A unique solvent assisted 'green' hydrotropic precipitation and response surface optimization for isolation of the dietary micronutrient β-Sitosterol -D- glucopyranoside from *Desmostachya bipinnata.*

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Electronic Supplementary Information

Experimental Methods

Chemicals and plant material

Roots of *D. bipinnata* were obtained along the river beds of River Cauvery in and around Thanjavur, India. The plant was authenticated by Dr. Jayendran, Department of Botany, Government Arts College, Ootacamund, India. A voucher specimen (JDB1422) after matching with authenticated herbarium sample RHT22739 (RAPINAT Herbarium, Trichy, India) was deposited in Government Arts College, Ootacamund, India. The roots were separated from aerial parts, shade dried and ground to semi-coarse powder and directly used for hydrotropic extraction. Sodium p-toluenesuphonate (Na-PTS) was purchased from Sigma-Aldrich, India. and Sodium cumene sulphonate (Na-CS) from Navdeep chemicals Ltd., Mumbai. Sodium salicylate (Na-Sal) was purchased from Merck Ltd., India. Pure β-Sitosterol-D-glucopyranoside (BSG) was obtained from Sigma-Aldrich Chemicals, India.

Analytical methods

Purity of the product obtained from hydrotropic experiments was analysed by High performance thin layer chromatography (HPTLC). Commercial grade of BSG was used as standard for the experiments. Amount of BSG present in extracted sample was quantified by HPTLC densitometric analysis, which was performed on aluminium-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 solvent system (Hexane-Ethyl Acetate 1:1) as 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 BSG and standards. 1H NMR and 13C NMR spectra were determined on a Bruker-300 NMR spectrometer and chemical shifts were expressed as part per million against TMS as internal reference. Liquid Chromatography observations were recorded on Agilent 1200 HPLC instrument.

Solubility studies

Solubility of Pure β-Sitosterol-D-glucopyranoside (Sigma-Aldrich®) (Fig.1) was studied in various concentrations of hydrotropic solutions of Na-PTS, Na-CTS, and Na-Sal. The solubility experiments were carried out in cylindrical glass vessel (50 ml) fitted with a six-bladed turbine impeller (i.d: 2 cm). Pure BSG was suspended in these vessels containing aqueous hydrotropic solutions at various concentrations (0.5-2.0 mol). Excess amount of BSG was equilibrated in these solutions and kept under vigorous stirring for 5 h at 35°C. After 5 h, the solution was allowed to settle and the clear solution was aspirated to analyse the amount of BSG solubilized. Also, the stability of BSG at different temperatures were studied and subsequently used in defining the optimum temperature range for extraction of BSG.

Effect of Solvent on Hydrotropic extraction

Solvents miscible with Na-PTS solution were used to initiate precipitation of desired compound in extracted solutions. Studies were performed with acetone, methanol, ethanol and acetonitrile on their efficiency in precipitation of BSG compared to water. Purity of precipitated BSG was also analysed at each experiment.

Hydrotropic precipitation

For hydrotropic extraction of BSG, roots of *D. bipinnata* were suspended in a completely baffled cylindrical glass vessel (500 ml) fitted with six-bladed turbine impeller (i.d: 2 cm). The concentration of hydrotrope varied according to optimization experiments. The suspension was vigorously agitated at 1000 rpm for 3 h. Samples were withdrawn at definite time intervals and analyzed for the BSG content. At the end of extraction, the clear solution containing the metabolites was filtered under vacuum. A slight yellow color filtrate was obtained. The insoluble sticky residue was washed with 10 ml of hydrotropic solution, filtered and mixed with the filtrate. The filtrate was diluted with pure water to bring down the concentration of hydrotrope to Minimum hydrotropic concentration (MHC), which afforded to produce precipitate. The precipitate was filtered, washed and analysed for amount and purity of BSG.

For solvent assisted hydrotropic precipitation (SAHP), water was replaced with solvents to dilute the hydrotropic filtrate which afforded to give precipitates which were later analysed for amount and purity of BSG.

