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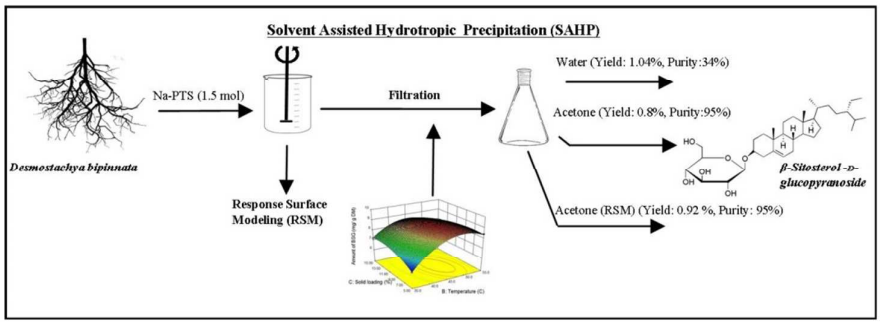
Novel Box-Behnken optimized isolation of the dietary micronutrient β-Sitosterol-D–glucopyranoside by solvent assisted 'green' hydrotropic precipitation from *Desmostachya bipinnata*.



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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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β-Sitosterol-D–glucopyranoside, a phytosterol glycoside is well known as dietary micronutrient and food supplement. The present work focuses on the novel investigation of solvent assisted 'green' hydrotropic precipitation of this molecule from *D. bipinnata* where the Box-Behnken optimized separation process improved the yield by 80.4% (Purity: 99.6%) than conventional extraction.

Natural products find their extensive use in food stuffs, since they enjoy the preference of consuming populations due to their plant origin and relative safety. Plant sterols are obligatory molecules in food stuffs and β-Sitosterol-D–glucopyranoside (BSG) forms an important ingredient of these phytosterols. BSG (Fig. 1) is found in fruits and vegetables, where dietary intake of this compound is recommended and it acts like an essential micronutrient and albeit, a vital cell constituent[1]. It is also used along with β-Sitosterol as a food supplement for older adults to enhance immune function[2]. The commercially available Moducare®, Harzol® and Sitosterin® are food supplements that contain BSG as a vital ingredient[3]. Dietary intake of BSG with other phytosterols has been evidently proved to improve immune system[4], in combating cancer and prostate abnormalities[1]. BSG is proved to be anti-microbial[5], antileukemic, antispasmodic, antitumour and Hypoglycemic[6] etc. They have been positively found to improve health of HIV infected patients when taken as daily dietary supplement[7]. Also, BSG has been proved to synergistically combat human pathogens when combined with other commercial antibiotics[5]. It is proved that oral absorption of BSG is not toxic to humans[8], and has been evidenced that daily oral supplementation with 60 mg β-sitosterol and 0.6 mg of its glucopyranoside (BSG) enhanced T-cell proliferative response after 4 weeks[9]. BSG combinations (Harzol™) have been used in Germany to treat benign prostrate hypertrophy (BPHand also has been proved to be adapted by humans to their dietary availability and incorporated them in their own metabolic needs over the course of evolution[10]. Like Vitamin C, lower availability of BSG and other sterols have to be dealt with supplements containing BSG and other sterols to counter the deficiency effects[11].

Since, oral absorption of BSG is less, it is imperative that to maintain a normal serum level of BSG in deficient humans, enormous amount of fruits and vegetables need to be consumed, which becomes practically difficult. Therefore, dietary supplements containing BSG and sterols need to be administered, which necessitates the need for pure isolation of BSG from plants. However, the variety and abundance of other plant sterols in edible plant parts, always results in meager yield of BSG through conventional extraction procedures. Therefore, non-conventional separation methods become necessary for rapid and better recovery of BSG. It was observed that stem and roots of *Desmostachya bipinnata* (L.) Stapf. (Fam. Poaceae) contain significant amount of BSG. Therefore, a unique process which gave rapid, pure and better yield of BSG has to be developed. The extraction of active principles from plants has been a daunting task since decades, as the process developed for complete isolation of a molecule varies with respect to plant and target molecule. Conventional isolation of molecules from plant matrices involves solvent extraction and subsequent purification through column chromatography[12].These techniques involve handling of large volumes of harmful volatile solvents which make the whole extraction process labour-intensive and time consuming. Therefore, non-conventional isolation processes which are environment friendly and easy to operate are now being developed and established.

