setting is noticed during April-May [5]. The extract of Mahua flowers is used in food industries for making jams, jellies, biscuits and other food products due to its nutritional components like vitamins, sugars, amino acids, organic acids, enzymes and other compounds (Betaine, tannins and crude pigments) and antioxidant activity [6]. *M. longifolia* seeds are of economic importance as they are good source of edible [3]. Mahua oil is used for manufacturer of laundry soaps and detergent, and also used as cooking oil in various tribal region of India [3].

The bark is used for rheumatism, chronic bronchitis, diabetes mellitus, ulcers, tonsillitis and bleedings [3, 7]. The flowers have been traditionally used as analgesic, diuretic, cooling agent, tonic, aphrodisiac, astringent, demulcent and for the treatment of helminthes, acute and chronic tonsillitis, pharyngitis and bronchitis [7]. Leaves are expectorant and also used for chronic bronchitis and Cushing's disease [7, 8]. Anticancer activity of *M. longifolia* was reported in Ayurvedic literature [9] and some *in vitro* cell line studies also confirmed its anti-proliferative property [2, 10, 11].

The presence of some bioactive substances in leaves supports the traditional medicinal uses of *M. longifolia* [12]. Different parts of *M. longifolia* were reported to contain sapogenins, triterpenoids, steroids, saponins, flavonoids, tannins, β-amyrin, betullic acid, ursolic acid, stigmasterol, β-carotene, xanthophylls, quercetin, dihyroquercetin, β-sitosterol, ethylcinnamate, sesquiterene alcohol, α-terpeneol, 3-β-monocaprylic ester of eythrodiol, 3-β-capryloxy oleanolic acid, myricetin, Mi-saponin A & B, erthrodiol and glycosides [3, 5, 13].

Since this plant has been used in Indian traditional systems of medicine to treat various diseases including cancer, preparation of extract from *M. longifolia* could be useful in developing herbal formulations and also effective in curing diseases than whole leaf powder. In this connection, as a

first step, we have optimized the suitable solvent and extraction conditions for the maximal recovery of phenolic compounds from *M. longifolia* leaf using response surface methodology and also we have characterized the physico-chemical properties and antioxidant potential of the extract.

MATERIALS AND METHODS

Plant materials

The leaf material of *M. longifolia* was collected from SASTRA Herbal Garden, Thachenkurichi, Thanjavur on July 2016. Samples were dried under shaded condition and finely powder using mixing grinder and used for further experiments.

Optimization of extraction

Effect of different solvents (Hexane, ethyl acetate, methanol, ethanol and water) on the recovery of phenolic compounds was investigated by taking 5 g sample in 50 ml of respective solvent and allowed to stand for 2 h at room temperature. Then the extract was filtered and the volume of filtrate was noted and analyzed for total phenolic content. Based on the total phenolic content, methanol was selected as a suitable solvent. Effect of different concentrations of methanol (50, 75 & 100%), extraction temperature (30, 50 & 70^{o}C) and extraction time (30, 75 & 120 min) were used to optimize the extraction conditions for the recovery of total phenol compounds from *M. longifolia* leaf using response surface methodology (Box-Behnken design). The experiments were designed and the results of analysis of total phenol content (TPC) were subjected to statistical analysis using ANOVA in Minitab software (Version 17) developed by Minitab Inc, Pennsylvania.

Totally 15 experiments were conducted in a randomized design and in each experiment 3 g of powdered sample was taken in a conical flask and extracted with 30 ml of different concentrations of methanol (50, 75 & 100%). Different temperatures (30, 50 and 70^{o}C) were maintained using a Universal Oven (Make: Memmert, Model: UF-30 Plus) and the extractions were carried out for three different timings (30, 75 and 120 min). At the end of each experiment, the contents were filtered and the filtrate was analyzed for TPC.

Total phenolic concentration

TPC was analyzed using Folin-Ciocalteu reagent method with some modifications [14]. The extract (0.1 ml) was added to 0.5 ml of Folin-Ciocalteau reagent and vortexed. Then, 2.0 ml of 2.2% sodium carbonate solution was added and the mixture was vortexed again. A blank was prepared with 0.1 ml methanol instead of the sample. The tubes were incubated at 40°C for 30 min in the dark and the absorbance was read at 720 nm against the blank using Spectrophotometer (Make: Perkin-Elmer). A calibration curve was prepared with standard gallic acid (7.81 – 500 mg/L, R2 = 0.958) and the concentration was calculated using the formula (y = 0.001x + 0.021) and the results were expressed as gallic acid equivalents (mg GAE / 100 g).

