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Whole extract optimization of *Adhatoda vasica,* Nees leaf by using Response Surface Methodology (RSM)

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ABSTRACT

Present work aimed at optimization of extraction process for various factors to get maximum extract yield for a drug, *Adusa* (leaf of *Adhatoda vasica* Nees). The drug is used in the form of powders or decoctions, so Soxhlet apparatus (hot percolation) was used to optimize the yield percentage. *Adusa* leaf was subjected to extraction process using Soxhlet apparatus. Response surface methodology (RSM) was used to design the number of random runs of the extracts for the drug with variation in the factors of temperature, concentration of ethanol in water, time for extraction for maximizing the yield concentration. The data thus obtained was analyzed for optimization for yield maximization. Minitab version 18 was used to design and analyze the data. Validation of the optimum conditions for maximum yield of the extract of *Adusa* leaf was carried out by re-run of the extract using optimum conditions and measuring the yield. The study showed that optimum condition for extracting *Adusa* leaf for temperature, solvent concentration, and time for extraction was 80^0C, 48.18% and 8 h respectively. *Adusa* leaf extract gave maximum yield of 22.07%. The optimum factors when reemployed yielded 23.73% of the extract validating the methodology. RSM used in present study is cheap and affordable method to optimize maximum yield% which may be reliably used by researchers for research in this area.

Keywords: Adusa leaf; Optimization; RSM; Soxhlet extraction.

INTRODUCTION

Medicinal plants are being utilized in the treatment of diseases from time immemorial. Plant based medicines are observed to be safe for utilization with lesser side effects than the synthetic drugs [1]. Plants as a source of medicinal compounds have continued to play a dominant role in the maintenance of human health since ancient times. According to the WHO, plant extracts or their active constituents are used as folk medicine in traditional therapies and 80% of the world drugs are of natural product origin [2, 3]. *Adusa*(*Adhatoda vasica* Nees) is a well known plant of Acanthaceae utilized as a drug ingredient in Unani, Siddha and Ayurvedic preparations [4-6]. It has been utilized in the indigenous system of medicine in India for more than 2000 years [7]. In Unani medicine, it is known as *Adusa* or *Bansa* [8]. The leaves, flowers, fruits and roots are extensively used for treating cold, cough, whooping cough, chronic bronchitis, asthma, as sedative expectorant, antispasmodic and anthelmintic. The drug is employed in different forms, such as fresh juice, decoction, infusion and powder form. It is also given as alcoholic extract and / or syrup [5]. The plant has been included in the WHO manual entitled '*The Use of Traditional Medicine in Primary Health Care'* [9] aimed to profit health workers in South-East Asia. It is used to helping them and informing the therapeutic utility of the plant and surrounding flora. The plant is mentioned as first aid medicine in primary health care and can be utilized in both adults and children and for a long period without any restriction of use [9]. The Phytochemicals studies of the various parts of *Adhatoda vasica* revealed the presence of alkaloids, phytosterols, polyphenolics and glycosides as a major class of compounds. Its principal constituents are quinazoline alkaloids with vasicine as its chief alkaloid. The leaves are rich in Vitamin C and carotene and yield an essential oil. Chemical compounds found in leaves and roots of this plant also includes essential oils, fats, resins, sugar, gum, amino acids, proteins, vitamin C etc. The leaves contain a very small amount of an essential oil and also a crystalline acid [10, 11].

Extraction and isolation of phytoconstituents from crude drug is the initial phase in analysis of medicinal plant constituents. It helps in assessing individual constituents for their pharmacological value. Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures.

There are variety of methods of extraction i.e., maceration, hot continuous extraction, percolation, decoction, ultrasound extraction, supercritical fluid extraction, micro wave assisted extraction etc. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations popularly have been called galenicals, named after Galen, the second century Greek physician. The purposes of standardized extraction procedures for crude drugs are to attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent known as menstruum. The extract thus obtained may be ready for use as a medicinal agent in the form of tinctures and fluid extracts, it may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities of some plants such as ajmalicine, hyoscine and vincristine and in case of selected drug *Adhatoda vasica* such as vasicine, bromhexine, etc. Standardization of extraction procedures contributes significantly to the final quality of the herbal drug [12]. If the plant was selected on the basis of traditional uses, then it is needed to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted.

