Box-Behnken designed Adsorption based elution – Unique separation process for commercially important acetyl shikonin from *Arnebia nobilis*.

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Supplementary Information

Experimental methods

Plant material and Isolation of compounds

Root bark of *Arnebia nobilis* was obtained from local market in Thanjavur, India. The plant was authenticated by Dr.Jayendran, Department of Botany, Government Arts College, Ootacamund, India. A voucher specimen (JDB1425) was deposited in Government Arts College, Ootacamund, India. The acquired root bark was ground to fine powder extracted using Hexane at room temperature for 24 h and repeated thrice with the residue. The extract was filtered through Whatman No.1 filter paper, and then all the filtrates were pooled up successively and concentrated under vacuum by Rotary evaporator (Buchi® Rotavap R-210). The total amount of pigments present in extract was determined by High Performance thin layer chromatography (HPTLC).

Adsorption studies

The efficiency of the developed separation technique (ABAE) was checked based on series of preliminary experiments and the whole process was optimized for maximum recovery of pure Acetyl shikonin.

Selection of adsorbent

Two grades of Commercial Activated carbon (AC) (Sigma-Aldrich) (A: Particle size: 200-325, B: 100-325; A: Surface Area: 750 m^2/g, B: 600 m^2/g) were used for the experiments and the grade that separated the acetyl shikonin with maximum recovery and purity was used for further studies. Since, plant extract is insoluble in water, various ratio of Isopropanol/water (v/v) were considered for solubilizing the extract maintaining the lowest concentration of Isopropanol possible which solubilized the extract completely. 20 ml of 30% isopropanol in water (v/v) containing 500 mg of plant extract was added to AC (10 g/l) and kept for stirring at 100 rpm until adsorbent reached saturation analysed by HPTLC. The time to reach saturation was recorded. The desorption rate and recovery of adsorbates were later observed by addition of 100% isopropanol at 100 rpm. The rate of desorption was noted and amount of compounds recovered after complete desorption was also recorded.

Isotherm Studies

Hexane extract (HE) of *A. nobilis* (1000 mg/ l) in 30% isopropanol in water (v/v) was prepared and appropriately diluted to the required initial concentrations. The initial concentrations (C_i) of HE and AS were obtained by HPTLC densitometric analysis. Precisely 50 ml of HE solution of known initial concentration (C_i: 50–1500 mg/l) was shaken at the constant agitation speed (100 rpm) with AC (10 g/l) for specific duration of 90 min in a thermostatic orbital incubator shaker (LABTECH, India). After equilibration, the final concentrations (C_e) were estimated again using HPTLC. A similar experiment under identical conditions was done with Pure Acetyl Shikonin (Standard) with initial concentrations (C_i: 50–750 mg/l). The isotherm models with single solute (AS) and multi-solute (HE) were compared and difference and adsorption capacities were observed. The various experimental conditions are given in Table 1. The amount adsorbed (in mg/g) were calculated using the following relationships

Amount adsorbed (q_e) = (C_i – C_e)/ m

where C_i and C_e are the initial and final concentrations (in mg/l) of dye, respectively and m is the mass of AC (in mg/l).

Affinity based Adsorptive Elution

500 mg of Hexane extract was solubilized in 100 ml of 30% isopropanol in water (v/v) and Activated carbon (10 g/l) (Particle Size: 200-325; Surface Area: 750 m^2/g) was added to the solution. The experiment was maintained at room temperature with agitation of 100 rpm for 90 min. After equilibration, excess solution was aspirated out and pure water was added. Separation of dye molecules was done by addition of increasing concentrations of isopropanol in water (10-100%). The contact time for desorption was maintained as 30 min. The dyes separated in different Isopropanol/Water ratio was analysed through HPTLC. The ratio which yielded pure Acetyl shikonin was used for modelling studies.

