STATISTICAL OPTIMIZATION OF AQUEOUS LEAF EXTRACT OF *AERVA LANATA* FOR CITRININ AND FUNGAL BIOMASS REDUCTION IN SUBMERGED FERMENTATION BY *ASPERGILLUS NIGER* USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Citrinin, a nephrotoxic mycotoxin produced by several fungal strains belonging to the genera *Penicillium*, *Aspergillus*, and *Monascus*. Generally found in stored grains and after their harvest. The objective of the present investigation was to study the antimicrobial activity (anti-fungal) of aqueous leaf extract of *Aerva lanata* and to optimize its conditions for the maximum inhibition of citrinin and fungal biomass by *Aspergillus niger*. Optimization of culture conditions was carried out using Box-Behnken method of response surface methodology. Extent of inhibition of citrinin was carried out using HPLC and reduction in fungal biomass was carried out using dry cell weight after comparing with controls. Optimized culture conditions as per the point prediction tool were found to be 11.27 mg/L for concentration of *Aerva lanata* extract, nine and half days of incubation period and temperature of 25.5 °C resulted in maximum inhibition of citrinin. These optimized values of tested parameters were and compared with control citrinin production (243.28 mg/L) and dry cell weight production (362.28mg).An average of 87.77±1.21% inhibition of citrinin and 80.02±1.42% of dry cell weight was obtained in an optimized medium at 9.5[th] d of fermentation with 97.82 % and 96.21% validity, respectively.

Keywords: *Aspergillus niger, Aerva lanata,* citrinin, fungal biomass, Box-Behnken design

INTRODUCTION

Mycotoxins, a group of structurally diverse secondary metabolites produced by various fungi, are toxic compounds. It contaminates various commodities of plant origin, cereals in particular, and is usually found together with another nephrotoxic mycotoxin, ochratoxin A (OTA). In addition to nephrotoxicity, citrinin is also embryocidal and fetotoxic **(Flajs and Peraica, 2009)**. Citrinin, a secondary metabolite of fungal metabolism was first isolated by Hetherington and Raistrick from a culture of *Penicillium citrinum* Thom **(Hetherington *et al*., 1931).** Several other fungal species within the three genera *Penicillium* **(Ei-Banna et al., 1987)**, *Aspergillus* **(Kurata, 1990)** and Monascus (Blanc *et al*., 1995; Li *et al*., 2003) were also found to produce citrinin **(Cigler *et al*., 1977; Bragulat *et al*., 2008)**. Due to its antibacterial potential, citrinin was used as an antibiotic [9]. Citrinin contaminates maize **(Nelson et al., 1985),** wheat, rye, barley, oats **(Scott et al., 1972),** and rice **(Tanaka et al., 2007).** Strains of *Monascus* are traditionally used in China to produce red and yellow pigments for food **(Xu et al., 1999).** Western countries limit the use of synthetic food colorants due to their toxicity and mutagenicity. Natural food pigments obtained from *Monascus* were good candidates for their substitution because reports on their toxic effects had been scarce for more than 1000 years.

A lanata belongs to the family Amaranthaceae, is one of the important medicinal plant that grows in the various parts of India, Pakistan, China and Saudi Arabia. It has antimicrobial properties **(Annie *et al*., 2004)**. The plant is used as a remedy for diabetes mellitus, urinary calculi, hematemesis, bronchitis, nasal bleeding, cough, scorpion stings, fractures, spermatorrhea, to clear uterus after delivery and al so to prevent the lactation **(Siveen and Kuttan, 2011)**. *A. lanata* plant has many medicinal properties due to the presence of numerous secondary metabolites. Sitosteryl palmitate, hentria contane, β-sitosterol and its D-glucoside, α-amyrin and betulin were isolated from the whole plant **(Aiyar *et al*., 1973; Chandra and Sastry, 1990)**. The glycosides like kaempferol 3-rhamnogalactoside and kaempferol 3-(6′′p-coumaryl) O-glucoside along with alkaloids, saponins and sugar like fructose, galactose, rhamnose and sucrose and also minerals are reported to occur in the plant **(Afaq *et al*., 1991)**. A many number of research work concerning pharmacological, phytochemical, toxicological studies has been reported **(Annie et al., 2004; Siveen and Kuttan, 2011; Nevin and Vijayammal, 2003; Nevin and Vijayammal, 2005;**

Chowdhary *et al*., 2002; Vetrichelvan and Jegadeesan, 2002; Selvam *et al*., 2001). But more work needs to be done regarding its medicinal importance, taxonomic and ecological aspects. As the plant of *A. lanata* has immense potential as an antimicrobial due to the rich source of phytochemicals it possesses, therefore, such studies on biological activities particularly anti-microbial are recommended in various parts of the countries of Asian subcontinent where it grows.