Unani system of medicine used crude forms of drugs consistently except decoctions, syrups, *Arqiyaat* (distillates) and some salts obtained from burning of various plant drug parts. In case of these forms the whole extract is more emphasized than isolated extracts. Even processing a crude plant drug part is considered as change its *Surat-e-Nu'iea*(specific form) and its *Taseer*(effect) may change, like extent of powdering can also do so [13].

Soxhlet extraction is a very useful tool for preparative purposes in which the analyte is concentrated from the matrix as a whole or separated from particular interfering substances. Sample preparation of environmental samples has been developed for decades using a wide variety of techniques [14]. Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds [15].

A lot of work has been carried out to describe optimum method to extract for maximum yield of isolated phytoconstituents like anthocyanins and total phenolic contents of barberry fruits (*Berberis vulgaris*) by using RSM [16]. Optimization of microwave assisted extraction of withanolides from roots of *Ashwgandaha* (*Withania somnifera*) [17], essential oils production from *Citrus latifolia* [18], conditions to isolate protein from germinated green gram (*Vigna radiata* L.) [19], microwave assisted extraction of inulin from chicory roots [20], extraction of bioactive compounds from *Feronia limonia* (wood apple) fruit [21], extraction of peanut proteins with water by RSM [22].

Since whole extracts are more important for the requisite *Taseer* as described in Unani medicine, so whole extract optimization for these drugs is the need of the time. No study has been carried yet for the optimization of whole extract. *Adusa* is important herbs in the Unani and other indigenous medical system. *Adusa* leaves have been recommended to be administered in the decoction form. So, the drug, *Adusa* has been selected for the present study by hot percolation (Soxhlet extraction) method to optimize various conditions of temperature and water: alcohol solvent ratio and time to maximizing the yield of the whole extract using RSM.

In statistics, RSM investigates the relationships between several explanatory variables and one or more response variables. The method was introduced by GEP Box and KB Wilson in 1951 [23]. The basic principle of RSM is to use a sequence of designed experiments to obtain an optimal response [23]. When many factors and interactions affect desired response, RSM is an effective tool for optimizing the process and the main advantage of it is the reduced number of experimental trials needed to optimize the parameters and the main advantage of it is the reduced number of experimental trials needed to optimize the parameters [24]. RSM is a statistical technique used for the development and optimization of complex processes. Many types of response surface designs are used for optimization like Central composite, Doehlert, and Box–Behnken. Box–Behnken design is preferable to the Central composite and Doehlert designs because it requires fewer test runs and is rotatable. A design is rotatable only when the experiments are roughly situated on a (hyper) sphere. By adequate selection of the number of centre points, it is possible to arrange that the precision of the response of a predicted design is similar over the whole domain. Such a design is said to have uniform precision. Box-Behnken design is advantageous because it does not contain any points at the extremes of the cubic region created by the two-level factorial level combinations [25]. In the present investigation the Box- Behnken design was selected and used to optimize, extraction of *Adusa* leaf with hydro-alcoholic solvent with different extraction conditions (Temp, Conc. and Time) by RSM, as the design provides three levels for each factor and requires fewer runs in the three-factor case than Central composite and Doehlert design.

MATERIAL AND METHODS

The response variable (yield%) was optimized by RSM using Minitab version 18. All the experiments were carried out in the Department of Ilmul Advia, NIUM, and Bangalore India.

