Effect of process parameters on the antioxidant activities of bioactive compounds from Harad (Terminalia chebula retz.)

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Abstract: Terminalia chebula retz (T. Chebula) is a herb known as king of medicinal plants in ayurvedic world. Presence of a large number of phytoconstituents makes T. Chebula a potential source of nutraceuticals and can become cynosure of modern nutraceutical world. Therefore, the objective of this study was to optimize the extraction conditions for bioactive constituents from T. Chebula fruits using Response Surface Methodology (RSM) and quantification of bioactive compounds in the optimized extract using GC-MS analysis. A central composite face centered design (CCD) was employed in this study. The extraction conditions for bioactive constituents from T. Chebula fruits were optimized by using three independent process variables, i.e. methanol concentration, 50%-90%, extraction temperature, 50 ℃-70 ℃ and extraction time, 30-60 min. Effect of extraction parameters was found to be significant. The optimum extraction conditions were identified as 90% in methanol concentration, 70 ℃ in temperature and 59.94 min for maximum total phenolic content (TPC), 3.87 GAE mg/g, total flavonoid content (TFC), 361.37 CE mg/g, total antioxidant activity(TAA), 0.158 AAE mM/g and α,α-diphenyl-β-picrylhydrazyl (DPPH) scavenging activity (DSA), 86.28%. Experimental values for response variables under the optimum conditions were found reasonably close to the predicted value. GC–MS analysis of methanol extract of T. Chebula fruits under the optimum conditions led to the identification of seven major bioactive compounds. The results showed that T. Chebula possesses wide range of bioactive compounds and can be utilized as a potential natural plant in the development of functional foods and nutraceutical supplements.

Keywords: T. Chebula, extraction, Total Phenolic Compound (TPC), Total Flavonoid Compound (TFC), Total Antioxidant Activity (TAA), α,α-diphenyl-β-picrylhydrazyl (DPPH) Scavenging Activity (DSA), Response Surface Methodology (RSM), GC-MS

Citation: Soni, S., H.K. Sharma, P. Kaushal, and C. Singh. 2015. Effect of process parameters on the antioxidant activities of bioactive compounds from Harad (Terminalia chebula retz.). AgricEngInt: CIGR Journal, 17(2):205-220.

1 Introduction

Terminalia is a genus of large flowering trees comprising around 250 species distributed in tropical region of the world. *Terminalia arjuna*, *Terminalia bellerica*, and *Terminalia chebula retz (T. chebula)* are namely three main species of Terminalia, which are highly valuable and are used for medicinal purpose. *T. chebula* is the most useful species of the family and is known for different vernacular names such as Black myroblan, Chebulic myroblan (English), hartika (Sanskrit) and harad (Hindi). *T. Chebula* is a flowering evergreen tree used in traditional medicines and is highly regarded as the 'King of medicines' in the Ayur-Vedic Medicines. Several pharmacological investigations for different biological activities of *T. Chebula* in different test models have been carried out. These studies showed that *T. Chebula* possesses antibacterial, antifungal, antimoebic, antiplasmoidal, antiviral, antimutagenic, antioxidant, antidiabetic, antiulcerogenic, wound healing, cryoprotective, radioprotective, anticarries, cardioprotective and chemomoprotective properties (Gupta, 2012).

Triphla churan, Abhayamodaka, Abhayarista, Vyaghn hartika, gandharva hartika etc. are different types of ayurvedic preparations of *T. Chebula*, which are popular

Received date: 2014-12-13 **Accepted date:** 2015-05-10
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and are used for different problems. *T. Chebula* is also used in food industry in the form of candy and murabba (preserve). The antioxidant property of harad is considered, not only to neutralize free radicals but also to help in cell regeneration. Moreover, it is known to cure eye diseases and alleviates back pain, chronic fever, gout, etc. All these simultaneous developments in world of functional foods have also drawn the attention towards *Terminalia Chebula.*

Saleem et al. (2001) examined the effect of 70% methanolic extract of *Terminalia chebula retz.* fruit, on the growth of several malignant cell lines. Methanolic extract (75%) of *Terminalia chebula retz.*, were found to inhibit lipid peroxide formation and to scavenge hydroxyl and superoxide radicals in vitro (Sabu and Kuttan, 2002). The extracts and pure compounds of Terminalia chebula retz exhibited antioxidant activity at different magnitudes of potency (Cheng et al., 2003). A significant antidiabetic and renoprotective effects with the chloroform extract of Terminalia chebula retz. has been reported (Rao and Nammi, 2006). Methanol extract of Terminalia chebula retz., potentially inhibit glycosylation by acting as a suppressor of intracellular glycosylation trafficking (Lee et al., 2011).

Response surface methodology (RSM) has been successfully used to model and optimize biochemical and biotechnological process related to food systems. The main advantage of RSM enables evaluation of the effects of several process variables and their interactions on response variables. RSM is a collection of statistical and mathematical techniques that has been successfully used for developing, improving and optimizing processes (Myers and Montgomery, 2002). It is faster and more informative than the classical one variable-at-a-time approach or the use of full factorial designs (Ozddemir et al., 2008). Bioactive constituent's extraction and their application are growing and are considered an imminent field of interest for research because of their preventive potential effects on the chronic diseases such as cardiovascular diseases and cancer, diabetes and aging. Many factors have been established to influence the extraction efficacy of bioactive constituents, such as extraction methods, particle size, solvent type, solvent concentration, extraction temperature, extraction time and pH (Dai and Mumper, 2010; Singh et al., 2012). Various solvent systems and methods have been tested for extraction of poly phenols from plant materials. Water, aqueous mixtures of ethanol, methanol and acetone are commonly used for the extraction. Methanol, ethanol and water have good antioxidant extraction ability from *T. Chebula* (Chang and Lin, 2010). *Terminalia chebula retz*, which is referred as king of herbs, possesses a number of medicinal properties. Therefore, the isolation, identification and quantification are important aspects and evaluation of their health benefits is an important area to study for its nutraceuticals and functional benefits. Researchers have shown that *Terminalia chebula retz.* have nutraceutical and functional benefits but a systematic study on the extraction is scarce as per the need, therefore the work was undertaken to optimize the process parameters for the maximum activity of bioactive constituents from *T. Chebula fruits* and its quantification by GC-MS.