Response surface methodology (RSM) is a statistical technique used for the development and optimization of complex processes **(Ferreira *et al*., 2007; Bezerra *et al*., 2008)**. It was selected and used to find the optimum conditions for maximum inhibition of citrinin and dry cell weight produced by fungus *A niger*. The technique has several advantages over conventional experimental or optimization methods in which one variable at a time is used. RSM provides a large amount of information and is more economical approach because; a small number of experiments are performed for monitoring the interaction of the independent variables on the response. In conventional optimization, the increase in the number of experiments necessary to carry out the research, leads to an increase in time and expenses as well as an increase in the utilization of reagents and materials for experiments. The equation of the model easily clarifies the effects for binary combinations of the independent variables. 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 that are prohibitively expensive or impossible to test because of physical constraints on experimentation **(Mason *et al*., 2003)**. Medicinal plants represent an important health and economic component of biodiversity. In the present investigation the Box Behnken design was selected and used to optimize the aqueous extracts of tested medicinal plant from Saudi Arabia on growth of *A niger* on citrinin production and fungal dry cell weight.

MATERIAL AND METHODS

Microorganisms and culture conditions

Citrinin-producing microorganisms namely *A niger* was obtained from the culture collection of the Microbiology Laboratory of King Khalid University Hospital, King Saud University, Riyadh; KSA. The fungal culture was

maintained on slants of potato dextrose agar medium at 4°C. The spore was suspended by growing the fungi on Petri dishes for 7 days at 25°C with potato dextrose agar (PDA) containing 50 mg l^{-1} of streptomycin. PDA powder (39.0 g) was dissolved in 1 L of distilled water, according to the product recommendations. Later Spores were harvested by adding 10 ml of sterilized distilled water on each plate. The spore suspension hence obtained was filtered using cheesecloth, and spores were counted using a haemocytometer and brought to a final concentration of 10^5 conidia/ml.

Collection and preparation of aqueous plant extracts

Literature survey was done and taxonomic studies of the herbarium specimens of the medicinal plants available at the National Herbarium of Saudi Arabia (RIYADH), and the herbarium of King Saud University (Pharmacy), was done by using long arm stereomicroscope. Leaves of the *A lanata* were collected and washed under tap water. Then the leaves were dried at 60°C in hot air oven for 5 days and ground to make a powder and passed through 20 mesh sieve. Ten grams of powdered leaves were made soluble with 100 ml distilled water at 200 rpm for 5 h at room temperature (**Razzak *et al*., 2009**). The remaining insoluble material was filtered by Whattman No.1 filter paper and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and again passed through 0.45 µm filter (Millipore) and stored at -20°C for further use.

Seed culture and fermentation

The culture of *A niger* was grown on potato dextrose agar (PDA) slants and spore suspension (4×10^6 spores per mL) was made in glycerol water solution (15 g l^{-1}). The seed culture media used in this study were glucose (20g l^{-1}), glycerol (30 g l^{-1}), peptone (8 g l^{-1}), NaNO$_3$ (2 g l^{-1}) and MgSO$_4$ (1 g l^{-1}) were dissolved in water-soluble extract of soybean meal. All fermentation experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of production media as per experimental design (**Ahmad *et al*., 2011**). Different concentrations of aqueous plant extract were added to liquid broth. 10 µl from suspension (4×10^6 spores per ml of *A niger*) was inoculated in each flask and kept in rotary shaker at 200 rpm. The control contained production medium and 10 µl of *A niger* suspension. The fungal mycelium was harvested by filtration by muslin cheese cloth to separate from liquid culture. The filtered biomass was then dried at 60°C for 24h and the dry weight of mycelium was determined. Filtrates were stored in 4°C for carrying out citrinin extraction. All experiments consisted of three replicates, and the averages were determined.