Plant material

Fresh leaves of *Adhatoda vasica* (*Adusa*) were collected from the herbal garden of NIUM, Bangalore campus, was identified and authenticated by the experts at the Institute of Trans-Disciplinary Health Sciences and Technology (FRLHT) Bengaluru with authentication number viz. *Adhatodavasica*, Nees leaf, 5213. Specimen of same was also submitted to the NIUM Herbarium with voucher specimen no 65/IA/Res/2019 for future reference.

Preparation of the extract

The leaves of *Adusa* were oven dried at 45 ^{0}C. The dried leaves were powdered using a grinder. The powder thus obtained, was extracted in hydro alcoholic solution.

For the extraction, 40g of sample powdered drug with 320 ml hydro alcohol solvent was used at different concentrations of ethanol, temperature as per the experimental plan (Table 1).

Experimental design for extraction of *Adusa* leaf

The Box-Behnken Design from RSM was used for designing the experimental combinations. The variables used were temperature (^{0}C), concentration of ethanol (%), (% of alcohol in hydro-alcoholic solvent) and time (h) [Table 1-4]. The tables were generated using Minitab version 18 which were followed to run the extracts with specified conditions.

METHOD FOR EXTRACTION

All the extractions were carried out according to randomized design (Table 4) generated through RSM by Minitab 18 for yield response with temperature and concentration variation. The extract was cooled, filtered through Whatman filter paper No 1. After that water bath was use to concentrate the extract then calculated yield in grams (g) for every extraction.

Plant extract yield (EY): The yield of the extract was calculated from the equation $\frac{W1}{W2} \times 100$ where, W_1 is the weight of extract after evaporation of solvent and W_2 is the dry weight of the plant sample.

Statistical Analysis

Statistical analysis was carried out by using RSM by software Minitab 18. After generating the random design, the yield obtained in various conditions of temperature and concentration was analyzed for optimization. The regression equation was generated and the optimization of the surface factors for obtaining maximum yield was noted. The results were generated in the form of tables and graphs.

Validation of the data obtained through RSM

The optimized values for response variable of yield were used for extraction and the yield percentage was observed and compared with the yield as generated from RSM. If the yield was similar to the value obtained from the RSM than the optimization procedure stands validated.

RESULTS

The extracts of the test drugs were prepared by using Soxhlet apparatus after designing the procedure using Minitab 18. The design created for the factors of temperature, concentration and time that influenced the yield% were randomly carried out. The random design was also charted out through the Minitab software. The input conditions were initially set as for temperature was 60 ^{0}C to 80 ^{0}C, concentration of ethanol in water was from 30% to 70% and time 6 h to 8 h. The drug and solvent ratio were kept constant for all the runs for extraction as 1: 8.

RSM was used for designing the experimental combinations. The variables used were temperature (^{0}C), concentration of ethanol (%) and time (h), depicted in Table 1 to 4. The random runs were also created for the drug through the software (Table 4).

The yield% for extracts is depicted in Table 5. The yield% is given against each of the runs.

For prediction Minitab 18 used for RSM for optimizing the extract conditions to get highest yield regression equations were generated as follows:

Yield% = 11 + 2.06(X) + 0.663(Y) - 25.9(Z) - 0.0189(*X*).(*X*) - 0.00329(*Y*).(*Y*)+0.71(*Z*).(*Z*) -0.00674(*X*).(*Y*)+0.190 (*X*).(*Z*)+0.0242 (Y).(Z);

Where X is temperature, Y is concentration and Z is time duration for extraction.

When the individual yield extracts were analyzed for optimum yield it was found that the following conditions for the factors gave maximum yield for *Adusa* leaf. For maximum yield of 22.07% with Standard Error (SE) of 2.01 for 95% confidence interval (16.89, 27.25) the predicted response factors need to be 80^{0}C, with ethanol concentration in water as 48.18% and time as 8 h.

The optimum conditions thus found through RSM for the drug was validated by again extracting the drugs with the predicted conditions. The yield% was practically found for *Adusa* leaf as 23.73%. This was very close to the predicted values (22.07%) with no significant difference and thus validates the model used.