Physico-chemical properties

Based on TPC, the optimal extraction conditions were predicted using RSM and the suitable conditions were used to prepare the antioxidant extract from *M. longifolia*. For this purpose, 20 g of powdered material was extracted with 200 ml of 88% methanol at 50^{o}C for 88 min. The contents were then filtered and the final volume was noted. The extract yield was calculated by taking 10 ml of extract in a pre-weighed crucible and heated at 105^{o}C in an oven for 1 h and then cooled to room temperature in a desiccator. Then, the gross weight was recorded and based on difference in weights, the extract yield was calculated and expressed on percentage basis. The solvent was evaporated using rotovapor (Make: Buchi, Model R-300) and the dry extract was re-dissolved in water in the ratio of 10 mg/ml and used for further experiments.

Physico-chemical properties of the extract like solubility and pH were investigated. The solubility of the extract was analyzed according to the method described by Joshi and Aeri [15]. The extract (100 mg) was taken in a 10 ml of water in a centrifuge tube (15 ml) and kept on magnetic stirrer for 30 min. Then the contents were centrifuged at 3,000 rpm for 10 min and the supernatant was collected in a pre-weighed crucible. Then the contents were dried at 105^{o}C for 48 h and the weight of the residue was noted. Based on the weight of the residue, the solubility of the extract was calculated and expressed on percentage basis. The pH of the extracts was measured using a pH meter after calibrating the instruments with buffers ranged from 4 to 9.

Antioxidant activity

The antioxidant activity of the extract was analyzed in terms of DPPH radical scavenging potential [16]. Different concentrations of 100 µl of extract was added to 3.9 ml of DPPH solution (0.025 g/L) and the reactants were incubated at 25°C for 30 min. Different concentrations of Butylated hydroxyl anisole (BHA) was used as a positive control and solvent was used instead of extract in blank. The decrease in absorbance was measured at 515 nm using a spectrophotometer. The radical scavenging activity of tested samples was calculated and expressed on percentage basis.

RESULTS AND DISCUSSION

Finding new and safe antioxidants from natural sources is of great interest for their applications in functional foods and nutraceuticals. There are many steps to obtain phytochemicals from plant source such as milling, grinding, homogenization and extraction. Among these steps, extraction is the main step for recovering phytochemicals from plant materials. Efficiency of the extraction process is affected by the chemical nature of compounds, extraction method used, sample particle size, solvent used as well as the presence of interfering substances. The yield of extraction depends on the solvent with varying polarity, pH, temperature, extraction time, and composition of the sample. Under the same extraction time and temperature, solvent type and sample composition are

known to be the most important parameters to determine the compound yield.

Effect of different solvents (hexane, ethylene acetate, methanol, ethanol and water) on the extraction of phenolic compounds from *M. longifolia* leaves was shown in the Figure 1. From the graph, it is revealed that methanol was effective for the extraction of total phenols (6744.24 mg GAE/100 g), which is followed by water (3568.32 mg GAE/100 g) and ethanol (2620.80 mg GAE/100 g). It is also noted that the low polar solvents such as hexane and ethyl acetate were not effective in releasing total phenols. Since, maximum level of phenols was quantified in methanolic extract, the compounds present in *M. longifolia* leaf might be high polar in nature and also highly soluble in methanol. Similarly, methanol was found as a best solvent for the extraction of phenols from *M. longifolia* bark [17]. Differences in the structure of phenolic compounds also determine their solubility in solvents of different polarity. Therefore type of extraction solvent as well as the isolation procedures may have a significant impact on the yield of extraction polyphenols from plants material. There are some reports concerning optimization of extraction conditions of phenolic compound content and antioxidant activities of some plants [18].

Yield of the compounds is not only depends on the extraction method, but also on the solvent used for extraction. The presence of various antioxidant compounds with different chemical characteristics and polarities may or may not be soluble in a particular solvent. Polar solvents are frequently used for recovering polyphenols from plant matrices. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate. Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption. Methanol has been generally found to be more efficient in extraction of low molecular weight polyphenols, whereas aqueous acetone is good for extraction of high molecular weight flavanols [19]. Since, methanol was noted as the efficient solvent in our work to extract polyphenols from *M. longifolia* leaves, majority of the phenols might be low molecular weight in nature, which needs to be identified and quantified using advanced analytical techniques like HPLC and LC-MS.