2 Materials and methods

2.1 Materials

T. Chebula fruits were procured from Hamirpur villages, Himachal Pradesh, India. The fruits of uniform shape and colour were selected whereas blemished and diseased seeds were eliminated. The chosen fruits had an average diameter of 2.5 cm and 5 cm in length. Standard ascorbic acid was procured from Hi-Media, Bombay. Gallic acid and catech in were procured from Sigma Aldrich, USA. All chemicals used in the study were either AR Grade or extra pure.

a) Proximate composition of fresh fruits

Moisture, fat, protein and total ash content were estimated by standard method (Ranganna, 1986). Total carbohydrate content of the samples was determined by difference.

b) Selection of experimental range of the design variables

i. Preliminary experiments for selection of extraction time

Extraction time plays an important role in the extraction of nutraceuticals therefore trials were conducted to select the range of time variable for optimization. The influence of extraction time on total phenolic content (TPC) was analyzed while other processing conditions (Solvent concentration, 70% and temperature, 50^0C and 70^0C) were kept constant. Higher limit of temperature range was used in trials for obtaining the lower limit of the time and vice-versa.

In determination of lower limit, the experimental results showed that as the extraction time increased from 15 to 30 min, total phenolic content (mg/g) was increased to 4.151 which remained constant till 60 min. After 60 min, further increase in extraction time did not significantly improve the recovery of total phenolics. In case of upper limit determination, increasing extraction time from 30 to 60 min, total phenolic content (mg/g) was increased to 5.87. After 60 min, further increase in extraction time did not significantly improve the recovery of total phenolics. A decrease in total phenolic content was observed after 60 min. Therefore the extraction time range, 30-60 min was decide to carry out the experimental design.

ii. Selection of solvent concentration and extraction temperature

Chang and Lin (2010) found that methanol, ethanol and water have good antioxidant extraction ability from *T. Chebula*. Methanol is usually recommended for the extraction of antioxidant compounds. Its effectiveness could be improved by adding water as co-solvent, particularly, in the protocols, where the extraction of bioactive compounds is multifarious in nature. Saleem et al. (2002) examined the efficacy of 70% methanol extract of *Terminalia chebula retz* fruit, for its effects on growth in several malignant cell lines. The experimental range for methanol concentration and extraction temperature was therefore selected in the range of 50%-90% v/v and 50^0C-70^0C, respectively.

c) Experimental design and statistical analysis

RSM is usually applied following a set of design experiments, which have the purpose of screening out the important factors. The primary purpose of the RSM methodology is to find the optimum settings for the factors that influence the response.

In general, the extraction of bioactive constituents is influenced by multiple parameters such as temperature, time and solvent polarity. Therefore, the independent variables were solvent concentration, 50%-90%, extraction temperature, 50 ℃-70 ℃, and extraction time, 30-60 min to find the optimum conditions for extractions. Central composite face centered design (CCD) of Response Surface Methodology (RSM) was used to understand the interactions of independent variables on bioactive constituent's extraction and to derive the optimum conditions of extraction.

The extraction conditions were optimized on the basis of maximum activity of the bioactive constituents, which was determined on the basis of total phenolic content (TPC), total flavonoid content (TFC), total antioxidant activity (TAA) and DPPH scavenging activity (DSA). The range and the levels of the experimental variables used in the coded and uncoded form in this study are given in Table 1. The central value (zero level) in experimental design was solvent concentration, 70% (v/v), extraction temperature, 60 ℃ and extraction time, 45 min. The test factors were coded according to the following Equation 1:

$$X_i = (X'_i - X^x_i) / \Delta X_i \quad (1)$$

where, X_i is the coded value of the i^{th} independent variable, X'_i is the natural value of the i^{th} independent variable, X^x_i is the natural value of the i^{th} independent variable at the centre point and ΔX_i is the step change value.

Table 1 The experimental design in coded and uncoded form for the optimization of variables using central composite face centered design

Standard order	Coded			Uncoded		
	Solvent Concentration (% v/v)	Temperature (^{0}C)	Time (min)	Solvent Concentration (% v/v)	Temperature (^{0}C)	Time (min)
1	-1	-1	-1	50	50	30
2	1	-1	-1	90	50	30
3	-1	1	-1	50	70	30
4	1	1	-1	90	70	30
5	-1	-1	1	50	50	60
6	1	-1	1	90	50	60
7	-1	1	1	50	70	60
8	1	1	1	90	70	60
9	-1	0	0	50	60	45
10	1	0	0	90	60	45
11	0	-1	0	70	50	45
12	0	1	0	70	70	45
13	0	0	-1	70	60	30
14	0	0	1	70	60	60
15	0	0	0	70	60	45
16	0	0	0	70	60	45
17	0	0	0	70	60	45
18	0	0	0	70	60	45
19	0	0	0	70	60	45
20	0	0	0	70	60	45

Experimental data were fitted to a second-order polynomial model and regression coefficients were obtained. The generalized second-order polynomial model used in the response surface analysis is expressed as:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ii} X_i X_j \ .$$

where, Y is the measured response, β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i, and X_j are the independent variables. The design expert software was used to generate response surfaces plots. A second order quadratic model was employed to correlate the independent process variables. The data were subjected to ANOVA and the effect of extraction parameters was determined at linear, quadratic and interactive level. The P values were used as a tool to check the significance of each of the coefficients, which, in turn are necessary to understand the pattern of the mutual interactions between the test variables.

2.5 Extraction of bioactive constituents

T. Chebula fruit pulp was dried in hot air oven at 60^{0}C and ground (particle size of dried powder was standardized by using 0.5 mm sieve size) to obtain dried powder of fruits. Dried powder, 10 g was weighed and

extracted in 150 ml of the solvent by soxhlet extraction method. Extraction was carried out under the set of designed conditions as per the CCD, Response Surface Methodology (Table 1). Each time, the mixture was filtered through Whatman filter paper (No. 41) and the supernatant was concentrated using a rotary evaporator at 40 ℃ and then the final volume was adjusted to 40 ml. The extracts were kept in the dark under refrigerated condition until further analysis.