Extraction and quantification of citrinin

The filtrates obtained from above experiment were used for extraction and estimation of citrinin. Briefly, the citrinin was extracted thrice with chloroform (1:1 *v/v*). All the three extractions were pooled and concentrated in vacuum at 40°C using a rotary evaporator. All the concentrates were then diluted in mobile phase (2ml) and citrinin was estimated by HPLC. All the samples were filtered through a 0.22µm disposable syringe filter (Micro Filtration Systems®) prior to injection into the chromatograph. Aliquots (30 µl) were injected on HPLC column and analysis were carried out using a Shimadzu® Liquid Chromatograph, equipped with an LC-20AD pump, a Rheodine® injector, an SPD-20A UV detector, a CBM-20 A-Communications Bus Module, and a LC Solutions Workstation system. A reverse-phase Atlantis® dC 18 column (150 × 3.9 mm, 5 µm) was used, at room temperature. The mobile phase used was acetonitrile-water (75:25 *v/v*) and formic acid (2%) with a flow rate of 1.5 ml/min for an isocratic run of 10 min. Absorbance of samples and standard was detected at 360 nm (**Abramson *et al*., 1999**). Retention times and peak areas were calculated by LC Solutions software. Evaluation of sample retention times with that of the standard identified the presence of citrinin in the samples. The relationships between peak area and the amount injected were linear over the ranges 2.5-50 µg.

OPTIMIZATION OF EXPERIMENTAL CONDITIONS (RESPONSE SURFACE METHODOLOGY)

Box–Behnken experimental design.

A response surface statistical experimental design was used to optimize the concentration of extract, incubation days and temperature. This design was based on a 3^3 factorial design, three replicates of the central run, leading to 15 sets of experiments, enabling each experimental response to be optimized. The responses were investigated using a Box–Behnken statistical experimental design. The optimization process involves evaluating the response of the statistically designed combinations, estimating the coefficients by fitting the experimental data to the response function, predicting the response of the fitted model, and checking the adequacy of the model. All experiments were performed in standard order to minimize the effects of uncontrolled factors that may introduce a bias in the response. Before starting an optimization procedure, it is important to identify the crucial factors affecting the quality of the derived outcomes. The levels of the three factors evaluated in this design are listed in (Tab 1). A three factor, three-Level Box–Behnken design was used for the optimization procedure, using the software Design Expert V 8.0.7.1. All other factors, for example volume of spore suspension, pH of the broth etc were maintained constant. The quality of the fitted model was expressed by the coefficient of determination R^2, and its statistical significance was checked by an *F*-test (analysis of variance) at the 5% significance level. The optimum processing conditions were obtained by using graphical and numerical analysis based on the criteria of the desirability function and the response surface. The experiment was finally repeated under the optimum values as per the point prediction tool of response surface methodology for concentration of extract, incubation period and temperature which should result in maximum inhibition of citrinin and dry cell weight production. These optimized values of tested parameters were validated (n=6) and compared with control for citrinin and dry cell weight inhibition.

Table 1 Levels of tested parameter for Box-Behnken design

| Independent factors	Unit	Symbol	Levels	
			Low	High
Extract Concentration	mg/L	A	2	16
Incubation Time	Days	B	8	24
Temperature	°C	C	20	30

RESULTS AND DISCUSSION

Design of the proposed assay and strategy for its development

The main aim of the optimization process was to maximize the response value in the extracts i.e., to optimize the most appropriate conditions for maximum inhibition of citrinin production and fungal biomass by using medicinal plant aqueous extract of *A lanata*. The suitability of the model equation for predicting the optimum response value was tested using the selected optimal conditions. The results for the three analyzed parameters and the maximum predicted yield and experimental yield citrinin and fungal biomass are given in Tab 2. The levels of inhibition of citrinin was assessed by HPLC in samples treated with extract of *A lanata* and the percentage of inhibition compared to the control shown in figure 1, similarly the inhibition in biomass was studied by dry cell weight and compared with control.

Table 2 The Box–Behnken Response surface design and corresponding response values