Studies on influential parameters

Before implementing a standardized experimental design protocol and progression of the study by RSM based modelling, maiden set of tests were performed by following the classical "one variable at a time" optimization approach to roughly select the applicable factors and their range extraction process. Parameters such as concentration of hydrotrope (X_1), temperature (X_2), solid loading (X_3), time of extraction (X_4), agitation (X_5) and amount of solvent (X_6) for induction of precipitation were studied

for their influence on final yield and purity of BSG. The experiments were conducted separately in fully baffled glass container (50 ml) with impeller (i.d: 2cm) and the parameters were identified for the possible influence. Most of the influential experimental parameters which increased yield of BSG obtained were analysed by HPTLC and were further considered for systematic experimental design to find the optimum parameter, set through RSM procedure.

Response surface methodology

The influential parameters were identified from preliminary experiments based on their effect on target response (yield of BSG). Consequently, only parameters such as Concentration of hydrotrope (mol), Temperature (°C) and Solid loading (%) (3 factor) and 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. 3 factors and 3 levels Box-Behnken design generated 15 set of experiments/runs which were carried out with 2 replicates and the average is depicted in Table 1. 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).

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

Where, Y is the predicted response factor, 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.

Statistical analysis

Results obtained were expressed as the mean ± standard deviation of the replications. Results obtained in experimental run generated by Box-Behnken design were 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 analysed for the dependent variables (responses): amount of BSG (Y_1). Mean values were analysed using least-square regression and fitted to the generalized second-order polynomial model (Equation 1) to all of the dependent Y response variables. 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 β-Sitosterol-D-glucopyranoside (BSG).

Experiments	Factors			BSG(mg/ g DM) (Y_1)
	Concentration of hydrotrope (mol) (X_1)	Temperature (°C) (X_2)	Solid loading (%) (X_3)	
1	1.5 (0)	55(1)	5 (-1)	8.4
2	1.5 (0)	45 (0)	10 (0)	9.1
3	2 (1)	35 (-1)	10 (0)	6.3
4	1.5 (0)	55 (1)	15 (1)	7.4
5	1.5 (0)	35 (-1)	15 (1)	7.2
6	2 (1)	45 (0)	15 (1)	8.3
7	1.5 (0)	45 (0)	10 (0)	9
8	1.5 (0)	45 (0)	10 (0)	9.1
9	2 (1)	45 (0)	5 (-1)	8.1
10	2 (1)	55 (1)	10 (0)	8.5
11	1.0 (-1)	45 (0)	15 (1)	8
12	1.0 (-1)	45 (0)	5 (-1)	8.1
13	1.5 (0)	35 (-1)	5 (-1)	6.2
14	1.0 (-1)	35 (-1)	10 (0)	7.2
15	1.0 (-1)	55 (1)	10 (0)	7.1

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

Table S2: Analysis of variance for response surface quadratic model

Source	BSG			
	DF	SS	MS	F Value
Model	9	12.23	1.36	281.13
A	1	0.08	0.08	16.55
B	1	2.53	2.53	523.71
C	1	0.06	0.06	0.26*
A^2	1	0.86	0.86	178.46
B^2	1	6.32	6.32	1307.64
C^2	1	0.78	0.78	160.48
AB	1	1.32	1.32	273.62
AC	1	0.022	0.022	4.66
BC	1	1	1	206.9

DF - Degrees of Freedom, SS – Sum of Squares, MS – Mean Square, All values are significant at 1%, * Not Significant. A- Concentration of hydrotrope (mol), B- Temperature (°C), C- Solid loading (%).

Table S3: Regression coefficients of the predicted second-order model for amount of β-Sitosterol-D- glucopyranoside

Model Parameters	BSG	
	Regression Coefficient	S.E
Intercept	9.07	0.04
A	0.1	0.025
B	0.56	0.025
C	0.012	0.025*
A^2	-0.48	0.036
B^2	-1.31	0.036
C^2	-0.46	0.036
AB	0.58	0.035
AC	0.075	0.035
BC	-0.5	0.035
S.E	0.032	
R^2	0.998	
Adj-R^2	0.995	
C.V.%	0.88	

S.E – Standard error, R^2 – Coefficient of multiple determinations, C.V – Coefficient of variance. All values are significant at 1%, * Not Significant.