2.6 Evaluation of nutraceuticals

2.6.1 Total phenolic content (TPC)

TPC was determined using Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (1965) with slight modifications. Reaction mixture contained 200 µl (0.2 ml) seed extracts, 800 µl (0.8 ml) freshly prepared diluted (1:10) Folin-Ciocalteu reagent, and 2 ml of 20% sodium carbonate. The volume of the resulting mixture was adjusted to 10 ml and placed in dark for 2 h to ensure completion of reaction. The absorbance of resulting blue-colour mixture was measured at 760 nm against sodium carbonate (20%) as a blank by using a spectrophotometer (Model, S2100UV+, Unico, USA). Each extract was analyzed in triplicates. Gallic acid was used as calibration standard and results were calculated as gallic acid equivalents (mg/g) of fruit powder.

2.6.2 Total flavonoid content (TFC)

Total flavonoid content was measured by method of Moreno et al. (2000), in which aliquots of diluted extracts (0.5 ml) were added to test tubes and mixed with 0.1 ml aluminum nitrate (10%), 0.1 ml aqueous potassium acetate (1M) and 4.3 ml of 80% methanol. The reaction mixture was kept for 40 min at room temperature and then the absorbance of the reaction mixtures was measured at 415 nm against 80% methanol as a blank by using a spectrophotometer (Model, S2100UV+, Unico, USA). Catechin was used as standard to construct a standard curve and results were calculated as catechin equivalent (mg/g) of fruit powder.

2.6.3 Total antioxidant activity (TAA)

The antioxidant activity of the *T. Chebula* fruit powder extracts was evaluated by the phosphomolybdenum method (Jayaramkumar et al., 2002). The assay is based on the reduction of Mo (VI) – Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The extract, 0.3 ml was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 ℃ for 90 min. Then the absorbance of the solution was measured at 695 nm using spectrophotometer (Model S2100UV+, Unico, USA) against blank (methanol) after cooling to room temperature. The antioxidant activity was expressed as mmol ascorbic acid equivalent per g of fruit powder.

2.6.4 DPPH scavenging activity

DPPH scavenging activity assay was carried out by following procedure of Gawron-Gzella et al. (2012). DPPH solution (0.0062 g/100 ml MeOH), 1.4 ml was mixed with 0.2 ml of the prepared extract. The reaction mixture was shaken and incubated in the dark at room temperature for 30 min. Absorbance (A) was measured at 536 nm using spectrophotometer (S2100UV+, Unico, USA). Inhibition of the DPPH radical by the sample was calculated according to the following Equation 2:

$$\text{DPPH scavenging activity (\%)} = [A_0 - A_1 / A_0] \times 100 \quad (2)$$

where, A_0 and A_1 are absorbance of the control and sample, respectively.

2.7 Optimization and validation of the model

'Design expert—7.0.0' software was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface plots. The verification of the validity and adequacy of the predictive extraction model was realized in these optimum conditions of solvent composition, temperature and time of contact.

2.8 Quantification of bioactive compounds using gas chromatography-mass spectrometry (GC-MS)

The extract of bioactive compounds was obtained under optimized extraction conditions and freeze dried (Lyophilizer-FD-S, using Allied Frost, Macflow Engineering Pvt. Ltd., India at -82^0C for 8 h). Optimized methanol extract, 2.5 mg was dissolved in acetone, using ultrasonic water bath. The sample was then loaded in the sample tray of GC-MS instrument. GC-MS analysis was carried out on a Thermo GC (Model TRACE 1300), which is an auto sampler and gas chromatograph interfaced to a mass spectrometer, Thermo MS [Model TSQ 8000 (triple quadrapole)]. GC-MS was employed with a column: Thermo TG 5MS (30 × 0.25 mm × 0.25μm, 5% phenyl methyl poly siloxane), operating in electron impact mode at 70 eV, helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 EI (electron ionization) was employed (split ratio of 10:1) with an injector temperature of 250 ℃ and ion-source temperature of 280 ℃. The oven temperature was programmed from 110 ℃ (isothermal for 2 min), with an increase of 10 ℃/min, to 200 ℃, then 5 ℃/min to 280 ℃, ending with a 9 min isothermal at 280 ℃. Mass spectra were taken at 70 eV with a scan interval of 0.5 s and fragments from 40 to 550 Da (Kumar et al., 2010).

2.9 Identification of components

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) libraries mass spectral database. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained. The name, molecular weight and structure of the components of the test materials were ascertained. Percentage of different nutraceutical components were determined with computer software by calculating the area acquired by different peaks.

3 Results and discussion

3.1 Proximate composition of T. Chebula

Proximate composition of unripened fresh *T. Chebula* fruits was found to contain 2.05% protein, 4.4% fat, 3.2% ash, 40.41% moisture and 49.94% carbohydrate content. Sundriyal and Sundriyal (2001) reported protein, 1.25%, fat, 3.90%, ash, 3.91 % and moisture, 53% in *T. Chebula* fruits procured from the Sikkim Himalayan region. The variations in the proximate parameters may be due to the difference in variety of plant, geographical area of cultivation, climate, ripening stage and harvesting time of the fruits.

3.2 Optimization of extraction conditions

3.2.1 Model fitting

The experimental values for different responses (TPC, TFC, TAA and DSA) under different combination of extraction conditions are given in Table 2. The results showed that TPC, TFC, TAA and DSA of *T. Chebula* fruits ranged from 2.83 to 4.33 mg (gallic acid equivalent- GAE)/g fruit powder, 143.75 to 349.87 mg catechin equivalent per g fruit powder, 0.024 to 0.39 mM ascorbic acid equivalent/g fruit powder and 63.19%-89.1%, respectively for the samples treated under different extraction conditions (Table 2).