Runs	Factors			Citrinin (mg/L)		Fungal biomass (mg)	
	Extract Conc. (mg/L)	Incubation Days	Temperature (ºC)	Actual	Predicted	Actual	Predicted
1	2	8	25	2	0.875	70	65.125
2	16	8	25	0	3.125	40	35.375
3	2	24	25	250	246.875	370	374.625
4	16	24	25	110	112.875	160	164.875
5	2	16	20	190	193.125	280	277.25
6	16	16	20	145	142.125	205	202
7	2	16	30	210	212.875	340	343
8	16	16	30	137	133.875	176	178.75
9	9	8	20	1	0.75	54	61.625
10	9	24	20	175	175	270	268.125
11	9	8	30	2	2	68	69.875
12	9	24	30	185	185.25	310	302.375
13	9	16	25	145	145.3333	196	199.3333
14	9	16	25	142	145.3333	199	199.3333
15	9	16	25	149	145.3333	203	199.3333
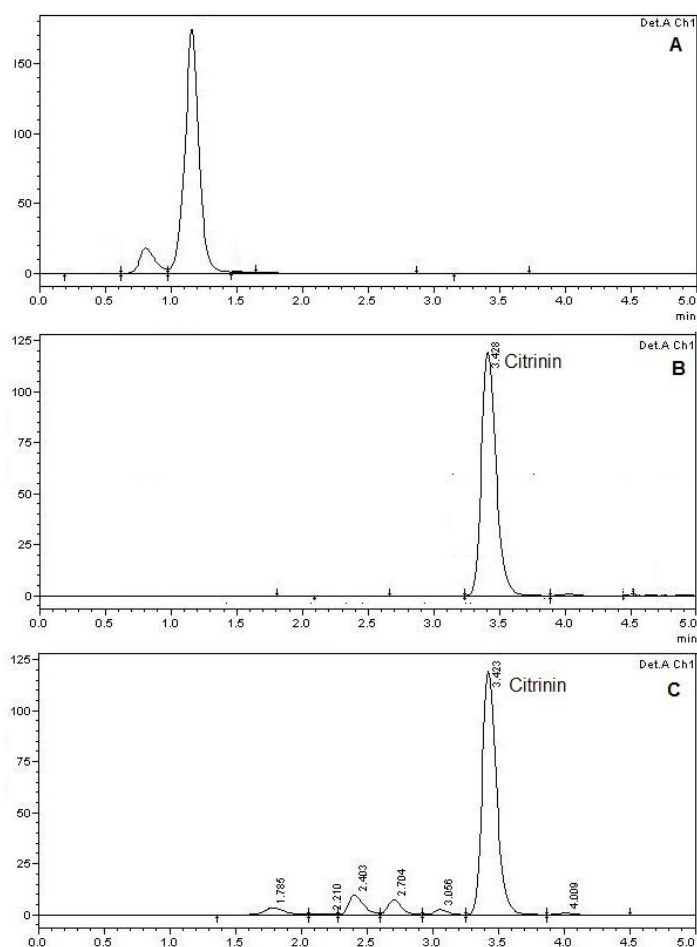
Figure 1 A typical HPLC chromatogram a) blank sample b) Standard citrinin (RT=3.42) c) fermented sample (RT=3.42)

Optimization of fermentation medium

The key parameters most influencing on the inhibition of citrinin and fungal biomass, *viz* concentration of plant extract, incubation days and temperature were studied. The results of experimental runs are summarized in (Tab 2). Data

collected from experimental runs were analyzed by using the software Design Expert V 8.0.7.1 and fitted to nonlinear quadratic models for citrinin inhibition and fungal biomass analysis. The model was validated by analysis of variance (ANOVA). The statistical analysis showed that the model represents the phenomenon quite well and the variation of the response was correctly related to the variation of the factors (Tab 3). In general, exploration and optimization of a fitted response surface may produce poor or misleading results, unless the model exhibits a good fit, which makes checking of the model adequacy essential **(Liyana-Pathirana and Shahidi, 2005)**. The F-ratio in this table is the ratio of the mean square error to the pure error obtained from the replicates at the design center. The significance of the *F*-value depends on the number of degrees of freedom (DF) in the model and is shown in the *P*-value column (95% confidence level). Thus, the effects lower than 0.05 are significant **(Morelli *et al.*, 2012)**. The *P*-value is used as a tool to check the significance of each coefficient and the interaction strength between variables **(Karacabey and Mazza, 2010)**. The higher the significance, the better the degree of correlation between the observed and predicted values **(Ravikumar *et al.*, 2006)**. Total extract concentration, incubation days and temperature content were significantly affected.

Table 3 Analysis of variance of calculated surface quadratic model for citrinin and fungal biomass

Parameters	Citrinin	Biomass
Model (Mean Square)	96302.81	154863.51
Residual	96.91	271.41
R^2	0.999	0.999
F	552.03	316.98
p-value	< 0.0001	<0.0001
± SD	4.40	7.36
RSD	3.58	3.75
*Adequate Precision	68.92	56.39

(*Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. This model can be used to navigate the design space).