DISCUSSION

The association between the response functions and the process variables were identified by three-factor recorded Box-Behnken design. Further, the extraction conditions of response variable were optimized. Active constituents were extracted by subjecting the plant material to acid hydrolysis in order to release the bound particles from their complex matrix, and then selectively separated with hydro alcohol, by and large a helpful solvent for preliminary extraction of chemical constituents from the plants [26].

The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods (e.g. finite element method or CFD analysis) and their associated numerical noise. Venter *et al*. (1996) have discussed the advantages of using RSM for design optimization applications. For example, in the case of the optimization of the extraction we want to find the of temperature(x1) time (x2) and solvent concentration (x3) that maximize the yield% (*y*) of the extract. The yield% is a function of the levels of temperature time and solvent concentration, as follows:

$y = f(x1, x2, x3) + \varepsilon$ (Where ε represents the noise or error observed in the response *y*)

The surface represented by *f* (*x*1, *x*2, x3) is called a response surface. The response can be represented graphically, either in the three dimensional space or as *contour plots* that help visualize the shape of the response surface.

Contours are curves of constant response drawn in the *xi*, *xj* plane keeping all other variables fixed. Each contour corresponds to a particular height of the response surface, as shown in Fig 1 to 3 as cited from present study.

Table 5 shows the results of the predicted and experimentally measured responses for the 15 runs according to the experimental design. The yield of the *Adusa* leaf ranged from 8.35% to 23.40%ondry weight basis and the maximum yield was obtained for the 6thrun under the experimental conditions of A= 80^{0}C; B = 50%; C = 8 h.

There was fair interaction between surface factors of temperature and concentration, temperature and time, and temperature, time and concentration. The summary table for ANOVA shows the p values of the same as 0.004, 0.477, and 0.139. The interaction though found is there but not significant (p>0.05). This employs that the three factors are independently acting on the yield. The surface plot as shown in the graph tends to exhibit the parabolic shape that opens on the X- axis signifies that there lie optimum conditions that result in the maximum or optimized yield. This is also evident from the un coded regression equation obtained for these factors against yield. It is a quadratic equation with positive sign for the 2nd degree terms of the form y = β0 + β1x1 + β2x2 + β12X1X2+ β11X12+ β22X22+ € as seen from the regression equation generated through Minitab 18

The yield percentage as response variable was seen that the optimum yield was at the conditions of temp 80^{0}C, concentration 48.18% and time 8 h. Yield% was found as 22.07%. The optimum yield at these conditions was validated by conducting the again a practical trial for extraction at these conditions and it was found to be 23.73 for *Adusa* leaf extract. The yield thus obtained was almost similar to the yield suggested by the RSM validating the procedure applied.

The present study aimed to optimize yield for whole extract. The reason behind it was that Unani medicine uses these drugs as decoctions, concoctions or powders. For exclusive concoctions or decoctions water would have been the ideal solvent, but for powders or powder-based dosage forms hydro alcohol was used as maximum ingredients are likely to dissolve in it. Thus, hydro alcoholic solvent may fulfill the need for all the three conditions. The drug selected for the study was of the nature that they have insignificant amount of volatile content. Apart from taking these precautions more of the surface factors may be chosen in future studies to fine tune and better set parameters for extraction process. Despite this, it seems that the study will save lot of solvent, drug material, time and cost if the drugs are selected for future studies for analyzing constituents. It may help pharmacies / laboratories to adopt the set-in conditions for maximum extract yield for these drugs to use them for pharmaceutical use.

The study observed that temperature has a definite effect on extraction process with increased temperature from 60 to 80 ^{0}C. It was found that there was consistent increase in the yield which saturated towards the higher end. The temperature enhancement therefore does not show any maximization trend of the yield after which it would tend to decrease the yield. Saturation or the maximum solubility of the extraction has an effect over the increased temperature. It may be explained as the boiling point of alcohol is 80 degree while as water 100 degree.