Table 2 Experimental values of TPC, TFC, TAA and DSA under different extraction conditions

Std. order	TPC[a]	TFC[b]	TAA[c]	DSA[d]
1	4.20	344.00	0.12	79.09
2	3.89	176.75	0.06	88.14
3	4.25	236.50	0.13	89.10
4	2.83	221.00	0.39	87.68
5	3.16	225.63	0.19	73.72
6	3.65	276.00	0.02	79.67
7	3.90	143.75	0.03	85.08
8	3.98	349.87	0.19	80.00
9	4.16	229.75	0.10	72.44
10	4.03	259.87	0.14	73.52
11	3.65	232.13	0.06	76.69
12	3.59	211.37	0.17	88.02
13	4.05	209.37	0.20	71.96
14	3.95	207.50	0.13	66.62
15	4.14	199.28	0.13	68.51
16	4.09	209.50	0.13	65.60
17	4.15	210.00	0.13	63.51
18	4.12	208.99	0.13	63.19
19	4.10	211.00	0.13	64.34
20	4.33	205.90	0.14	63.55

Note: [a] Total phenolic content in mg GAE/g dried fruit powder

[b]Total Flavonoid content in mg CE/g dried fruit powder

[c] Total antioxidant activity in mM AAE/g dried fruit powder

[d] DPPH scavenging activity in percentage

Data expressed as average of triplicate measurements

The fitness and adequacy of the model was judged by the coefficient of determination (R^2) and lack-of-fit. R^2, which can be defined as the ratio of the explained variation to the total variation is a measure of the degree of fit. The closer the R^2 value to unity, the better the empirical model fits the actual data. The coefficient of determination (R^2) were 0.940, 0.994, 0.997 and 0.936 for the regressed models predicting the TPC, TFC, TAA and DSA respectively, suggesting a good fit. The coefficient of regression equations obtained by fitting of TPC, TFC, TAA and DSA data are given in Table 3.

Table 3 Regression coefficients of predicted quadratic polynomial models for the responses TPC, TFC, TAA, AND DSA

Coefficient	TPC	TFC	TAA	DSA
Intercept	-6.138[a]	2433.982[a]	0.30823[a]	511.000[a]
Linear				
A	-0.013[b]	-30.618[a]	-0.01522[a]	-0.329
B	0.488	-27.756[b]	3.03E-03[a]	-14.555[b]
C	-0.161	-13.917	6.13E-03[a]	-0.137[b]
Quadratic				
A^2	-9.50E-04	0.192[a]	4.03E-04[b]	-0.013
B^2	9.58E-04[b]	0.183[b]	-8.88E-05[b]	-2.82E-03[a]
C^2	1.73E-03	0.046	-2.68E-04[a]	1.78E-03
Interactive				
A*B	1.47E-04[b]	0.081[a]	-2.69E-05[a]	9.36E-03[b]
A*C	-4.16E-03[a]	0.094[a]	-1.23E-04[a]	0.131
B*C	-1.60E-04[b]	-0.017[b]	1.54E-04[a]	2.34E-04
R^2	0.9400	0.9945	0.9970	0.9369
Adjusted R^2	0.8861	0.9895	0.9944	0.8802

Note: Statistically significant at [a] $p<0.001$, [b] $p<0.05$, and [c] $p<0.10$

The significance which was determined using F-test and p-value are presented in Table 3. The corresponding variables would be more significant if the absolute F value becomes greater and the p-value becomes smaller. The F-value for all derived models was greater than the tabulated F value, indicating the adequacy of the models to predict responses at different extraction conditions. The predicted models seemed to be reasonably representing the observed values. Thus, the responses were sufficiently explained by the models. The non significance of lack of fit for all responses also strengthened the reliability of the models. The adjusted R^2 is a corrected value for R^2 after 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 (Chan et al., 2009).

In the study, the adjusted R^2 was for all responses very close to their corresponding R^2 value. High values of adjusted R^2 also advocated significance of the model for all responses. The coefficient of variation (CV) describes the extent to which the data are dispersed (Liyana-Pathirana and Shahidi, 2005). The coefficient of variation is a measure of residual variation of the data relative to the size of the mean; the small values of CV give better reproducibility. The CV (Table 3) revealed that the experimental results were precise and reliable.

3.3 Total phenolic content (TPC)

Polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Phenolic compounds contribute to the overall antioxidant activities of plants mainly due to their redox properties. Generally, the mechanisms of phenolic compounds for antioxidant activity are neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.

Interactive effect of solvent concentration and temperature showed that, at lower limit of temperature, TPC did not show any significant change till 70% solvent concentration and then it began to increase (Figure 1a). At high extraction temperature, TPC decreased with the increase in solvent concentration (Figure 1a). Rodtjer et al. (2006) reported that quantification of total amount of phenolics in the extract showed that 70% solvent-water

mixtures extracted the phenolic more efficiently and contained more complex mixtures of phenolic compounds than the pure solvent extracts. Several researchers established that the extraction yield of phenols greatly depends on solvent polarity. Chirinos et al. (2007) mentioned that solvents with high polarity do not give good extraction results. The use of water in combination with other organic solvents contributes to creation of moderately polar medium that ensures the extraction of polyphenols. Chebulinic acid is a major component in *Terminalia Chebula Retz.* In comparison to non – polar solvents, polar solvents could extract chebulinic acid at higher yield. This could be the reason that TPC increased with the increase in solvent concentration.