Classical optimization studies

Amount of Adsorbent needed for complete adsorption and selective desorption of AS was studied with varying amount of AC (2.5-12.5 g/l) at RT and 100 rpm agitation for 90 min adsorption and 30 min desorption. The minimum amount of adsorbent needed to completely separate pure AS was noted. Desorption time required to selectively separate AS from AC was studied by analysing the amount of pure AS desorbed by HPTLC at different desorption time (5-60 min). Experiments were done at 8.75 g/l of AC at RT at 100 rpm agitation. The time which yielded maximum amount of pure AS was recorded. With optimized values of AC (8.75 g/l) and desorption time (30 min), experiments were repeated to find exact ratio of Isopropanol/Water that yielded maximum amount of pure AS. Finally, Effect of agitation was studied in desorption patterns by maintaining the experimental sets containing AC (8.75 g/l) for 30 min at different agitation speeds (50-200 rpm). Since, agitation speed had no effect on yield and purity of AS but only affected time needed to completely desorb pure AS, agitation speeds with respect to time were recorded to reach desired yield of AS. The amount of HE was kept at 500 mg for all the above experiments. The optimized values were later used for Response surface modelling.

Response surface modelling

The influential parameters were identified from classical optimization experiments based on their effect on target response (yield and purity of AS). Consequently, only parameters such as Amount of Activated Carbon (AC), Desorption time and Isopropanol/water (3 factor) at 3 levels (-1, 0, +1) from their scanned range were considered for Box-Behnken method based experimental design to obtain the standard set of experiments for RSM based modelling and optimization. Stirring speed was maintained at 150 rpm and neutral pH for all the experiments. 3 factors and 3 levels Box-Behnken design generated 15 set of experiments/runs which were carried out with 3 replicates and the average is depicted in Table S1.To minimize the effects of inexplicable variability in the observed response due to inessential factors, the order of experiments were randomized. Experimental data thus obtained were fitted in second-order polynomial model and regression coefficients were determined as in Equation (1)

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \qquad ----- (1)$$

Where, Y is the predicted response, β_0 is the intercept and β_i, β_{ii}, β_{ij} are regression coefficients for linear effects, regression coefficients for squared effects, regression coefficients for interaction effects and X_i and X_j are the parameters, respectively.

A final run of experiment with the RSM optimized parameters was performed and the yield and purity of acetyl shikonin was analysed.

Characterization

Yield and Purity of AS obtained from experiments was analyzed by High performance thin layer chromatography (HPTLC). Commercial grade of AS was used as standard for the experiments. Amount of AS present in extracted sample was quantified by HPTLC densitometric analysis, which was performed on aluminum-backed plates (20 × 20 cm) coated with 0.2 mm layer of Silica gel 60 F_{254} (E-Merck, Germany). Sample application was done as 6 mm bands using CAMAG Automatic TLC Sampler 4 (ATS4) applicator (Switzerland) fitted with a CAMAG microliter syringe. Constant application rate of 150 nL/s was maintained. Linear ascending technique was used to develop the plates to a distance of 80 mm with pure chloroform as solvent system for mobile phase in CAMAG Automatic Developing Chamber 2 (ADC2), which was previously saturated with mobile phase vapor for 30 min at 25°C. Developed plates were scanned using CAMAG TLC scanner 4, and visualized under UV light at 254 nm and 365nm. The scanned images were later processed for densitometric analysis using VisionCATS v1.4.0. Concentration of 100 – 800 ng/spot was checked for linearity of both the compounds and concentration was plotted against peak area and concentration curve was obtained. Specificity of this method was confirmed by analysing and comparing the R_f values of spots of AS and standards. 1 mg of AS was ground along with 100mg of Potassium Bromide (KBr- pre-ground and desiccated at 500°C for 12hr) and the disc was prepared by Hydraulic pressure method. FTIR (Perkin Elmer) analysis was performed at wave number range of 400-4000 cm^{-1}.1H NMR and 13C NMR spectra were determined on a Bruker-400 NMR spectrometer and chemical shifts were expressed as part per million against TMS as internal reference. Optical Rotation values $[\propto]_D^{20}$ values were measured by Autopol IV (Rudolph Research, USA) and given in 10^{-1}deg cm^2 g^{-1}. Mass spectra were recorded on Agilent 1200 (Liquid Chromatography), Agilent 6320 (Quadrupole Mass Analyser) spectrophotometer.