The concentration of alcohol and water has got a sure optimizing effect on the extract as seen from the results. The optimization concentration is 48.18 for *Adusa* leaf extract whereas concentrations ranged from 30% to 70%. In case for time factor the optimizing condition was 8 h, whereas temperature variation used was 6 h to 8 h.

From the ANOVA summary the p values for interaction variables of temperature and concentration, temperature and time, and concentration and time, were 0.298, 0.162, and 0.693, respectively. Though they were not significant at p <0.05, but at 80% significant level the results may be interpreted to have fair interaction except time and concentration as the temperature is vital in the method of extraction adopted.

RSM being an effective tool for accounting surface factors for an outcome has been used here to save labour, cost and standard procedure for the starting drug material for analysis. The optimization of extract is need of time. There are no documented references for the exact starting conditions to get maximum yield in extraction process. The present study is helpful as a reference for getting maximum yield when extraction of the tested drug is carried out through Soxhlet apparatus. This apparatus has remained as indispensable tool for extraction of the plant drug especially those which are used in decoction or concoction form and have insignificant heat labile ingredients. Moreover, the traditional system pharmacies when manufacture solid dosage forms like tablets need to minimize the size of the tablet for better compliance. Extractable used as fillers and accommodation of the dosage of drug can go a far to accomplish the same. The similar means have been adopted by various pharmaceutical companies may be a way forward for others.

CONCLUSION

The results showed that ethanol concentration and temperature and time affected the measured responses significantly. Under the optimal condition of 48.18% ethanol, 80 ^{0}C temperature and 8 h predicted yield% values were 22.07% for *Adusa* leaf extract. Overall, the study suggests that the model obtained in the present study can be applied for large scale production of extract for further use in pharmacy/food industries. The present study is helpful as a reference for getting maximum yield when extraction of the drug is carried out through Soxhlet apparatus.

Table 1: Boundaries of the experimental domain and spacing of the compositional variable levels for *Adusa* leaf

Independent Variables	Symbol code	Low variables	High variables
Temperature (^{0}C)	A	60	80
Concentration of ethanol (%)	B	30	70
Time (h)	C	6	8

Table 2: Randomized design for running extract of *Adusa* leaf

Run	Block	A	B	C
1	1	-1	-1	0
2	1	1	1	0
3	1	-1	0	-1
4	1	1	0	-1
5	1	-1	1	0
6	1	1	0	1
7	1	0	-1	1
8	1	0	1	1
9	1	0	0	0
10	1	-1	0	1
11	1	0	1	-1
12	1	1	-1	0
13	1	0	0	0
14	1	0	0	0
15	1	0	-1	-1

Table 3: Design executed for surface factors of Temperature, Conc. and Time for yield percentage for *Adusa* leaf

Std. Order	Run Order.	Pt. Type	Blocks	Temp. (^{0}C)	Conc. (%)	Time (Hrs)
1	1	2	1	60	30	7
4	2	2	1	80	70	7
5	3	2	1	60	50	6
6	4	2	1	80	50	6
3	5	2	1	60	70	7
8	6	2	1	80	50	8
11	7	2	1	70	30	8
12	8	2	1	70	70	8
15	9	0	1	70	50	7
7	10	2	1	60	50	8
10	11	2	1	70	70	6
2	12	2	1	80	30	7
14	13	0	1	70	50	7
13	14	0	1	70	50	7
9	15	2	1	70	30	6

Table 4: Yield percentage for *Adusa* leaf after Soxhlet extraction

Std. order	Run order	Pt. Type	Blocks	Temp.	Conc.	Time	Yield
1	1	2	1	60	30	7	10.82
4	2	2	1	80	70	7	17.52
5	3	2	1	60	50	6	15.50
6	4	2	1	80	50	6	22.93
3	5	2	1	60	70	7	15.25
8	6	2	1	80	50	8	23.40
11	7	2	1	70	30	8	16.02
12	8	2	1	70	70	8	17.78
15	9	0	1	70	50	7	19.50
7	10	2	1	60	50	8	8.35
10	11	2	1	70	70	6	19.25
2	12	2	1	80	30	7	18.48
14	13	0	1	70	50	7	20.00
13	14	0	1	70	50	7	16.68
9	15	2	1	70	30	6	19.43

