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STANDARDIZATION OF CONDITIONS FOR EXTRACTION OF FLAVONOIDS FROM GLYCYRRHIZA GLABRA CALLUS CULTURES.

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ABSTRACT

Glycyrrhiza glabra is a perennial herb of Fabaceae family containing wide range of biologically active substances including flavonoids. The medicinal properties of the plant are mainly attributed to the presence of flavonoids. Extraction is an important step in isolation of these flavonoids, as it is a crucial process requiring intense standardization. The aim of the current research was to determine the optimized conditions for efficient and simultaneous extraction of major flavonoids from *Glycyrrhiza glabra* callus. Callus extracts were prepared with ethanol using Heat stirred extraction (HSE) method. Optimization was done by using L16 orthogonal design of experiment. The effect of factors such as Temperature, extraction time, solvent concentration material ratio, number of extractions on content of flavonoids was investigated. The maximum flavonoid content (10.2 mg/g) was obtained under optimum conditions of 1:30 material ratio, using 70% ethanol as solvent for 4 hrs extraction duration at temperature 85 ºC in three cycles of extractions. Ethanol concentration and extraction time were found to be the most significant factors influencing the flavonoid yield, and had a positive effect on flavonoid contents.

KEYWORDS: ***Extraction, Flavonoids, Glycyrrhiza glabra, Standardization, Orthogonal array design***

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INTRODUCTION

Glycyrrhiza glabra Linn. commonly known as Licorice is one of the most widely used herb from the ancient medical history of Ayurveda, both as a medicine and as a flavoring agent. It is popularly used worldwide in food, confectionery and pharmaceutical products, such as cough syrups, herbal supplements, chewing gums, drinks, candies etc. Licorice roots are extensively used in herbal medicines for their emollient, anti-inflammatory, anti-viral, anti-allergic, gastro-protective, and anti-cancerous properties attributed mainly to the presence of wide range of flavonoids. Various antioxidant properties of *Glycyrrhiza glabra* roots has also been reported [1-3] which are attributed to the phenolic compounds such as Glabridin, Licoisoflavone B, Licochalcone and Liquirtigenin [4]. These flavonoids of *Glycyrrhiza glabra* have also been reported to exhibit multiple pharmacological properties like antiparasitic, anti-inflammatory, antibacterial and anti-tumor and choleretic properties [5-9]. However, the extraction of these compounds from field grown plants requires uprooting of the plant leading to complete loss of the plant. Plant tissue culture is therefore seen as a good alternative where these valuable flavonoids can be produced under controlled environment. There is not much scientific documentation on the optimization of extraction conditions of these flavonoids from *Glycyrrhiza glabra* tissue cultures especially for pharmaceutical use. Extraction is an important step in the isolation and later in the identification and quantification of flavonoids. In many cases, prediction of extraction conditions for such plant metabolites are subject to several factors and are certainly not straight forward [10]. Therefore, good experimental design and optimization of extraction conditions is necessary for efficient extraction of metabolites. Since the phenolic compounds of different plants differ structurally, it is very difficult to develop a standardized extraction method that would simultaneously extract all inherent flavonoids [11]. The extractability of the phenolic compounds depends on the type of the solvent extraction method employed, extraction time, nature and preparation of material to be extracted, chemical structure of phenolic compounds, temperature, solid-liquid ratio, and possible presence of interfering substances. At present, there is a renewed interest in developing new processes based on the use of different variables like temperature, solvent, and material ratio for the extraction of low molecular weight components that may be environment friendly and benign. Previous research documentation authenticates that temperature-assisted, enzyme-assisted, supercritical-fluid-based, and semi bionic-based extractions are superior over conventional extraction [12]. But, It is quite complex to predict the suitable experimental conditions for a given separation task, and therefore good experimental design becomes significantly important. Such experiments are often executed in the form of orthogonal arrays. Orthogonal array design (OAD), also known as Taguchi design, is believed to incorporate the advantages of simplex method and factorial design. It arranges different factors for effective optimization of experimental conditions. The results of the OAD experiment can be statistically treated by two ways: analysis of variance (ANOVA) and direct observation analysis [13-14]. In ANOVA, the effects of different factors on response functions can be evaluated by both significance (*F* ratio) and percentage contribution (PC%) value. In other words, the importance of a factor or the interaction among different factors can be estimated from the significance and PC% value. Direct observation analysis is also called range analysis. From ANOVA and direct observation analysis of experimental results, factors that significantly affect the output responses can be found and optimal parameters for an analytical procedure can be obtained. The use of OAD can simplify the experiment procedure without affecting the quality of results. Adopted as a chemo metric method, OAD has been widely applied for the optimization of analytical procedures in recent years [15-18]. There are very few reports available on the respective extraction of flavonoids like Glabridin, Licochalcone but a method for simultaneous extraction of these compounds or optimization of process for extraction of total flavonoids especially from G.glabra callus has not been established yet. Therefore, this paper reports about the development of optimal process for extraction of flavonoids from *Glycyrrhiza glabra* callus cultures. In the present study, a L16 (4^5) OAD procedure is performed with five factors at four levels that are important influence parameters in extraction of flavonoids and the impact of most influential factors on content of flavonoids of *Glycyrrhiza glabra*, has also been studied through response surface methodology.