Table S4: Analysis of variance for the lack of fit testing for β-Sitosterol-D- glucopyranoside

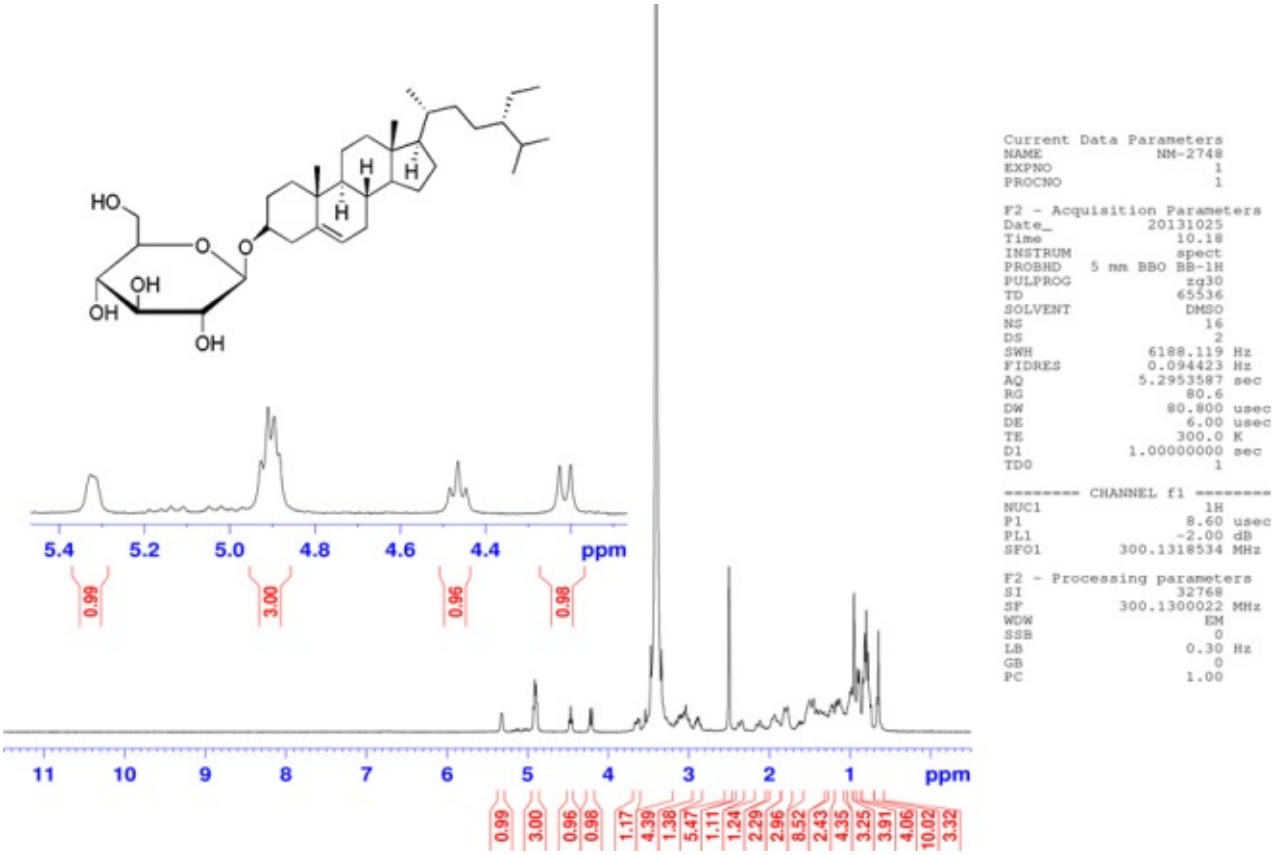
Source	DF	SS	MS	F Value
Lack of fit	3	0.18	0.29	1.75*
Pure error	2	0.33	0.17	
Total error	5	0.024	0.24	

DF - Degrees of Freedom, SS – Sum of Squares, MS – Mean Square, *Not significant

Table S5: 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	Concentration of hydrotrope (mol)	1.97	1.5
X_2	Temperature (°C)	49.5	45
X_3	Solid loading (%)	9.73	10
X_4	Extraction time (h)	NC	3
X_5	Agitation (rpm)	NC	1000
X_6	Amount of Acetone for precipitation (ml/ml of hydrotropic solution)	NC	2.8
	Predicted Values*	9.2 ± 0.05	-
	Observed values**	9.14 ± 0.03	-

*Mean ± 95% Confidence interval, ** Mean ± Standard deviation (n=3). NC- not considered for optimization. BSG- β-Sitosterol- D – glucopyranoside