Acetyl Shikonin (1): $C_{18}H_{18}O_6$; red powder; $[\alpha]_D^{20}$ = +456 (c 0.1, CHCl$_3$); mp: 104-106 °C; ESI-MS m/z = 329[M-1]$^-$; FTIR(v_{max}^{KBr}): 3401, 2364, 1742, 1613, 1231, 1050 cm^{-1}**H-NMR (400MHz, CDCl$_3$):** δ 12.56 (s, 1H, –OH), 12.40 (s, 1H, –OH), 7.19(s, 2H), 6.98 (d, J = 1.2 Hz, 1H), 6.03 (ddd, J = 1.2, 4.6, 7.2 Hz, 1H), 5.13 (t, J = 7.26 Hz, 1H), 2.59 (m, 1H), 2.46(m, 1H), 2.13 (s, 3H), 1.69 (s, 3H), 1.57 (s, 3H); **^{13}C-NMR (100MHz, CDCl$_3$):** δ 178.32, 176.82, 169.87, 167.61, 167.08, 148.35, 136.23, 132.84, 133.00, 131.84, 117.82, 111.70, 111.96, 69.65, 32.97, 25.88, 21.06, 18.06.

Statistics

Results obtained were expressed as the mean ± standard deviation of the replications. Results obtained in experimental run generated by Box-Behnken design was expressed as mean of replicates. Response surface methodology based model fitting and statistical analysis was performed using Design Expert (release 9.0.3.1; State-Ease, Inc., Minneapolis, MN, USA). An analysis of variance (ANOVA) was performed to determine the significant levels defined at $p < 0.05$, $p < 0.01$ and $p < 0.001$.
Extractions were performed in replicates at all points in the study design. The corresponding extracts were analyzed for the dependent variables (responses): amount of AS (Y_1). Mean values were analyzed using least-square regression and fitted to the generalized second-order polynomial model (Equation 1) to all of the dependent Y response variables [36]. Response surfaces plots were developed using reduced fitted polynomial models which allows the relationship between the experimental levels and the response of each factor to be examined and the optimum conditions to be recognized.

Table S1: Box Behnken design setting of the independent variables and experimental results for the amount of Acetyl shikonin.

Experiments	Factors			AS (mg/ g DM) (Y_1)
	Amount of Adsorbent (g/l) (X_1)	Desorption time (min) (X_2)	Isopropanol (%) (X_3)	
1	7.5 (-1)	35(1)	70(0)	3.2
2	8.75 (0)	35 (1)	60 (-1)	4.2
3	10 (1)	30 (0)	80 (1)	3.8
4	10 (1)	30 (0)	60 (-1)	3.4
5	8.75 (0)	30 (0)	70 (0)	5.1
6	10 (1)	25 (-1)	70 (0)	3.3
7	8.75 (0)	35 (1)	80 (1)	4.6
8	8.75 (0)	25 (-1)	60 (-1)	4.1
9	8.75(0)	25 (-1)	80 (1)	4.3
10	8.75 (0)	30 (0)	70 (0)	5.2
11	10 (1)	35 (1)	70 (0)	4.2
12	8.75 (0)	30 (0)	70 (0)	5.2
13	7.5(-1)	25 (-1)	70 (0)	3.7
14	7.5 (-1)	30 (0)	60 (-1)	3.4
15	7.5 (-1)	30 (0)	80 (1)	3.2