Phenols are secondary metabolites found in plants which are useful in defence mechanism against UV radiation and oxidative stress. Natural polyphenols are the most abundant antioxidants in human diets, and their radical scavenging activities are related to substitution of hydroxyl groups in the aromatic rings of phenolics. Total phenolic content and total antioxidant activity in phytochemical extracts of different plants may have a direct relationship. The results on recovery of polyphenols from *M. longifolia* at various conditions designed by RSM tool were given in Table 1. Data revealed that the phenolic recovery from *M. longifolia* was maximum (7792.4 mg GAE/100 g) at extraction temperature (50^{o}C), time (75 min) and solvent concentration (75%). Similarly, phenolic extraction method was optimized for other plants such as *Origanum vulgare* and *Jatropha curcas* [20, 21].

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, we could optimize the response (phenolic yield) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Regression analysis was performed at the end based on the experimental data. ANOVA tables were generated, and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined and the significant value was set at $p < 0.05$.

In this work, good fit was achieved and most of the response variability was explained by the model and the R^2 of currently done experiment was 94.29. The R^2 value for these response variables was remarkably close to 100, denoting that the regression models provide excellent explanations of the relationship between the independent factors and the responses. Gan et al. [22] suggested that the R^2 value should be at least 0.80 for a good fit of a model. The adjusted R^2 is a corrected value for R^2 after the elimination of the unnecessary model terms. If there were many non-significant terms have been included in the model, the adjusted R^2 would be remarkably smaller than the R^2.

When we looked into the effect of each of individual factor on the phenolic yield, extraction temperature alone was found to be significant ($p < 0.05$), whereas solvent concentration and extraction time were does not have major impact (Table 2). Interaction of two factors such as Temperature x Temperature was only noted to has significant effect, whereas temperature x solvent concentration, temperature x time, time x time and solvent concentration x solvent concentration were not significant (Table 3). Similarly, temperature and time where reported as significant factors that affects phenolic extraction in pearl millet [23].

By considering two variables at one time while keeping the third one at the middle level, the response surface plots of the solvent concentration (X1), extraction time (X2) and extraction temperature (X3) on the TPC were generated to aid in visualization (Figure 3). From this study, it is revealed that the plot between TPC vs time, temperature and solvent, explains that the TPC of *M. longifolia* leaf was steadily increased and attained maximum level at the temperature of 50^{o}C, extraction time of 75 min and solvent concentration of 75%. Figure 4 revealed that the maximum yield of total phenols (7755 mg GAE/100 g) was achieved under the optimal conditions such as extraction temperature (50^{o}C), extraction time (88 min) and solvent concentration (88%). Similarly the optimum condition was reported for *Orthosiphon stamineus* and *Lawsonia inermis* leaves [24, 25].

A free radical is any chemical species that has at least one unpaired electron in the outermost shell [26]. These uncoupled electrons are very reactive with adjacent molecules such as lipids, proteins, and carbohydrates and can cause cellular damage. Presence of free radicals within the body can also have a significant role in the development

and progression of many disease processes like heart disease, congestive heart failure, hypertension, cerebro vascular accidents and diabetic complications. Over exposure to environmental factors such as smoking, ultraviolet radiation and pollutants can generate excess of free radicals which result in tissue damage and hypoxia. When the production of free radicals exceeds the capacity of the body's antioxidant defenses system, a condition known as oxidative stress occurs.

Antioxidants are substances that capable of counteracting the damaging effects of oxidation in body tissues. Antioxidants are divided into two classes based on mechanism of action: (1) chain-breaking antioxidants (Eg. Vitamin E and beta-carotene, which ''break the chain'' of free radical formation by donating an electron to stabilize an existing free radical) and (2) preventive antioxidants are enzymes that scavenge initiating radicals before they start an oxidation chain. Apart from these endogenous antioxidants, certain phytochemicals like polyphenols from external plant sources also could act as strong antioxidants in our body. In the present work, we have demonstrated the antioxidant effect of *M. longifolia* leaf extract using DPPH assay. Antioxidant activity of the plant extracts was measured on the basis of scavenging activities of the stable 2,2-diphenyl-1- picrylhydraziyl (DPPH$^{\bullet}$) radical. DPPH radical is an organic free radical with an absorption maximum at 517 nm. It loses this absorption when accepting an electron and results in a visually noticeable discoloration from purple to yellow. This experiment can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations [19].