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DE                    6.00 usec
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Fig. S1 Proton Nuclear Magnetic Resonance spectra of BSG

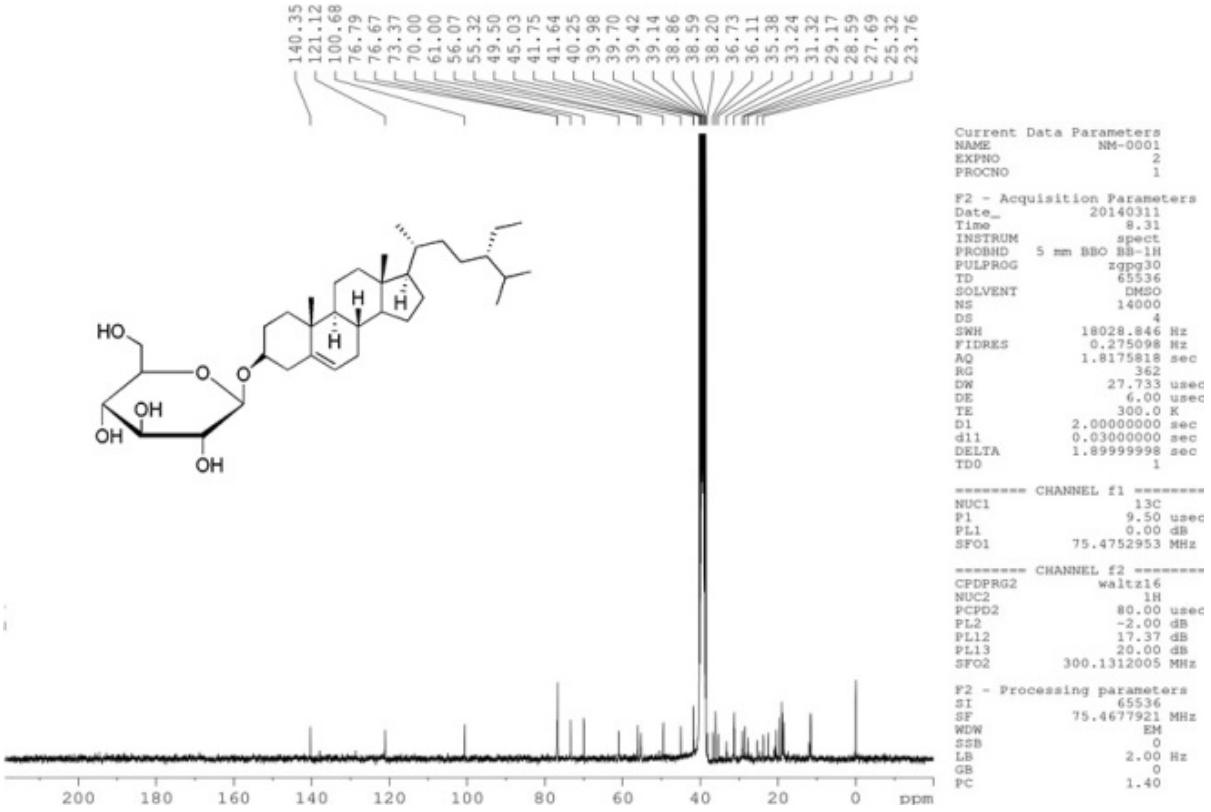

Fig S2 Carbon Nuclear Magnetic Resonance spectra of BSG

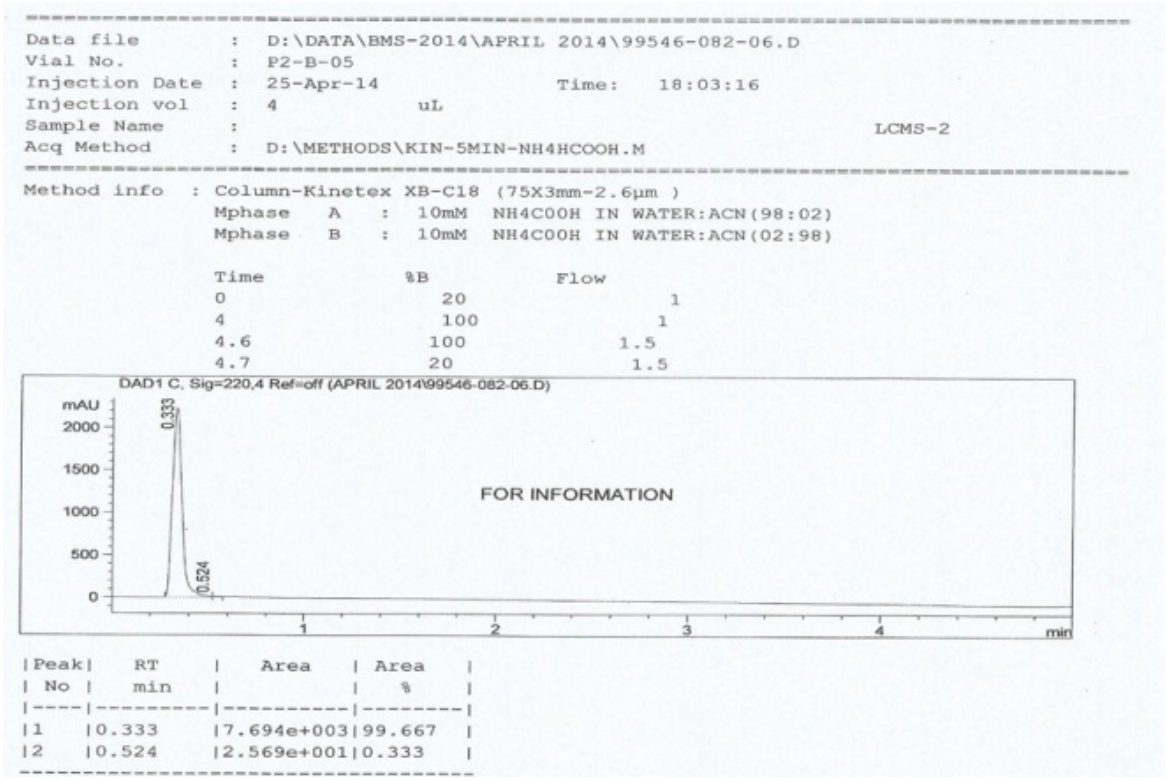