Values in parentheses are coded form of variables, DM- Dry Material

Table S2: Analysis of variance for response surface quadratic model

Source	AS				
	DF	SS	MS	F Value	P value
Model	9	7.17	0.8	149.36	<0.0001
A	1	0.18	0.18	33.75	0.0021
B	1	0.08	0.08	15	0.0017
C	1	0.08	0.08	15	0.0017
A^2	1	5.39	5.39	1010.82	<0.0001
B^2	1	0.47	0.47	88.89	0.0001
C^2	1	0.95	0.95	178.89	<0.0001
AB	1	0.49	0.49	91.87	0.0002
AC	1	0.09	0.09	16.87	0.0093
BC	1	0.135	0.135	1.87	0.02*

DF - Degrees of Freedom, SS – Sum of Squares, MS – Mean Square, Model issignificant at p<0.0001, * Significant at p<0.02. A- Amount of adsorbent (g/l), B- Desorption time (min), C- Isopropanol (%).

Table S3:Regression coefficients of the predicted second-order model for amount ofAcetyl shikonin

Model Parameters	AS	
	Regression Coefficient	S.E
Intercept	5.17	0.042
A	0.15	0.026
B	0.1	0.026
C	0.1	0.026
A^2	-1.21	0.038
B^2	-0.36	0.038
C^2	-0.51	0.038
AB	0.35	0.037
AC	0.15	0.037
BC	0.05	0.037*
S.E	0.034	
R^2	0.99	
Adj-R^2	0.98	
CV %	1.8	
Adeq Precision	32.98	

S.E – Standard error, R^2 – Coefficient of multiple determinations, C.V % – Percentage of Coefficient of variance.Model issignificant at p<0.0001, * Significant at p<0.02.A- Amount of adsorbent (g/l), B- Desorption time (min), C- Isopropanol (%).

Table S4: Optimum conditions obtained from RSM and one variable at a time methods

	Variable name	Optimum values obtained	
		Response Surface Modeling	One variable at a time
X_1	Amount of adsorbent (g/l)	8.93	8.75
X_2	Desorption time (min)	31	30
X_3	Isopropanol (% in water [v/v])	72	70
X_4	Agitation (rpm)	NC	150
	Predicted Values* (mg/g DM)	5.26 ± 0.2	-
	Observed values**(mg/g DM)	5.25 ± 0.3	5.2