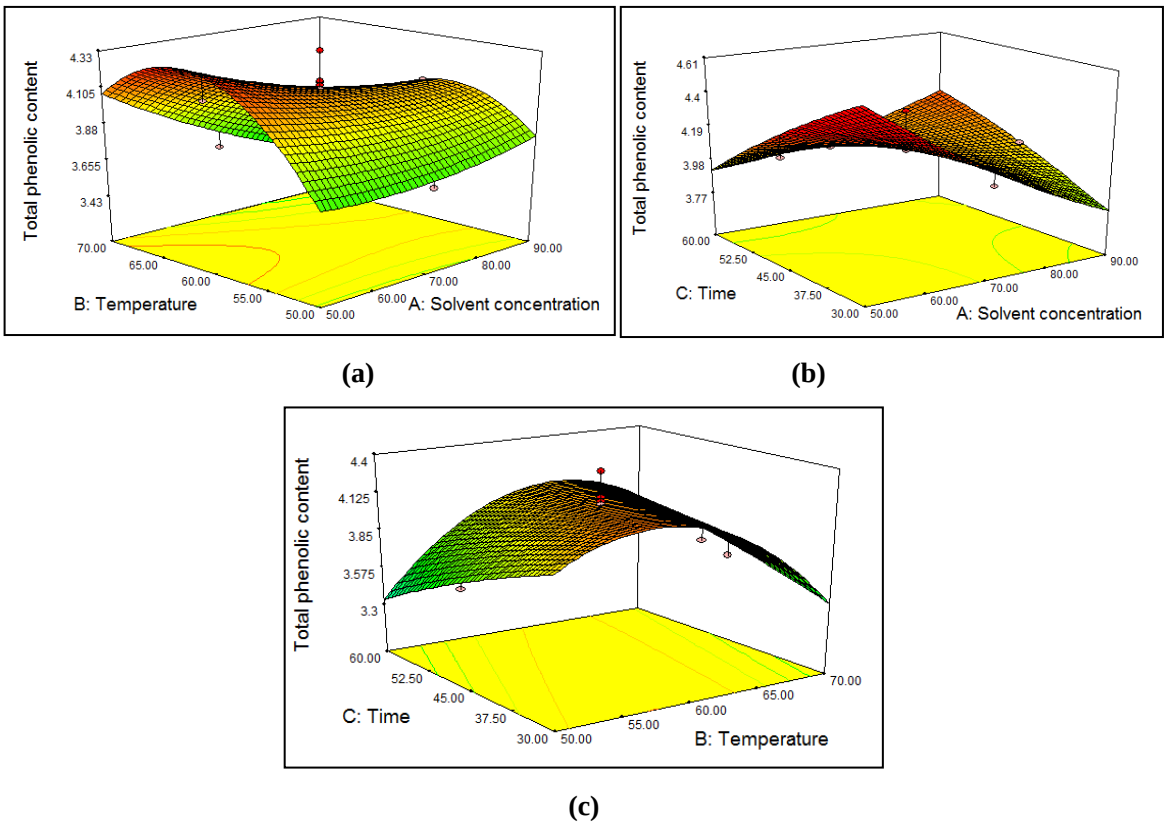


Figure 1 Response surface plots for total phenolic content (mg/g) as a function of (a) solvent concentration (%) and temperature (^{0}C) (b) solvent concentration (%) and time (min) (c) temperature (^{0}C) and time (min). The value of the third independent variables in each plot was kept at the center point.

Total phenolic content first increased and then decreased with the increase in extraction time (Figures 1b and 1c). This observation was well explained by Fick's second law of diffusion, which stated that final equilibrium will be achieved between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) after a certain time, hence, an excessive extraction time was not useful to extract more phenolic antioxidants (Silva et al., 2007). TPC increased with the increase in temperature up to 65^{0}C and further increase in temperature led to deceleration of phenolic extraction (Figure 1c). Increasing temperature may favor extraction by enhancing the solubility of phenolic compounds in the solvent. The increase in temperature may be due to the increase in rate of extraction thereby decreasing the extraction time.

3.4 Total flavonoid content (TFC)

At higher extraction temperature, TFC increased gradually with the increase in solvent concentration (Figure 2a). Figure 2a also depicts that low extraction

temperature was suitable for higher TFC at low solvent concentration whereas high extraction temperature was suitable at high solvent concentration. Ramos et al. (2002) reported that high extraction temperature enhances extraction efficiency of phenolic compounds as it ameliorates the mass transfer, improves the solubilization of the solutes in the solvent and reduces the surface tension and viscosity. Total flavonoid content did not show appreciable changes increase in methanol concentration at lesser extraction time (Figure 2b). At longer extraction time, TFC increased with the increase in solvent concentration (Figure 2b) because large period of time was required to achieve final equilibrium between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent), hence, longer extraction time was found increase TFC significantly (Silva et al., 2007). TFC was maximum at high solvent concentration and larger extraction time. Total flavonoid content increased with the increase in temperature then declined (Figure 2c). At low extraction time, TPC decreased with the increase in temperature whereas at longer extraction time, TFC initially decreased and then it increased with the increase in temperature (Figure 2c).