Fig S3 High Performance Liquid Chromatography analysis of BSG obtained from RSM optimized Solvent assisted hydrotropic precipitation. Purity % (Area %) = 99.6 %.

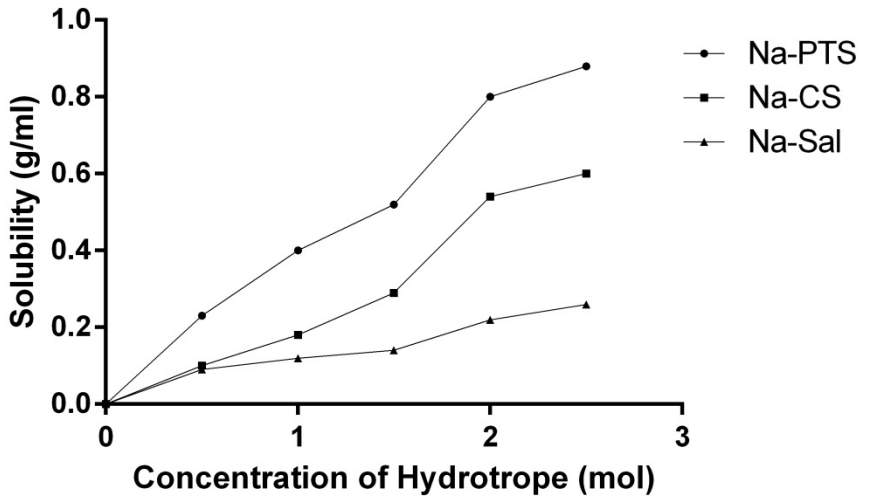

Fig. S4 Solubility of β-Sitosterol -D- glucopyranoside (BSG) in various hydrotropes at 45°C. Na-PTS – Sodium p toluene sulphonate, Na-CS – Sodium cumene sulphonate, Na-Sal – Sodium salicylate.