An experimental design of 15 runs containing 3 central points was made according to Box-Behnken of response surface methodology to optimize these medium parameters. The individual and interactive effects of these parameters were studied during fermentation. The response was measured in terms of actual factors of inhibition of citrinin and dry cell weight. Data collected from experimental runs were analyzed by using the software and fitted to nonlinear quadratic models for citrinin and dry cell weight inhibition. The regression analysis was carried out to fit mathematical models to the experimental data aiming at an optimal region for the responses studied. Predicted response for the yield of each response could be expressed by the following polynomial quadratic equation in terms of actual values: The significance of each coefficient is listed in Table 4. The fitted model equation is:

Citrinin inhibition (mg l^{-1}) $=-48.0841 + 5.637A + 48.95B - 23.94C - 0.616AB - 0.2AC + 0.05625BC + 0.25A^2 - 1.05B^2 + 0.51C^2$.

Biomass (mg) $= +441.250 + 13.69A + 45.4092B - 61.170C - 0.803 AB - 0.63571AC + 0.16250BC + 0.36139A^2 - 0.89128B^2 + 1.32833 C^2$

Table 4 Regression coefficients and their significance in the quadratic model

Terms	Citrinin		Biomass	
	Coefficient Estimate	*P-value*	Coefficient Estimate	*P-value*
Intercept	145.33	< 0.0001	199.33	< 0.0001
A- Extract Conc.	32.5	< 0.0001	59.87	< 0.0001
B- Incubation Time	89.37	< 0.0001	109.75	< 0.0001
C- Temperature	2.87	0.1240	10.62	0.0096
AB	34.5	< 0.0001	45	< 0.0001
AC	7	0.0245	22.25	0.0018
BC	2.25	0.3536	6.5	0.1379
A^2	12.45	0.0029	17.70	0.0057
B^2	67.29	< 0.0001	57.04	< 0.0001
C^2	12.70	0.0026	33.20	0.0003

This multiple nonlinear model resulted in three response surface graphs each for citrinin and dry cell weight. Point prediction tool of the software was used to calculate maximum inhibition of citrinin and dry cell weight. Finally the optimum values as per the point prediction tool were found to be 11.27 mg/L for concentration of extract, nine and half days of incubation period and temperature 25.5°C which resulted in maximum inhibition or minimum production of citrinin (33.51 mg/L) and dry cell weight production (72.36 gm). These optimized values of tested parameters were validated under similar conditions (n=6) as discussed in material and methods and compared with control citrinin production (243.28mg/L) and dry cell weight production (362.28mg). An average of 87.77±1.21% inhibition of citrinin and80.02±1.42% of dry cell weight was obtained in an optimized medium at 9.5th d of fermentation with97.82% and 96.21% validity, respectively.