*Mean ± 95% Confidence interval, ** Mean ± Standard deviation (n=3). NC- not considered for optimization.
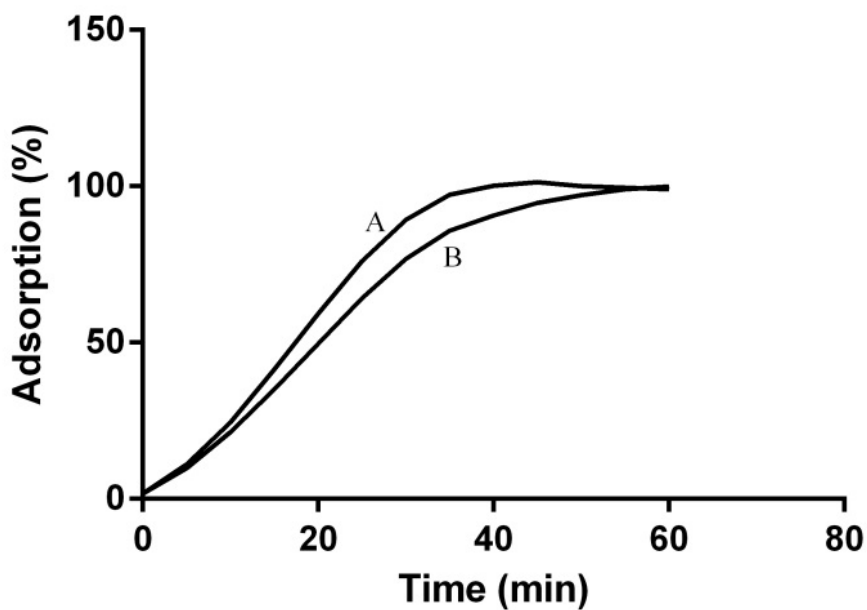
Fig. S1 Adsorption efficiency based on type of adsorbent, commercial Activated Carbon (AC) (Sigma-Aldrich) A: Particle size: 200-325, Area: 750 m^2/g B: Particle size: 100-325; Area:600 m^2/g.
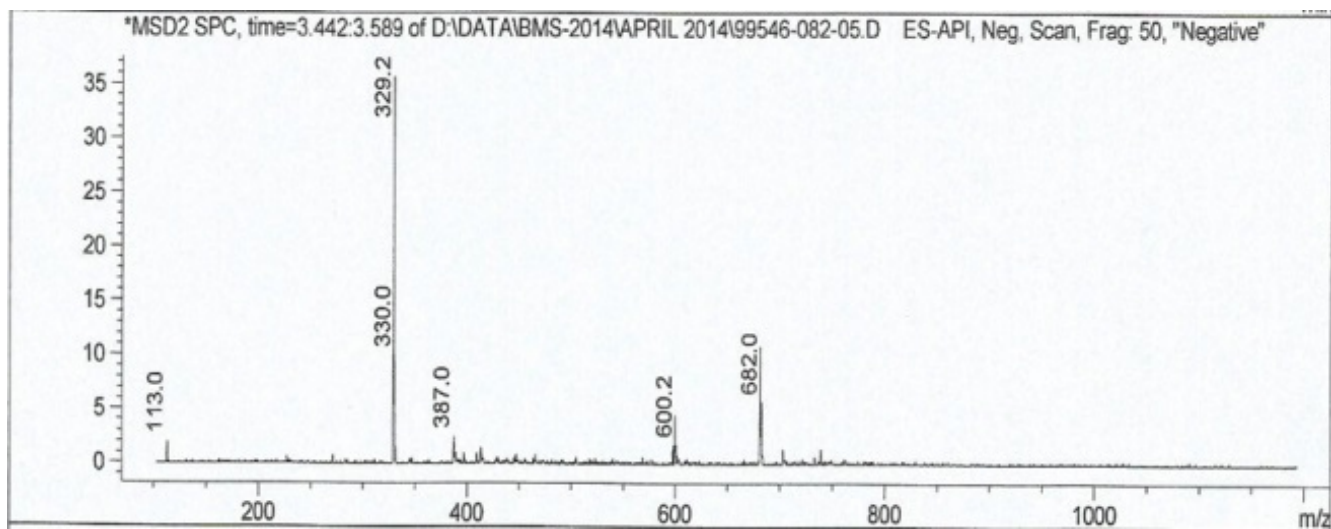
(a)