MATERIALS AND METHODS

Chemicals

Ethanol, Sodium hydroxide, Aluminium chloride, $NaNo_2$, Quercetin were obtained from Scientific agencies, Faridabad, India.

Plant material and Callus induction

Glycyrrhiza glabra plants were collected from botanical garden at Jamia Humdard University, New Delhi. The young leaves obtained from the field grown plants were used as explants for callus induction. The explants were washed thoroughly in water and were surface sterilized with 70% ethanol and 0.1% mercuric chloride and were placed in sterile petriplates for inoculation. Callus was grown on previously standardized medium consisting of Murashige and Skoog (1962) basal salts and vitamins with 3% (w/v) sucrose, 1% agar and filter sterilized growth hormones NAA (1.0 mg/l) and BAP (0.5 mg/l). Callus cultures were maintained on this medium and sub cultured at frequent intervals and used for further studies.

Experimental design of extraction process

Extraction of flavonoids was carried out through heated stirred extraction (HSE) method using ethanol as a solvent. The main factors that affect the extraction of flavonoids like temperature, extraction time, materials ratio, extracting agent (%) and the no. of extractions were studied and the optimum extraction conditions were then determined by L16 (4^5) orthogonal design of experiment. The Experimental data of Orthogonal Array Design (OAD) direct observation analysis (Range analysis) and A single factor analysis of variance (One way ANOVA) was carried out to investigate the effect of

each factor in the extraction of flavonoids. All the statistical analysis was carried out with Stat ease Design expert 9 trial version software.

Estimation of total flavonoids content (TFC)

Total flavonoids content (TFC) was estimated spectrophotometrically by Aluminium chloride with slight modifications [19]. About 0.1 ml of the leaf extract added 4.9 ml of distilled water was added to make the volume to 5ml. to this, 0.3 ml 5% NaNO2 was added and kept for 5 minutes. Then, 3 ml of 10% AlCl3 was added. After 6 minutes, 2 ml of 1 M NaOH was added and the absorbance was measured at 510 nm. Quercetin was used as a standard for constructing a calibration curve.

Response surface analysis

The most influencing factors among the five factors affecting the flavonoid extraction was known from the range analysis of OAD and their effect on content of flavonoids of Glycyrrhiza glabra was graphically represented and analyzed by 3D response surface plots.

RESULTS AND DISCUSSION

Extraction of Flavonoids from the callus cultures of Glycyrrhiza glabra

Flavonoids, a broad group of secondary metabolites have gained recent interest, because of their broad pharmacological activity. Extraction of flavonoids is a crucial process requiring intense standardization. A suitable extraction method and extraction solvent is essential to enhance the extractability of flavonoids from a particular plant material. In this study, Flavonoids were extracted from the callus cultures of *Glycyrrhiza glabra* through heated stirred reactor method using ethanol as a solvent. Solvent extraction is an efficient method of obtaining plant derived chemicals. In this process the solid material comes in contact with a solvent and its soluble components move to the solvent. Thus, solvent extraction of plant material results in the mass transfer of soluble compounds to the solvent. Since mass transfer of the compound also depends on its solubility in the solvent, heating and stirring the solvent enhances the mass transfer. Claudia *et. al* [20] have reported the heat stirred reactor method to be the best method for obtaining higher yields of phenols and flavonoids as compared other methods. The recovery of flavonoids from plant material is greatly influenced by the solubility of the flavonoids in the solvent used for the extraction process; hence, ethanol was selected as it interacts with the flavonoids probably through non-covalent interactions and promotes rapid diffusion of flavonoids into the solution.

Standardization of Flavonoid Extraction Using Orthogonal Design of Experiment

The parameters and the orthogonal design of experiment for the extraction of flavonoids are given in the Table-1.

Table 1
Levels and factors affecting the Flavonoid yield.