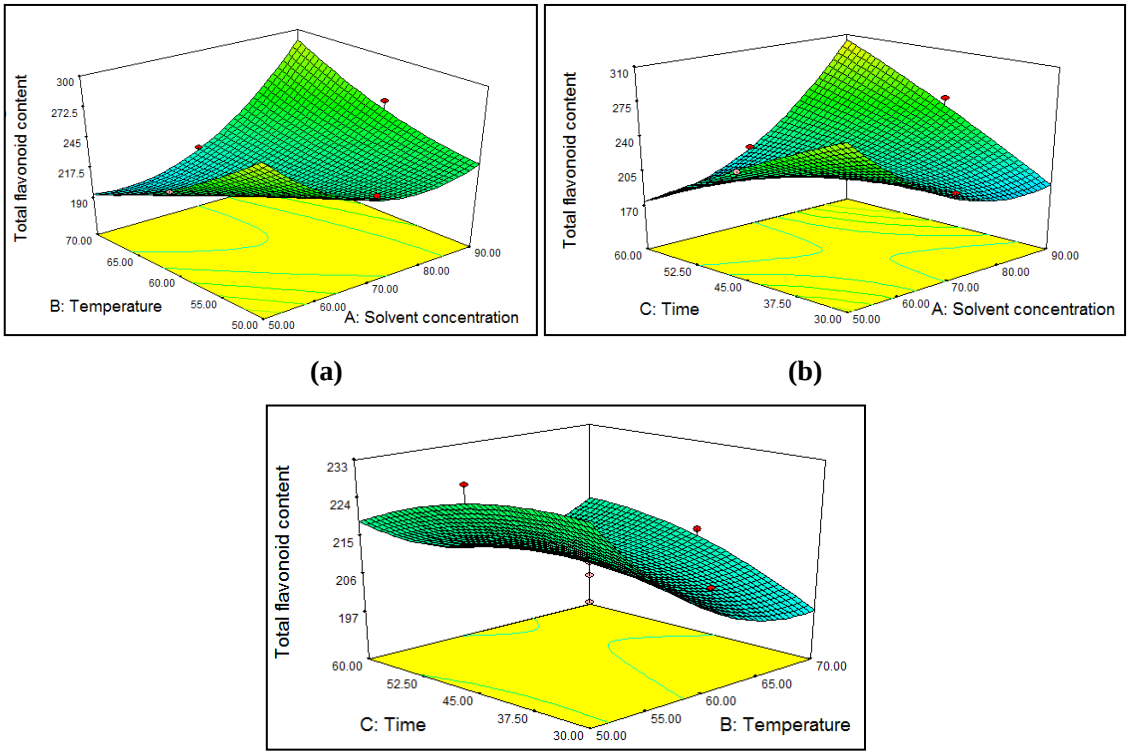


Figure 2 Response surface plots for total flavonoid content (mg/g) as a function of (a) solvent concentration (%) and temperature (^{0}C) (b) solvent concentration (%) and time (min) (c) temperature (^{0}C) and time (min). The value of the third independent variables in each plot was kept at the center point.

3.5 Total antioxidant activity (TAA)

Antioxidants are widely used as ingredients in dietary supplements for maintaining health and preventing diseases. Higher extraction temperature resulted in gradual increase in total antioxidant activity (Figure 3a). Ramos et al. (2002) reported that high extraction temperature enhances extraction efficiency of phenolic compounds. However, in a complex polyphenol system, a temperature increase can promote molecular collisions, favouring polymerisation and reducing antioxidant capacity (Pinelo et al., 2004). Highest TAA was attained by interactive effect of solvent concentration and

temperature, at their upper limits. TAA has been reported to reach a maximum followed by a decrease in further increase in proportion of the organic solvent in the extraction medium (Liyana and Shahidi, 2006). The difference in the results may be due to the different value of antioxidants present in T. Chebula. Figure 3a depicts that the total antioxidant activity decreased with the increase in extraction time whereas TAA increased with the increase in methanol concentration. TAA increased with the increase in solvent concentration (Figure 3b) and extraction temperature (Figure 3c). Bimakar et al. (2011) observed that polar compounds in the plant matrix would be easier to extract with a more polar solvent while lower polarity solvents enable to obtain the extracts with higher concentration of bioactive compounds. According to Rajha et al. (2014), short periods of extraction time are required at elevated extraction temperature to avoid the degradation of the bioactive compounds.

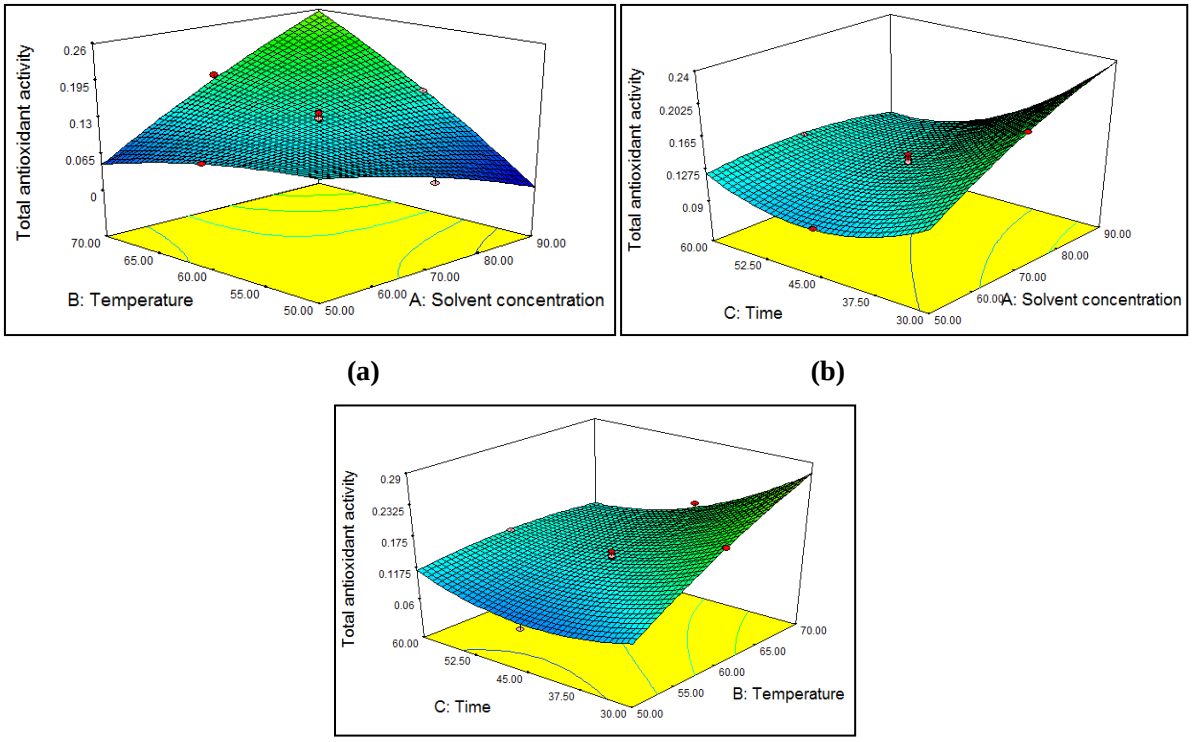


Figure 3 Response surface plots for total antioxidant activity (mm/g), as a function of (a) solvent concentration (%) and temperature (^{0}C) (b) solvent concentration (%) and time (min) (c) temperature (^{0}C) and time (min). The value of the third independent variables in each plot was kept at the center point.

2.10 DPPH scavenging activity (DSA)

α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging (DSA) method offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological sources. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine (Contreras-Guzman and Srong, 1982).