Hydrotropes are low molecular weight organic salts that have an amazing ability to solubilize water insoluble organic compounds into water. They achieve this feat by achieving a desirable concentration in aqueous solutions usually above their characteristic minimum hydrotrope concentration (MHC). Thus, at high concentrations of hydrotrope, solutes can be solubilized in water and could be later recovered as precipitates just by diluting the solution and bringing down the hydrotropic concentration below MHC. This provides an easy method to extract, enrich or concentrate compounds from plant matrices. The hydrotrope has an advantage of reusability as they are chemically inert during extraction process[13].Initially developed in 1950s, Response surface methodology (RSM) is now extensively applied to solve, model and enhance tedious optimization problems involving numerous influential factors[14]. Box–Behnken design (BBD) is a well-known RSM method and effective design practice involving lower number of experimental runs and model fitting thus economizing the time and resources needed for experimental processes as is routinely used in many optimization studies[15]. It is therefore widely applied for developing extraction processes of molecules from plants[16].

The novel technique developed in the current study, specifically isolates the target molecule (BSG) with high purity from other impurities using a green process. The identification of influential factors and process parameters involved in hydrotropic extractions through RSM which could aid in optimizing the extraction process and to obtain maximum recovery of target compounds, besides reducing the number of steps in isolation of pure molecule, has also been attempted.

Initial solubility studies of BSG were studied in various concentrations of hydrotropic solutions of Sodium p-toluenesuphonate (Na-PTS), Sodium cumene sulphonate (Na-CTS), and Sodium salicylate (Na-Sal) (0.5-2.0 mol). The solubility experiments were carried out in cylindrical glass vessel (50 ml) fitted with a six-bladed turbine impeller (i.d: 2 cm) under vigorous stirring for 5 h at 35°C. The amount of BSG solubilized was analysed and plotted†. The stability of BSG at different temperatures was also evaluated†. Studies were performed with acetone, methanol, ethanol and acetonitrile on their efficiency in precipitation of BSG from hydrotropic extract compared to water. Purity of precipitated BSG was also analysed at each experiment†.

Authenticated† plant material (stem and roots) of *D. bipinnata* collected from river beds of River Cauvery in Thanjavur 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 analysed for the BSG content. At the end of extraction, the clear solution containing the metabolites was filtered under vacuum. A slight yellow colour 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 through High Performance Thin Layer Chromatography (HPTLC).

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.

On the basis of the spectral data, Fig S1-S2†, the structure of the compound was identified and confirmed as β-Sitosterol-D-glucopyranoside. All the spectral data were in complete concurrence with the literature[17]. The BSG obtained from final optimized process was analysed through HPLC and its purity was found to be 99.6% (Fig. S3†).

The influential parameters were identified from classical optimization 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) 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. 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 S1†.

Experimental data thus obtained were fitted in second-order polynomial model and regression coefficients were determined as in Equation (1).

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

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.

A final run of experiment with the RSM optimized parameters was performed and the yield of BSG was analysed through HPTLC.

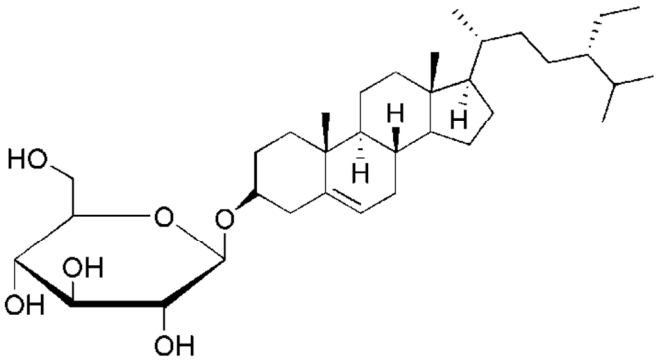

Fig. 1 β-Sitosterol -D- glucopyranoside.

To successfully understand the mechanism of hydrotropic extraction and to optimize the influential parameters, the knowledge of its solubility in different hydrotropic solutions at different temperatures and concentration is essential. The solubility of pure BSG in different hydrotropes as a function of hydrotrope concentration is given in Fig. S4. The trend showed increase in solubility of BSG with increase in hydrotrope concentration. Na-PTS (2.5 mol) works best in solubilizing BSG (0.88g/ml) compared to Na-CS (0.6g/ml) and Na-Sal (0.26g/ml). Based on these observations Na-PTS which is also considered as 'green' hydrotrope[18] was taken for further optimization and extraction studies due its high solubilizing capacity of BSG.