	A	B	C	D	E
Levels	Temperature (˚C)	Extraction time (hrs)	Ethanol concentration (% V/V)	Material ratio (W:V)	No. of. Extractions
1	55	2	50	1:30	1
2	65	4	60	1: 45	2
3	75	6	70	1:60	3
4	85	8	80	1:75	4

Range analysis was used to indicate the effect of each factor and determine the optimal level of different factors. The mean value of the sum of the evaluation indexes of all levels in each factor (*K*) was used to determine the optimal level and the optimal combination of factors. The range (R) was defined as the range between the maximum and minimum value of the mean values and used to evaluate the importance of the factors. The optimal level for each factor could be obtained when *K* is the largest and larger R means greater significance of the factor [21]. According to L16 (4^5) orthogonal array matrix, sixteen experiments were carried out. The results are shown in table 2. The optimal extraction conditions of highest flavonoid recovery using ethanol were found at a temperature 85˚C (A_4), 4 hours extraction time (B_2), 70% ethanol concentration (C_3), 1:30 material ratio (D_1) and 3 times of extraction cycle (E_3). Since 'k' at these combinations (A_4 B_2 C_3 D_1 E_3) was the highest. Since larger range values (R) means greater impact on the product yield, by comparing the range values of different factors, the order of significant factors was Extraction time (0.43) > Solvent percentage (0.34) > Temperature (0.32) > Number of extraction (0.25) > Material ratio (0.23).It can be seen from the results that extraction time and solvent percentage are the most influencing factors among the five factors affecting the flavonoid yield.

Table 2
L_{16} (4^5) OAD matrix with the experimental results.

Experiment	A	B	C	D	E	Flavonoids (mg/g)
1	1	1	2	3	4	5.1
2	1	2	1	4	3	3.8
3	1	3	4	1	2	6.5
4	1	4	3	2	1	7.3
5	2	1	1	1	1	5.3
6	2	2	2	2	2	9.4
7	2	3	3	3	3	6.1
8	2	4	4	4	4	8
9	3	1	3	4	2	6.8
10	3	2	4	3	1	7.2
11	3	3	1	2	4	8.1
12	3	4	2	1	3	5.8
13	4	1	4	2	3	3.9
14	**4**	**2**	**3**	**1**	**4**	**10.2**
15	4	3	2	4	1	6.3
16	4	4	1	3	2	6.8
K_1	5.68	5.25	6	7.15	6.53	
K_2	7.2	7.65	6.65	6.25	7.38	
K_3	6.78	6.75	7.6	4.63	7.88	
K_4	6.98	6.98	7.35	6.95	7.53	
k_1	1.42	1.32	1.5	**1.79**	1.63	
k_2	1.8	**1.91**	1.66	1.74	1.84	
k_3	1.69	1.68	**1.9**	1.16	0.97	
k_4	**1.74**	1.74	1.83	1.56	**1.88**	
R	0.32	0.43	0.34	0.23	0.25	

ANOVA of the Orthogonal Array Design

Although the optimal value of different factors can be easily determined by the range analysis, this method cannot distinguish whether the difference between the data fluctuation of each factor level was caused by experimental conditions or by experimental errors. Therefore, analysis of variance was necessary to obtain the magnitudes of the factor affecting the result [22]. In the ANOVA, the data were analyzed by a F-test and PC% (Percent contribution). The F value of each factor (F) implies the ratio of the variance for the each factor to that of the experimental error [14, 21]. During the F-test, Fα was a constant and defined as a critical value of the F-value for different inspection levels and can be found from the distribution table of the F-values. F ratio analysis indicates that which parameter has the significant effect on the yield of flavonoids. When F is larger than Fα, the factor effect for the results is prominent. It can be seen from the results that the factors Extraction time, solvent percentage, Material ratio and Number of extraction are statistically significant at $\alpha < 0.05$ where as Temperature is shown to be the insignificant factor at at $\alpha < 0.05$.These results are further confirmed by the analysis of percentage contribution (PC%) contained in Table-3.

Table 3
ANOVA and percent contribution for flavonoids yield in the L_{16} (4^5) OAD.

Factors	SS	Degrees of freedom	Mean square	F value	F α (3,3) = 0.35	SS'	PC%
A	45.9	3	1.84	0.24	<	43.7	19
B	57.8	3	4	1.42	>	52.1	24.1
C	54.3	3	2.08	0.61	>	55.6	22.5
D	31.7	3	2.53	1.26	>	29.5	13
E	45.9	3	4.2	1.11	>	43.7	19
error	2.25					13	2.6
Total	**231**	**15**				**231**	**100**