DPPH scavenging activity is not solely dependent on a single group of antioxidant compounds it also include capacity of existing compounds to scavenge DPPH radicals (Prior et al., 2005). DPPH scavenging activity increased linearly with the increase in solvent concentration but DPPH scavenging activity initially did not show appreciable changes with increase in temperature till 55^{0}C then gradually increased (Figure 4a). According to Wettasinghe and Shahidi (1999), high temperature may mobilize certain antioxidants while promoting possible concurrent decomposition of antioxidants which were already mobilized at lower

temperature. It was also stated that the rate of extraction of thermally stable antioxidants at elevated temperature is higher than the rate of decomposition of antioxidants of less soluble antioxidants. This has been suggested by relatively high antioxidant activities possessed by extracts prepared at higher temperatures. Increasing temperature may favor extraction by enhancing solubility of antioxidant in solvent. DPPH scavenging activity decreased with increase in extraction time, which may be due to phenolics oxidation due to light or oxygen exposure (Figure 4b). Figure 4c showed that DSA decreased with increase in the extraction time but increased with increase in the extraction temperature. Thoo et al. (2010) explained the potential extraction time increase by the loss of antioxidants following heat or oxygen exposure, whereas in a complex poly phenol system, a temperature increase can promote molecular collisions, favouring polymerisation and reducing antioxidant capacity (Pinelo et al., 2004).

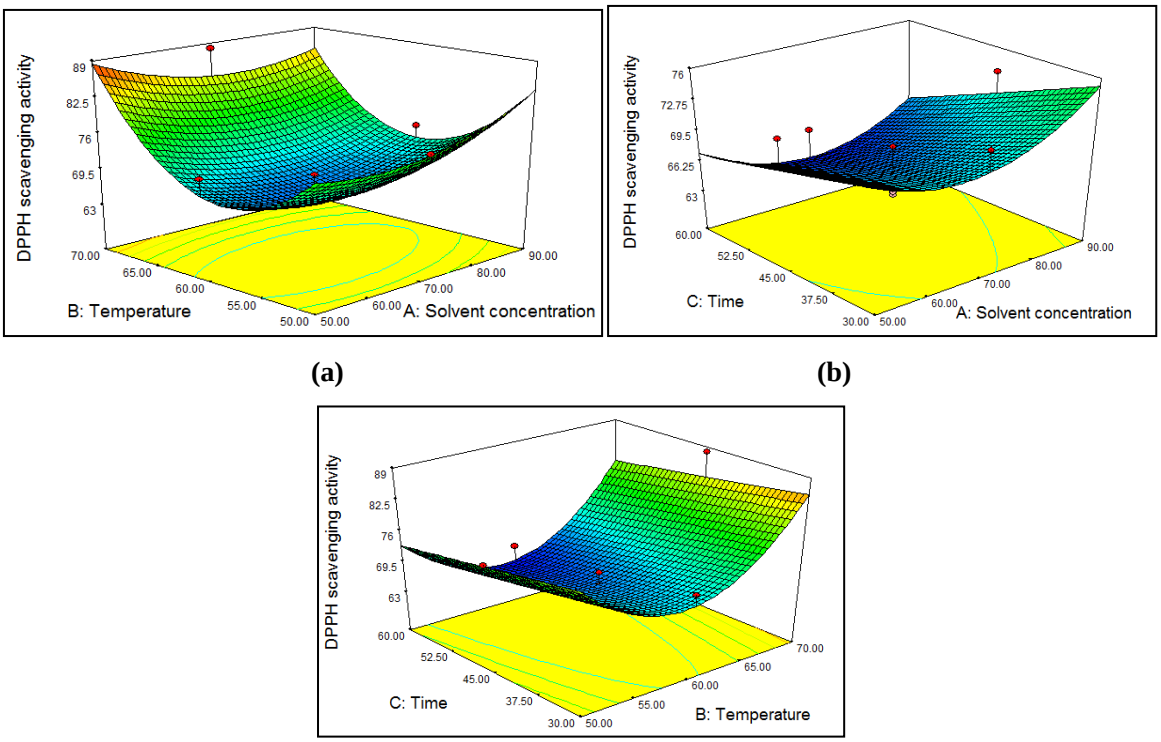


Figure 4 Response surface plots for DPPH scavenging activity (%), as a function of (a) solvent concentration (%) and temperature (^{0}C) (b) solvent concentration (%) and time (min) (c) temperature (^{0}c) and time (min). The value of the third independent variables in each plot was kept at the center point.

2.11 **Optimization and verification of processing conditions to maximize extraction of bioactive compounds**

Design expert software 7.0.0 was used to optimize the processing conditions like solvent concentration, temperature, time to maximize extraction of bioactive constituents. The software uses second order model to optimize the responses. When constraints in the range were selected then the optimum processing conditions were found as 90% v/v solvent concentration, 70 ℃ temperature and 59.54 min time. But in practice, it is difficult to maintain the predicted conditions during processing and some deviation is expected. Therefore, optimum conditions were targeted as 90% v/v solvent concentration, 70 ℃ temperature and 60 min time.

In order to verify the predictive optimum conditions, the experiments were conducted. Table 4 showed that the experimental results were very close to the predicted

one. The difference in the predicted and actual experimental values was statistically assessed by determining the coefficient of variation (CV). The coefficient of variation was found to be 0.27 for TPC, 2.18 for TFC, 1.34 for TAA and 0.057 for DSA, respectively. The data showed that the experimental results were very close to the predicted one. Therefore, a fit degree between the experimental values and predicted values were obtained. Therefore, the derived models may be used to predict the vales with precision.