The relationship between independent and dependent variables is illustrated in three-dimensional representation of the response surfaces and one factor plots generated by the models for citrinin and biomass (Figure 2). Figure 2A and B shows the effect of extract concentration and incubation days on citrinin and fungal dry cell weight at a constant temperature 25°C. It is apparent from the figure that citrinin and fungal dry cell weight inhibition increased with increasing extract concentration and number of incubation days. The effect of temperature and concentration of extract in figure 2C and D shows on inhibition of citrinin and fungal dry cell weight at fixed incubation period of 16 days. Fig 2E and F shows the effect of temperature and incubation days of citrinin and fungal dry cell weight at fixed concentration plant extract (9 mg/L). The effect of individual factors for extract concentration, incubation days and fermentation temperature of both fungal and fungal dry cell weight is evident from the figures 3AI, AI1; BI, BI1and CI, CI1 that both these variables have direct effect citrinin concentration and fungal biomass. The results of analysis confirmed that the response model was adequate for reflecting the expected optimization, and the model was satisfactory and accurate.
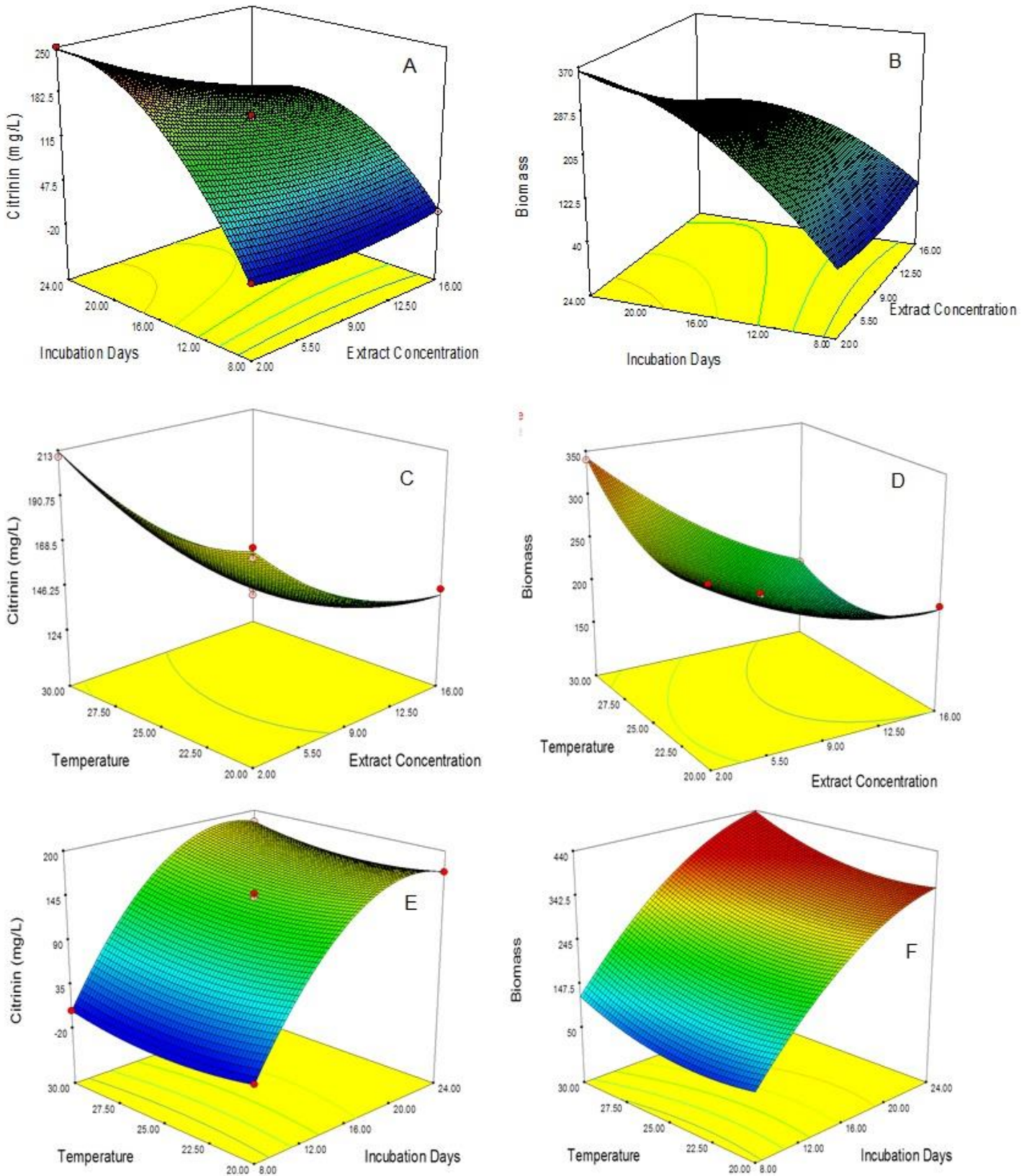
Figure 2 Response-surface graphs showing the effect of extract concentration, incubation time and temperature on the responses: (AI and A1I) Relative effect of extract concentration and incubation time on citrinin and biomass production, respectively at constant temperature. (BI and B1I) Relative effect of temperature and extract concentration on citrinin and biomass production, respectively at constant concentration of extract.