Table 5: RSM Model Summary for the *Adusa* leaf extract

S	R-sq	R-sq(adj)	R-sq(pred)
2.32153	87.89%	66.08%	0.00%

Table 6: A. Response Optimization: yield parameters

Response	Goal	Lower	Target	Upper	Weight	Importance
Yield	Maximum	8.35	23.4		1	1

Table 6: B. Solution

Solution	Temp.	Conc.	Time	Yield Fit	Composite Desirability
1	80	48.1818	8	22.0664	0.911388

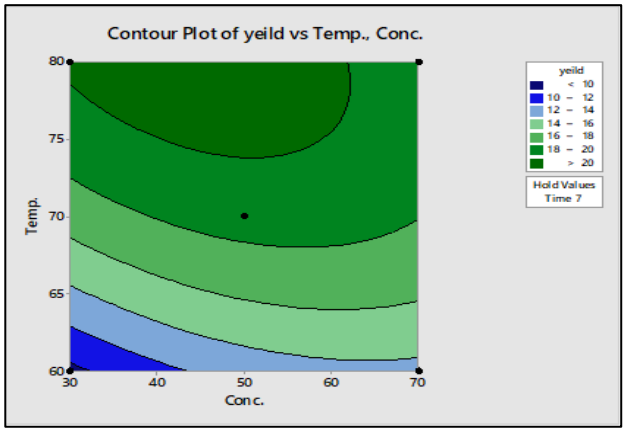

Figure 1: Contour plot of yield v/s temp and concentration

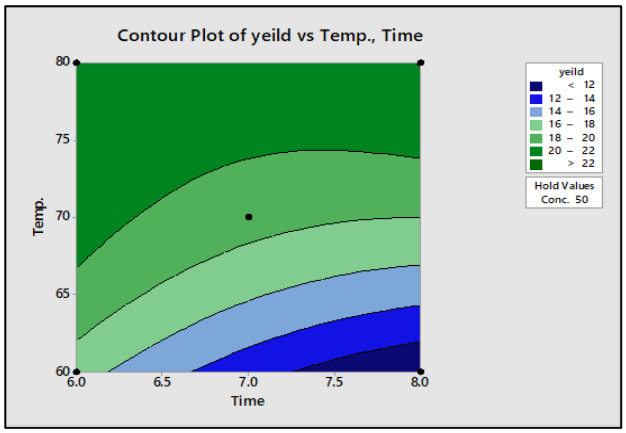

Figure 2: Contour plot of yield v/s temp and time

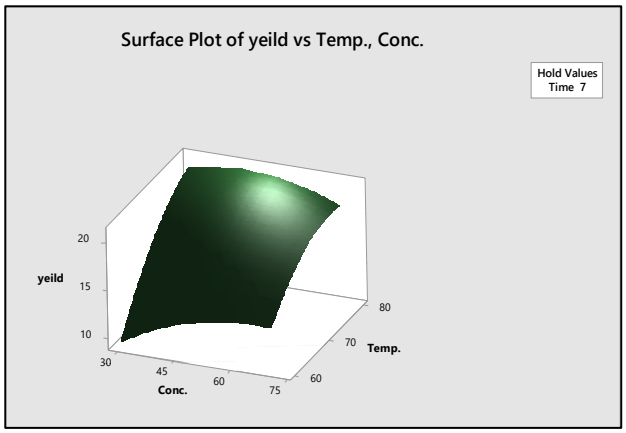