From this Figure 5, the DPPH radical scavenging activity of methanolic extract of *M. longifolia* re-dissolved in water was found to be comparable to the standard (BHA). But, the IC-50 values indicating that the methanolic extract of *M. longifolia* (62.45 mg/L) was weaker when compared to the commercial antioxidant (IC-50: 16.21 mg/L). The IC-50 of a compound is inversely related to its antioxidant capacity, as it expresses the amount of antioxidants required to decrease the DPPH concentration by 50%, which is obtained by interpolation from a linear regression analysis. A lower IC50 indicates a higher antioxidant activity of a compound. But, higher the IC-50 value (3750 mg/L) was reported in plant *Lepidium apetalum* leaves [27] and when compared to this plant, *M. longifolia* has better antioxidant power. *M. longifolia* leaf extract has a colour of dark green, charactristic odour and has a astringent taste. When the extract is dissolved in water, it has low water solubility (36%) and slightly acidic (pH 5.8) in nature. The yield of metholic extract was 21.71% (Table 4).

These results revealed that the methanolic extract of *M. longifolia* has a remarkbale level of DPPH scavenging activity and hence it can act as strong antioxidant in biological system and thereby could prevent the oxidative damage caused by free radicals. Free radicals are considered to be involved in the multistage carcinogenic process. Peroxyl radicals and lipid peroxidation can independently cause mutations on DNA, which are crucial for the initiation of the carcinogenic process. Antioxidant phytochemicals may modulate the initiation of carcinogenesis process by protecting against DNA damage. Thus, the polyphenols of *M. longifolia* could play an important role in anti-cancer activity of this plant material, which gives the scientific basis for the usage of this plant in Indian traditional medicine for cancer therapy [28].

Table 1 Box–Behnken design matrix, experimental data and predicted values for level-three-factor response surface analysis for the recovery of total phenolic compounds from *Madhuca longifolia*.

Std Order	Run Order	Pt Type	Blocks	Variables			Total phenolic content (mg GAE / 100 g)	
				Solvent Conc. (%)	Temp. (^{o}C)	Time (Min)	Observed values	Predicted values
1	1	2	1	50	30	75	5959.80 ± 598.92	5951.82 ± 1201.33
13	2	0	1	75	50	75	7635.60 ± 0.00	7678.54 ± 800.89
3	3	2	1	50	70	75	5845.03 ± 98.52	5443.57 ± 1201.33
2	4	2	1	100	30	75	5419.33 ± 414.84	5820.80 ± 1201.33
11	5	2	1	75	30	120	6152.67 ± 103.71	5867.03 ± 1201.33
5	6	2	1	50	50	30	6622.63 ± 312.54	6738.46 ± 1201.33
4	7	2	1	100	70	75	6152.67 ± 311.13	6160.65 ± 1201.33
15	8	0	1	75	50	75	7792.40 ± 536.69	7678.54 ± 800.89
8	9	2	1	100	50	120	7647.20 ± 497.80	7531.38 ± 1201.33
10	10	2	1	75	70	30	4997.30 ± 108.89	5282.94 ± 1201.33
14	11	0	1	75	50	75	7607.60 ± 523.97	7678.54 ± 800.89
12	12	2	1	75	70	120	5760.00 ± 47.14	5867.84 ± 1201.33
6	13	2	1	100	50	30	7509.60 ± 534.57	7215.98 ± 1201.33
7	14	2	1	50	50	120	7129.20 ± 165.46	7422.83 ± 1201.33
9	15	2	1	75	30	30	5560.00 ± 141.42	5452.16 ± 1201.33

Table 2 Estimated Regression Coefficients for the recovery of total phenolic compounds from *Madhuca longifolia*.

Term	Coef	SE Coef	T-Value	P-Value
Constant	-4481.47	2954.38	-1.517	0.190
Solvent Conc	17.77	53.16	0.334	0.752
Temp	393.05	59.85	6.567	0.001
Time	34.46	22.35	1.542	0.184
Conc. x Conc.	-0.18	0.32	-0.566	0.596
Temp x Temp	-4.30	0.50	-8.671	0.000
Time x Time	-0.17	0.10	-1.707	0.148
Solvent Conc. x Temp	0.42	0.38	1.111	0.317
Solvent Conc. x Time	-0.08	0.17	-0.483	0.649
Temp x Time	0.05	0.21	0.223	0.833
Model Summary				
S	381.582			
PRESS	11375562			
R-Sq	94.29%			
R-Sq(pred	10.76%			
R-Sq(adj	84.01%			

Table 3 Analysis of variance of the response surface analysis on the recovery of total phenolic compounds from *Madhuca longifolia*.