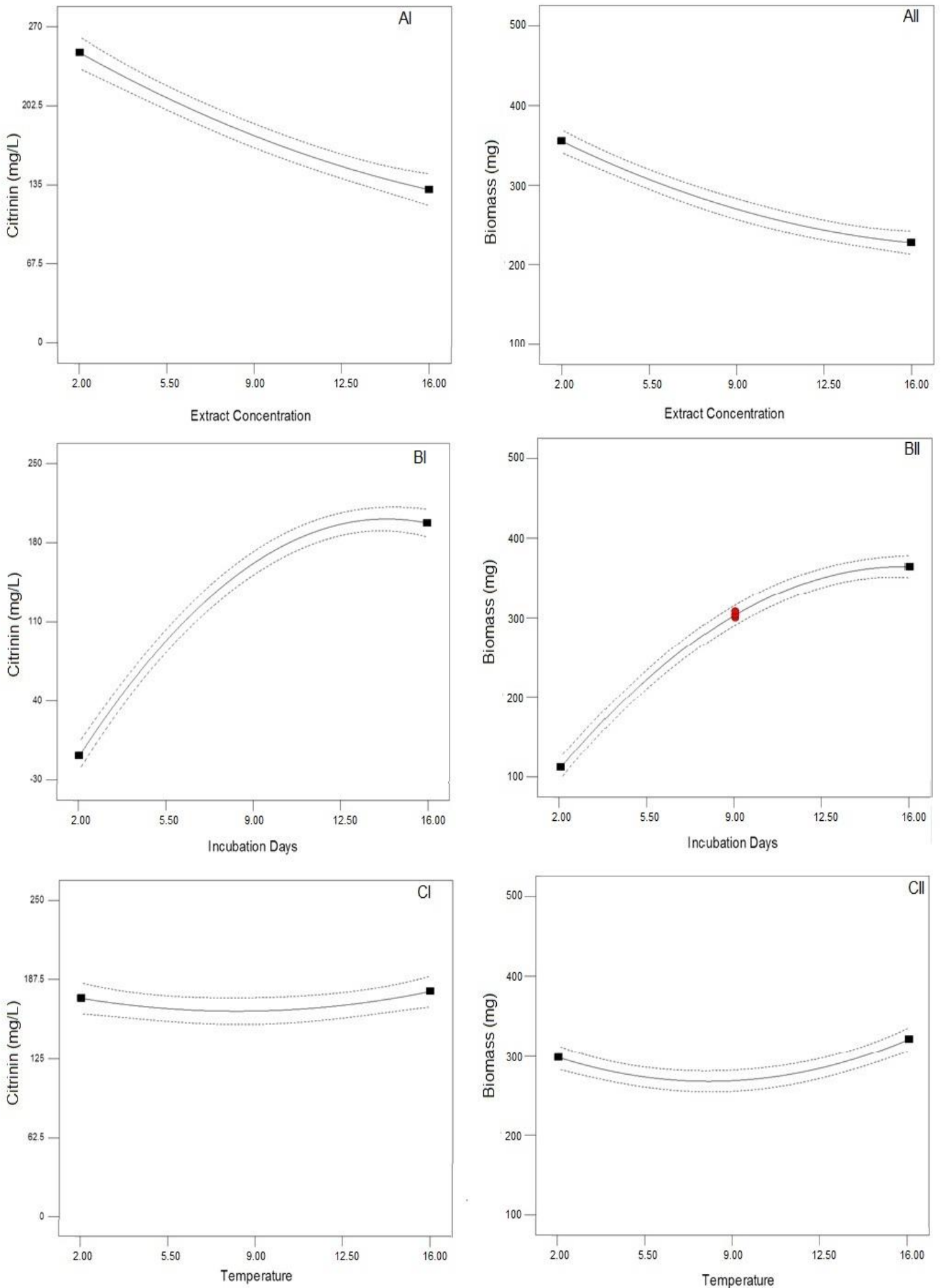
Figure 3 Effects of individual factors extract concentration (AI and AII), incubation time (BI and BII) and temperature (CI and CII) on citrinin and biomass production

CONCLUSION

The present research study was carried out to identify the antimicrobial activity of natural products for inhibition of mycotoxins in food grains. The antifungal potential of the *A lanata* was studied for the first time for preservation of food grains from production of citrinin, a known mycotoxin. Citrinin is a proven hepato-nephrotoxic agent and hence need to be inhibited. The effect of individual variables on inhibition of citrinin and fungal dry cell weight was also studied using Box-Benkhen response surface methodology. Optimum conditions for maximum inhibition of mycotoxin citrinin were also developed. The results obtained using response surface predictions were in good agreement with the experimental results. Therefore, Box–Behnken statistical design used in determining the optimum experimental conditions such as extract concentration, incubation days and temperature was reliable and effective. Hence extract of *A lanata* can be used as a preservative in food grains for the management of pest control and prevention of any toxic fungal secondary metabolite because of its potential antimicrobial activity. Present investigation also offers the possibility of developing methods for monitoring citrinin production by fungi coupled to novel environmentally safe agrochemicals of plant origin.

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