It reflects the factor's influence and the percentage contribution due to experimental error providing an estimate of the adequacy of the whole experiments. Larger percentage contribution means more significant factor influence. When the percentage contribution due to error is low, say 15 % or less, it can be assumed that no important factor has been omitted and the whole experimental results are reliable [14-23]. The PC% is calculated by Eq. (1):

$$PC\% = SS'/ SStotal \times 100 \quad (1)$$

Where SS' is the purified sum of squares and is given by SS−SSerror, SS is the individual sum of squares, SSerror is the sum of squares of error and SS total is the total sum of squares. It should be noted that the value of SS'_{total} is equal to SS_{total} in statistics, while SS'error is obtained from the difference of SS_{total} and the sum of SS' of the considered factors. The results indicate that Extraction time (24.1%) is the most important factor contributing to the flavonoid yield followed by Solvent percentage (22.5%). Temperature (19%) and Number of extraction (19%) have equal contribution and Material ratio (13%) is the least

contributing factor. Obviously, the data analysis of PC% is in good agreement with the conclusion obtained from the significance analysis discussed above. Since the percentage contribution due to error was low (2.6 %), it was assumed that no important factor had been omitted and the whole experimental results were reliable.

Impact of influencing factors on the yield of flavonoids

Through the range analysis and ANOVA of the experimental data in orthogonal array design, it is clear that the extraction time and solvent (ethanol) percentage is the most significant factors having a major impact on the yield of flavonoids. Therefore, the effect of different levels of these two factors on the yield of flavonoids was also studied further using 3D response surface plots. The relationship between variables was graphically represented by 3D response surface plots generated by the model. Different shapes of the plots indicate different interactions between the dependent and independent variables. Figure 1 shows the interaction between ethanol concentration and extraction time on the yield of flavonoids. It was observed that there was a linear relationship between ethanol concentration and the extraction yield from 50 to 70% of ethanol concentration. The yield of flavonoids maximized at 70 % ethanol at 4 hours of extraction time.

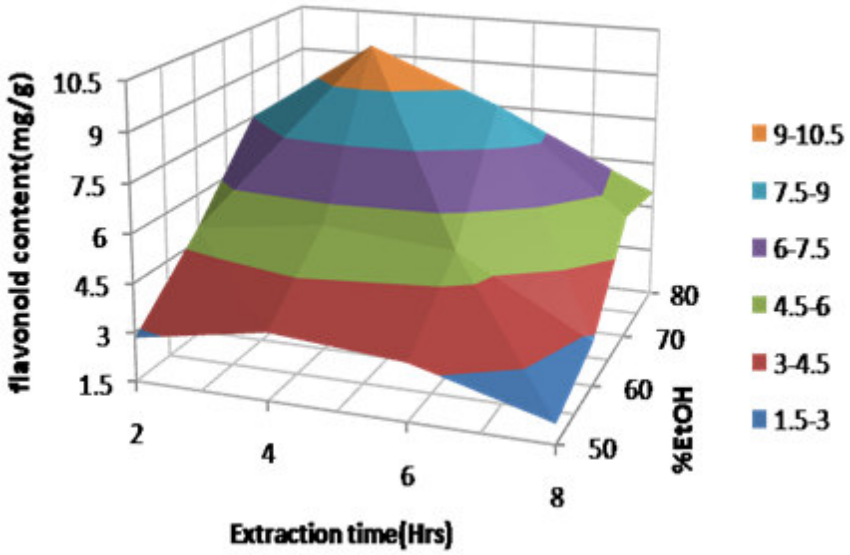

Figure 1
Response surface plots indicating the effect of different concentrations of ethanol (%) on flavonoid content at different extraction times (Hrs).

However, when the extraction time was than 4 hours, there was a slight decline in the response. Similarly, ethanol concentration was above 70% did not show any obvious effect on extraction yield but increase in the percentage of ethanol in aqueous solutions up to 70% had a positive influence on the response. The results revealed that the contents of these compounds in *Glycyrrhiza* callus varied considerably as a function of solvent composition and results were in agreement with previous studies which showed that solvent nature exert a great power in flavonoid extraction capacities in many species [24, 25]. Furthermore, these results were in accordance with previous reports suggesting that a binary solvent system is more efficient than mono-solvent system (water or pure ethanol) in the extraction of phenolic compounds in regard to their relative polarity [26, 27]. In all the cases, It was observed that the yields of flavonoids suddenly decreased with increase in extraction time beyond 4 hours probably because the increase extraction time might have accelerated chemical degradation of bioactive compound in extraction process, which resulted in the lower extraction yield [28].

CONCLUSION

The L16 orthogonal design and response surface methodology were successfully employed to optimize the extraction conditions of flavonoids from the callus cultures of *Glycyrrhiza glabra*. The maximum yield of flavonoids obtained was 10 mg/g under heated stirred extraction with 1:30 material ratio, using 70% ethanol as solvent for 4 hrs extraction duration at temperature 85 ºC in three cycles of extractions. The factors that significantly affected the extraction of flavonoids were ethanol concentration and extraction time, while the influence of material ratio and extraction cycle were not so pronounced.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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