Source	DF	Seq SS	Adj SS	Adj MS	F-Value	P-Value
Regression	9	12018511	12018511	1335390	9.17	0.013
Linear	3	685683	6547495	2182498	14.99	0.006
Solvent Conc.	1	171737	16261	16261	0.11	0.752
Temp	1	14179	6279766	6279766	43.13	0.001
Time	1	499767	346090	346090	2.38	0.184
Square	3	11111747	11111747	3703916	25.44	0.002
Conc. x Conc.	1	4544	46589	46589	0.32	0.596
Temp x Temp	1	10682765	10948688	10948688	75.19	0.000
Time x Time	1	424438	424438	424438	2.92	0.148
Interaction	3	221080	221080	73693	0.51	0.695
Conc. x Temp	1	179818	179818	179818	1.23	0.317
Conc. x Time	1	34034	34034	34034	0.23	0.649
Temp x Time	1	7228	7228	7228	0.05	0.833
Residual Error	5	728023	728023	145605		
Lack-of-Fit	3	708183	708183	236061	23.80	0.041
Pure Error	2	19840	19840	9920		
Total	14	12746534				

Table 4 Physico-chemical properties of solvent extract of *Madhuca longifolia* leaf.

S. No.	Physico-chemical properties	Solvent extract
1	Colour	Dark green
2	Odour	Charactristic odour
3	Extract yield on dry weight basis (%)	21.71 ± 0.69
4	pH	5.80 ± 0.02
5	Water solubility (%)	36.21 ± 0.59

Figure 1. Effect of different solvents on the recovery of total phenolic compounds from *Madhuca longifolia* leaf

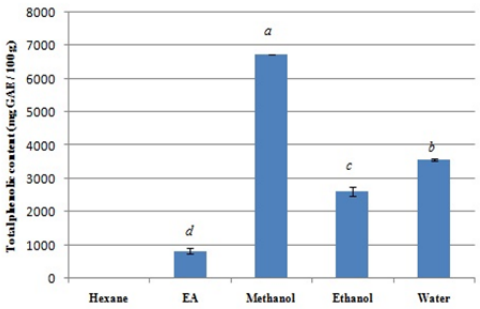


Figure 2. Effect of independent factors (solvent concentration, temperature and time) on the TPC level of *Madhuca longifolia* leaf

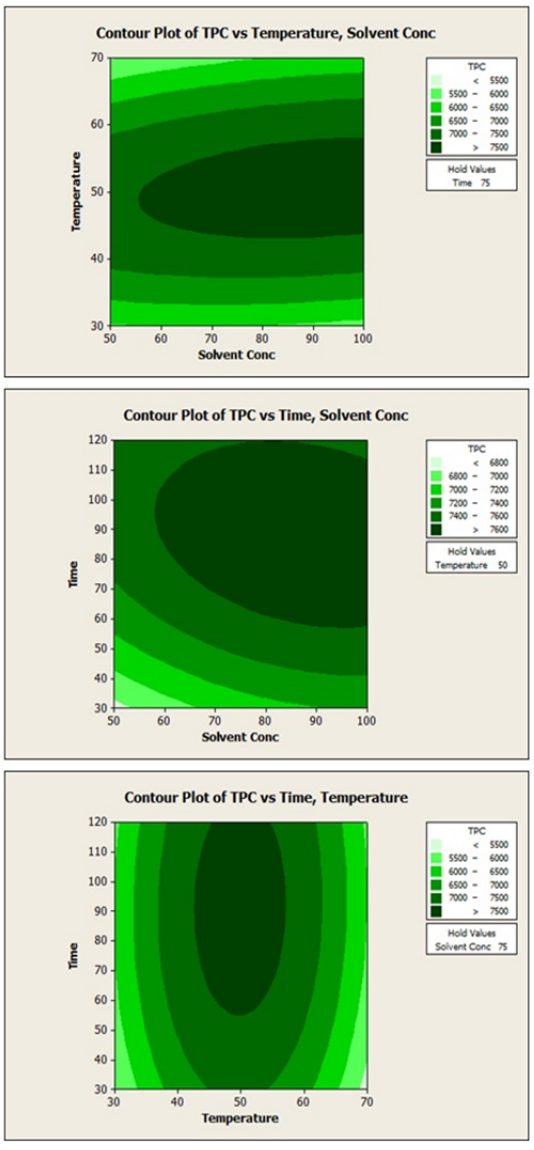


Figure 3. Effect of interaction of factors (solvent concentration, temperature and time) on the TPC level of *Madhuca longifolia* leaf