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------------------------------------------------------------------------------------
Data file        :  D:\DATA\BMS-2014\APRIL 2014\99546-082-05.D
Vial No.         :  P2-B-04
Injection Date   :  25-Apr-14              Time:    17:57:04
Injection vol    :  1              uL
Sample Name      :                                              LCMS-2
Acq Method       :  D:\METHODS\KIN-5MIN-NH4HCOOH.M
------------------------------------------------------------------------------------
Method info   :  Column-Ascentis Express C18 (50X2.1mm-2.7µm )
                 Mphase   A   :   10mM  NH4COOH IN WATER:ACN(98:02)
                 Mphase   B   :   10mM  NH4COOH IN WATER:ACN(02:98)
                 Flow = 1ML/MIN
                 Time             %B
                 0.0              0.0
                 1.5              100.0
                 3.2              100.0
```


| Peak | RT | Area | Area |
No	min		%
1	2.105	1.730e+001	2.373
2	3.470	7.117e+002	97.627

(b)

Fig S2 Liquid Chromatography – Mass spectroscopy analysis of AS obtained from RSM optimized Adsorption based elution. (a) LC chromatogram and the Purity % (b) Mass spectra of AS.
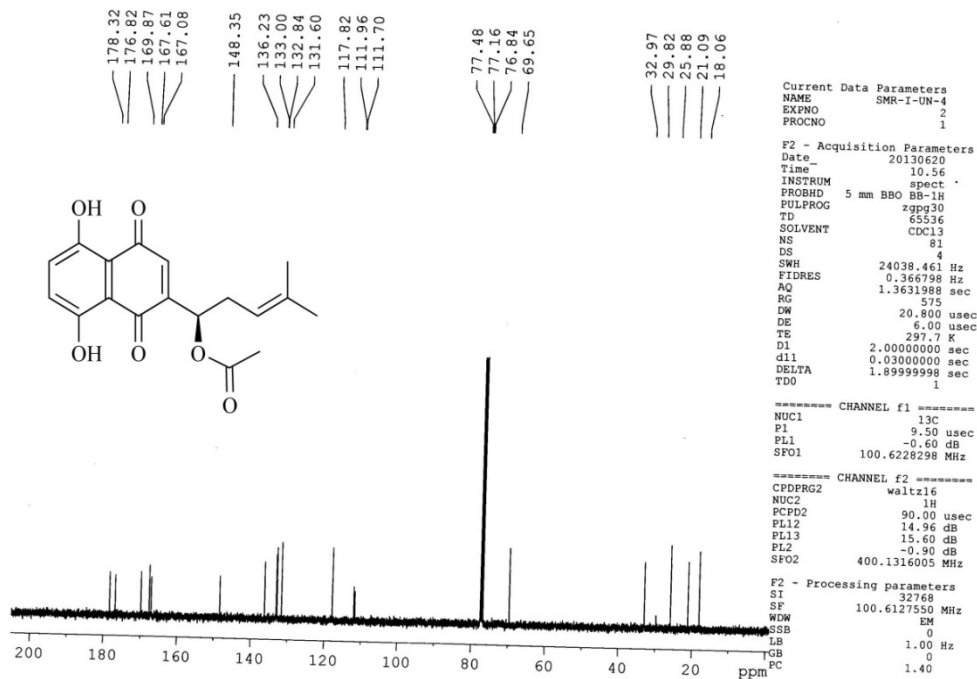
(a)
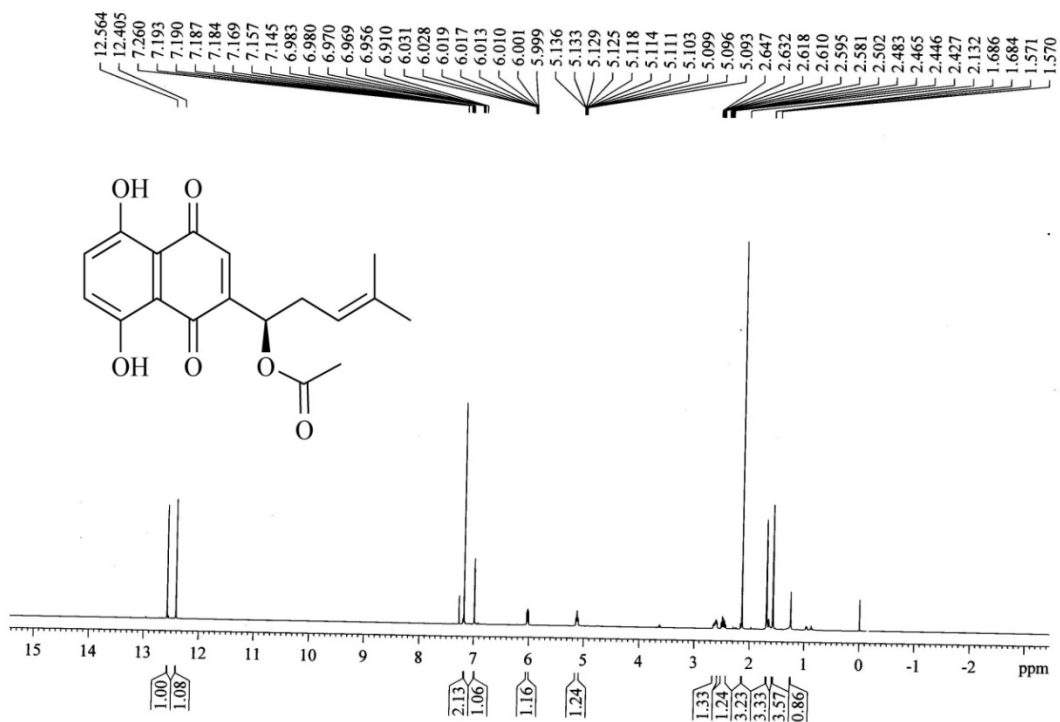
(b)

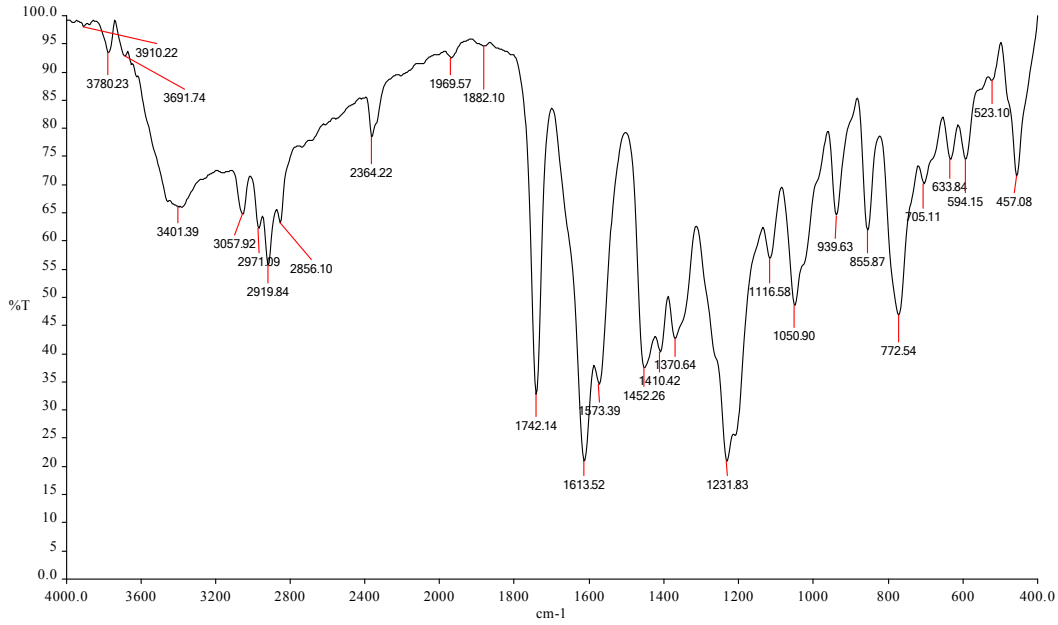**Fig S3** Nuclear Magnetic Resonance spectra of AS (a) Proton NMR (b) Carbon NMR