Figure 3: Surface plot of yield v/s temp and concentration

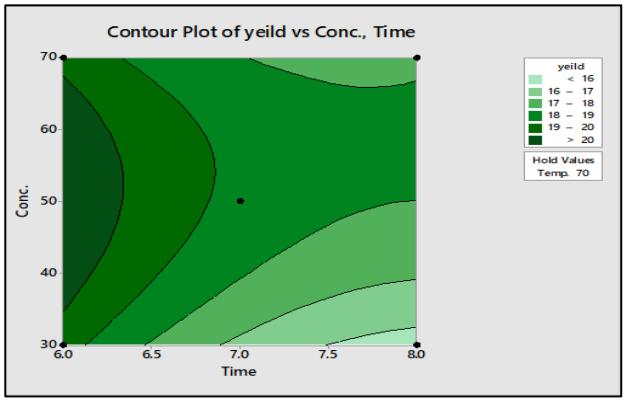

Figure 4: Contour plot of yield v/s concentration and time

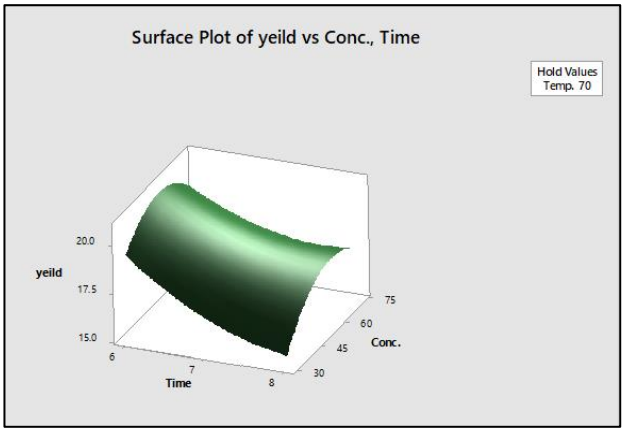

Figure 5: Surface plot of yield v/s concentration and time

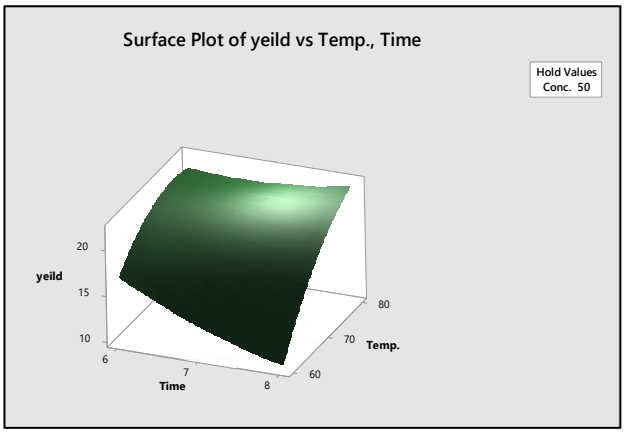

Figure 6: Surface plot of yield v/s temp and time

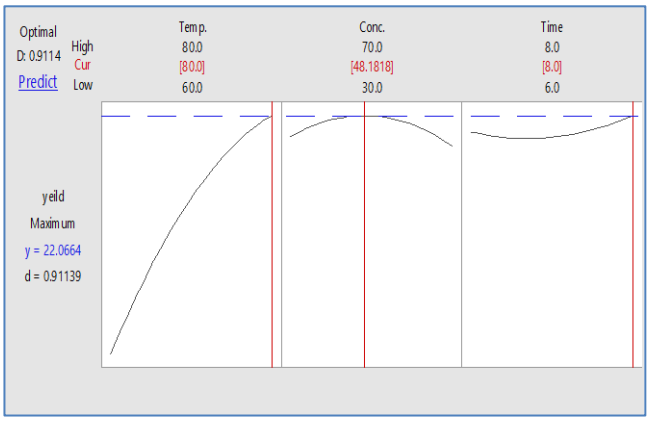

Figure 7: Optimization plot of yield v/s temp, time and concentration for *Adusa* leaf extract

Conflicts of interest

There are no conflicts of interest

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