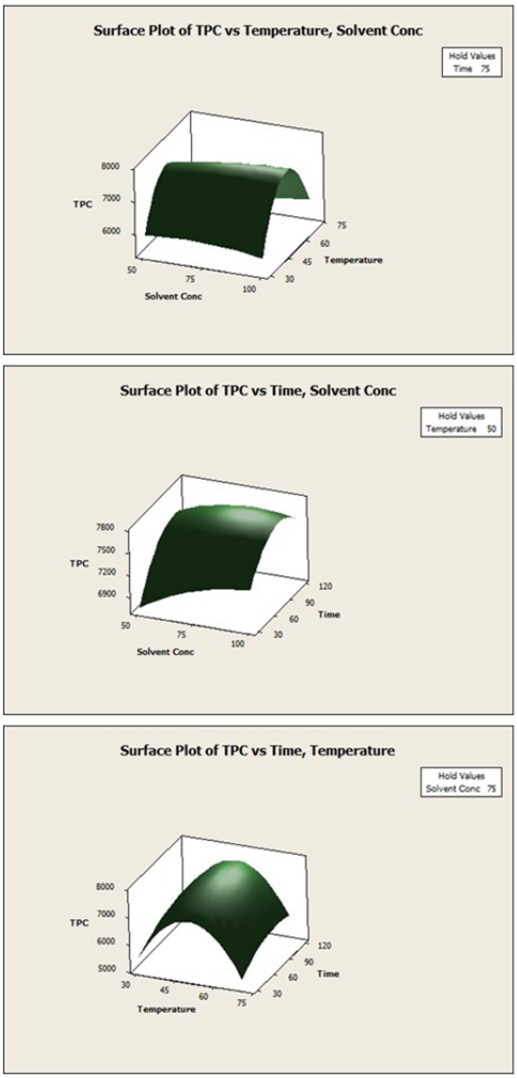


Figure 4. Optimal conditions for the recovery of TPC from *Madhuca longifolia* leaf

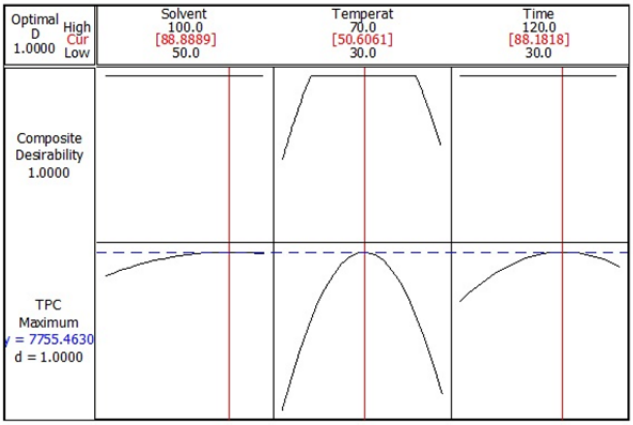
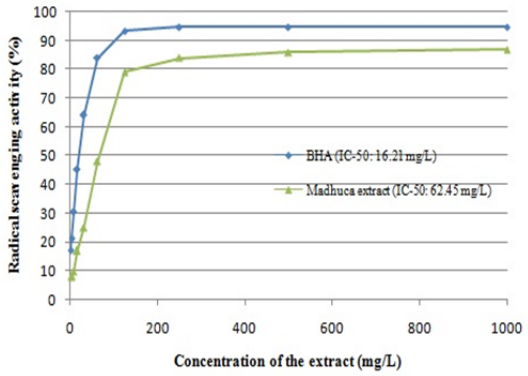


Figure 5. Antioxidant activity of methanolic extract of *Madhuca longifolia* leaf



CONCLUSIONS

In the present work, we have identified the suitable solvent and optimal conditions for the extraction of antioxidant phytochemical compounds from *M. longifolia* leaves using RSM tool. Such studies are very much useful in preparing the bioactive extract at large-scale for industrial use. Results of physico-chemical and antioxidant properties indicates the suitability of the *M. longifolia* extracts in the formulation of novel drugs. Development of therapeutics from such traditional medicinal source could be a vital step in improving the health conditions of the livelihoods especially in India, where people still believes on herbal therapy.

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