Table 4 Optimization and verification of the processing conditions and experimental value of TPC, TFC, TAA, DSA and desirability

		Optimum Value			
		(In range)	**(Targeted)**		
Variables	Solvent Concentration (%)	90	90		
	Temperature (^{0}C)	70	70		
	Time(min)	59.94	60		
		Predicted Value		*Experimental Values*	*Coefficient of Variation*
Responses	Total Phenolic content(mg/g)	3.885		3.87	0.27%
	Total flavonoid content(mg/g)	350.37		361.37	2.18%
	Total Antioxidant Activity (mM/g)	0.1913		0.158	13.4%
	DPPH scavenging activity (%)	86.35		86.28	0.057%
	Desirability (%)	77.3			

Note: *As 59.94 min is difficult to regulate hence time was considered 60 min

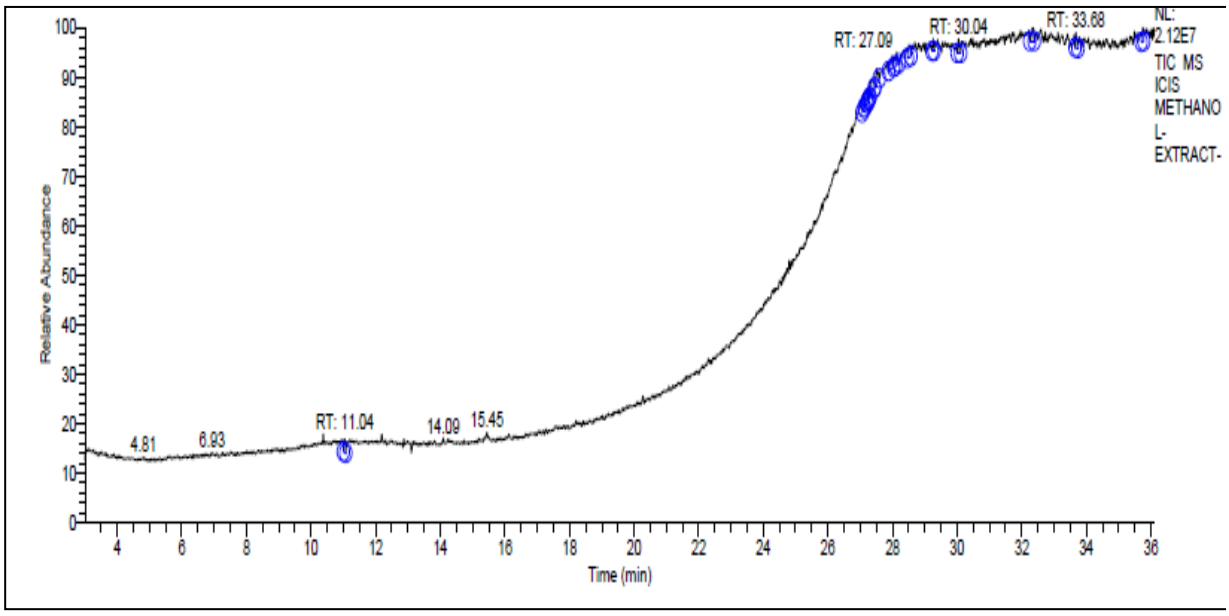
2.12 **Quantification of bioactive constituents**

GC-MS analysis of bioactive constituents is shown in Figure 5 whereas major active compounds with their retention time (RT), active principles and peak area (%) in the methanol extract of *Terminalia Chebula Retz are* presented in Table 5. Methanol extract consisted ethyl iso-allocholate , 1-Monolinoleoyl glycerol trimethylsilyl ether , Rhodopin etc., which possesses antiasthma, anti-inflammatory, anticancer, remove toxins, anticoronary, heptaprotective, hypocholesterollemic, antieczemic, nematicide diuretic, antimicrobial, antioxidant, antifungal and cytotoxic activity. The presence of various bioactive compounds confirms the importance of *T. Chebula* in various ailments which is generally prescribed by the traditional practitioners. Number of phenolics compounds such as 1-stachydrine, n-pentacose, n-triaconatne, n-triacontanol, n-nonacosane, pelargonidin-3-galactoside, glucocappasalin, beta-sito-sterol and phthalic acid (Khare, 2004).

Table 5 Bioactive compounds identified in the methanol extract of *T. Chebula* extract by GC-MS analysis

RT	Compound name	Peak Area (%)	Activity
11.04	Ethyl iso-allocholate	4.81	Antimicrobial, antiasthma, anti-inflammatory, anticancer, diuretic
27.27	1-Monolinoleoylglycerol trimethylsilyl ether	42.88	Antimicrobial steroid
28.00	Rhodopin	9.15	Carotenoid antioxidant
28.13	âDGlucopyranosiduronic acid, 3(5ethylhexahydro2,4,6trioxo5pyrimidinyl)-1,1-dimethylpropyl-2,3,4trisO(trimethylsilyl),methyl ester	5.49	Antioxidant, remove toxins, reduce fat, anti-inflammatory, anticancer, diuretic, wound healing
28.51	9,12,15Octadecatrienoic acid, 2,3bis[(trimethylsilyl)oxy]propyl ester	8.46	Anticoronary, Heptaprotective, hypocholesterollemic, antieczemic, nematicide
29.26	.psi.,.psi.Carotene, 3,4-didehydro-1,1',2,2'-Tetrahydro1' hydroxy1methoxy	3.31	Antibacterial, Antioxidant
30.04	4-Normethy-l9,19-cyclolanoststan-7-one, 3-acetoxy	5.72	Antifungal, cytotoxic

Figure 5 GC MS chromatogram of the methanol extract of *T. Chebula* fruits



3.9 Conclusions

The higher solvent concentration was found to be suitable for bioactive extraction of *T. Chebula*. The optimal conditions for the antioxidants extraction were found to be methanol concentration, 90%v/v, extraction temperature, 70℃, and extraction time, 60 min, for T. Chebula. The optimum conditions were total phenolic content, 3.87 mg/g, total flavonoid content, 361.37 mg/g, total antioxidant activity, 0.158 mM/g and DPPH scavenging activity, 86.28%. T. Chebula fruits extract mainly had seven bioactive compounds under the optimum conditions, which possesses antiasthma,

anti-inflammatory, anticancer, diuretic, antimicrobial, antioxidant, antifungal and cytotoxic activity. It can be concluded that *T. Chebula* is a potential source of wide variety of phytochemical constituents and has high therapeutic value and can be utilized as a potential nutraceutical source in food industry. Furthermore, the isolation of the bioactive compounds identified in GC-MS analysis, responsible for the phytochemical activity can be taken up which may result in development of modern drugs, nutraceutical supplements and functional foods from *T. Chebula* plants source.

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