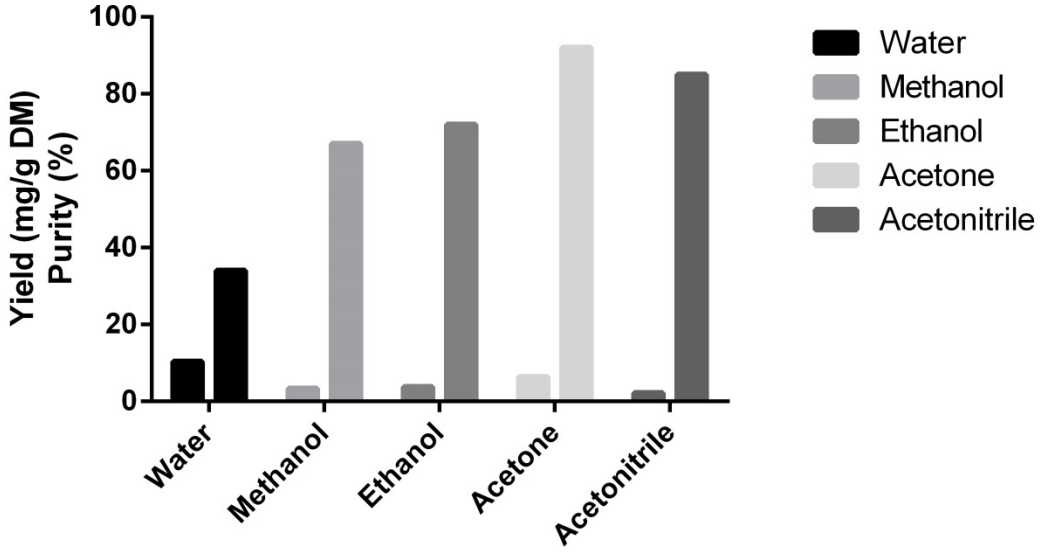

Fig S5 Effect of different solvents on yield and purity of precipitated BSG.

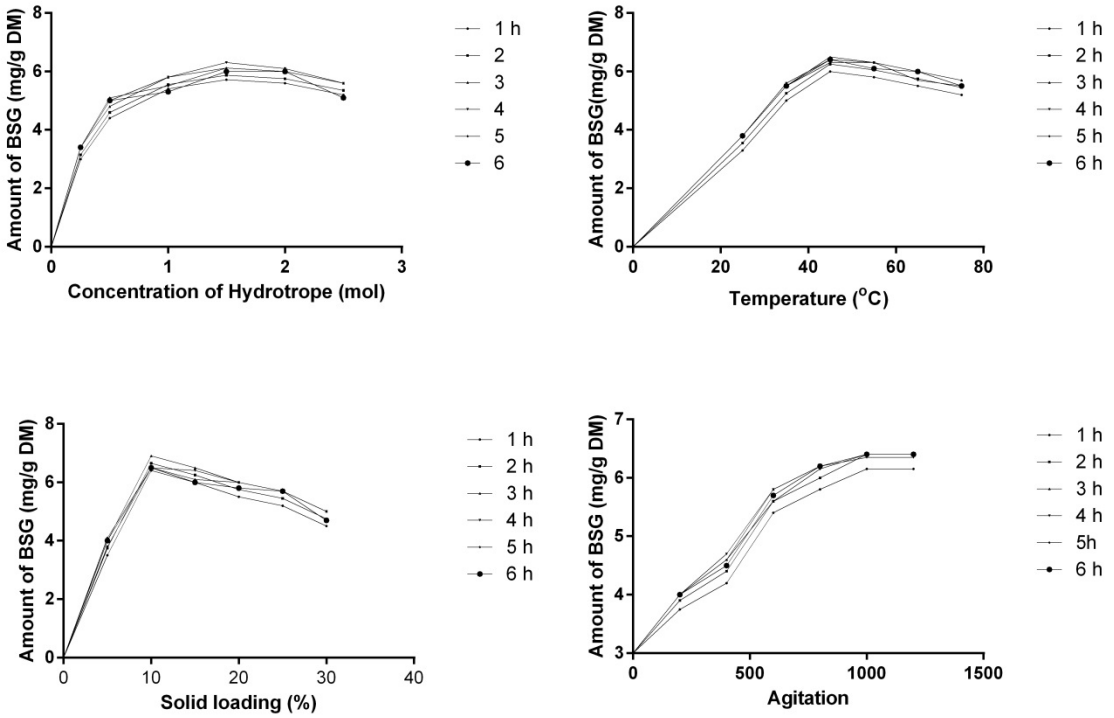

Fig. S6 Factors affecting the hydrotropic extraction of BSG from *D. bipinnata.*