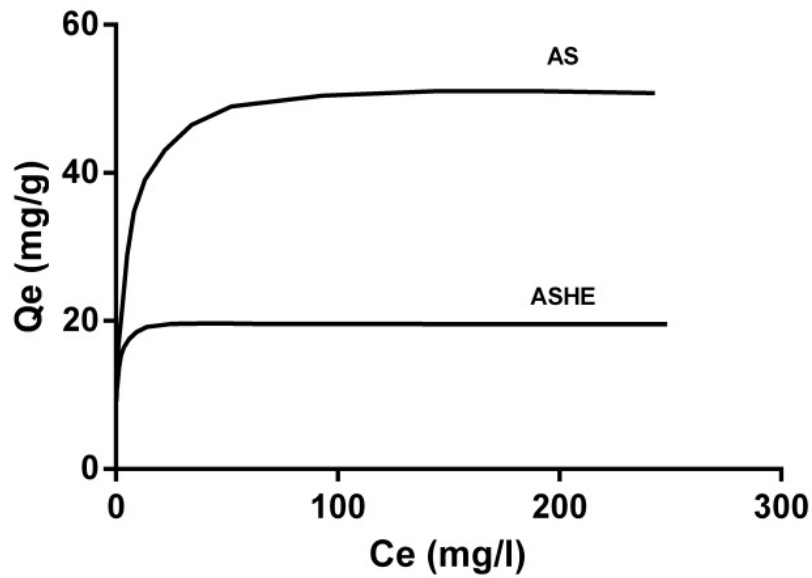**Fig S4** Fourier Transformed Infrared Spectra (FT-IR) of AS

Fig. S5 Equilibrium adsorption isotherms for adsorption of Acetyl Shikonin (AS) and Acetyl shikonin containing Hexane extract (ASHE) onto Activated Carbon, C_e – Final concentration of Dye in solution after adsorption (mg/l), Q_e – Amount of dye adsorbed by adsorbent (mg/g).
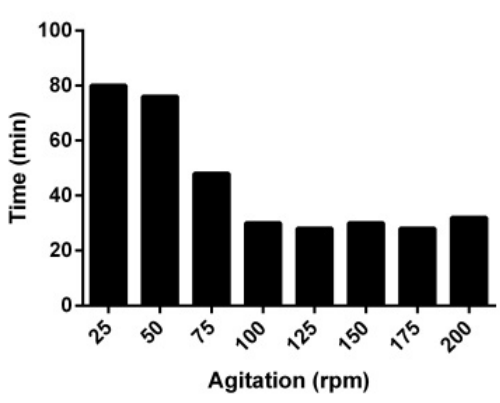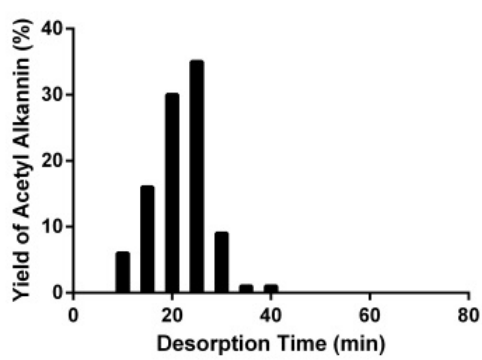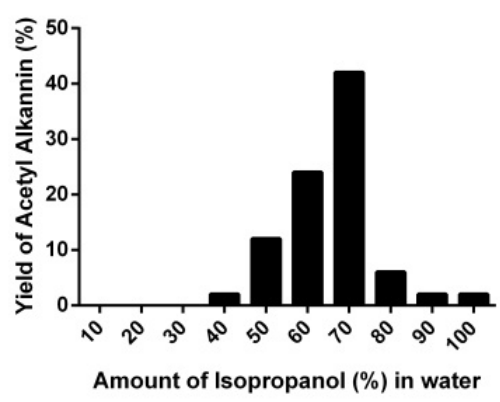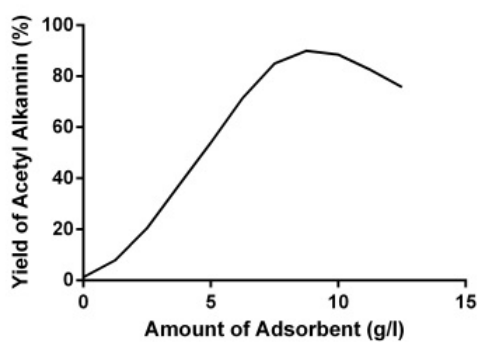
Fig. S6 Effect of different parameters on separation of acetyl shikonin, Amount of Adsorbent, Isopropanol/water ratio and Time had significant effect on Yield and purity of AS, whereas agitation only affected the desorption time.