The major disadvantage when this hydrotropic extraction technique[19] is applied in phyto-molecule isolation is that when water is added to dilute the hydrotropic solution, along with target/desired compounds, the other non-essential compounds also get precipitated which lowers the purity of desired metabolite. In the present study, dilution with water precipitated other low polar molecules along with BSG which got solubilized in the extraction process due to the action of hydrotrope. Now, to circumvent this problem, a new approach was developed and applied in which solvents, soluble in water and which solubilize hydrotropes were used instead of water for dilution. The fact that Na-PTS is soluble in methanol, ethanol, and acetone, makes them ideal for precipitation. Fig. S5† shows the yield of precipitate and purity of BS in the precipitate using various solvents for inducing precipitation in hydrotropic extract. Observations reveal that water has the highest yield (10.4 mg/g DM) but purity of BSG is less (34%). This is due to the fact that other low-polar metabolites are also precipitated along with BSG which adds up to the precipitate amount. Whereas, for methanol and ethanol, the purity of BSG is considerable (67, 72 %) but the yield is less (3.4, 3.8 mg/ g DM). This is due to the fact that BSG is partially soluble in both the alcohols and hence considerable amount of BSG dissolved in solvent while only little precipitates. Acetone works best for precipitating BSG (yield: 6.4 mg/g DM; Purity: 92 %) because of two advantages; firstly, it dissolves low-polar compounds that are precipitated along with BSG during dilution of hydrotrope, thus precipitating pure BSG. This is possible because of least solubility of BSG in acetone. Secondly, it readily brings down the concentration of Na-PTS below MHC as Na-PTS is easily soluble in acetone. Acetonitrile has an advantage of solubility with water but its capacity to precipitate water insoluble molecules like BSG was limited substantiating its non-consideration to be an ideal solvent. Thus, acetone formed as an

ideal solvent for pure isolation of BSG through hydrotropic extraction.

Parameters such as time of extraction, concentration of hydrotrope, temperature, solid loading, and agitation were studied for their influence on final yield and amount of solvent for precipitation for its influence on purity of BSG. Experiments were done varying a primary parameter and analysing the yield with respect to time. In Fig. S6†, plots of agitation versus yield of BSG at different time intervals showed that, over 1000 rpm, the extraction yield remains constant. The trend depicts linearity in increase of yield with increase in speed of agitation, until it reaches 1000 rpm. Therefore, 1000 rpm could be used as an optimized speed for extraction. From Fig. S6† it is observed that over three hours of extraction time; the yield remains same in all the plots containing a different primary parameter such as temperature, solid loading etc. Thus, total extraction time could be set to 3 h for efficient extraction of BSG. The amount of solvent added to induce precipitation had a linear effect on precipitation. The solvent such as acetone is added to the filtrate obtained from hydrotropic extraction to bring down the hydrotropic concentration to MHC thus inducing precipitation of non-water soluble compounds. It was observed that 2.85 ml of diluting solvent was required per ml of hydrotropic extract solution to bring down the solution below MHC. MHC of Na-PTS is 0.35 mol. Therefore, this volume (2.85 ml) was made constant in all the extraction experiments. Similarly, MHCs of Na-CS and Na-Sal are 0.65, 0.1 mol respectively[20]. Due to clear linearity observed in their effects on extraction process; time, agitation and precipitation solvent amounts were fixed to be 3 h, 1000 rpm and 2.8 ml/ml of Na-PTS extract solution in all the extraction experiments. Fig. S6† also shows effect of hydrotrope concentration over extraction of BSG. As concentration increased, the extraction yield also increased until a concentration (1.5 mol) and then decreased mildly. Such an observed response might actually affect the extraction kinetics and efficiency. Similarly, BSG yield increased with increase in temperature until 45°C and then decreased thus affecting the extraction efficiency. BSG was also found to be stable at 45°C as analysed by HPTLC.

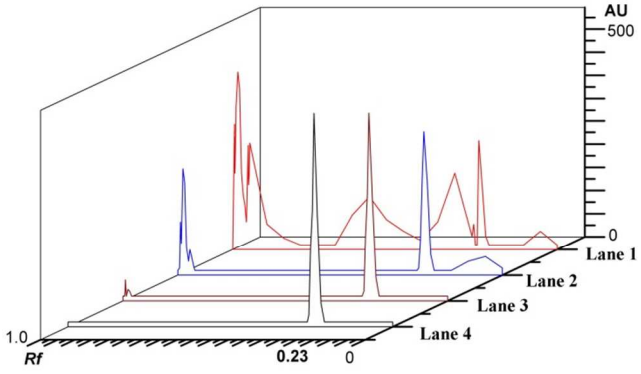

Fig 2.High Performance Thin Layer Chromatography (HPTLC) analysis. Lane 1 - Methanolic extract from stem and roots of *D. bipinnata*, Lane 2 - Precipitate obtained using conventional hydrotropic extraction, Lane 3 - Precipitate obtained using acetone assisted hydrotropic extraction, Lane 4 - Standard BSG.

Amount of plant material loaded for extraction had direct effect on extraction yield. A solid loading greater than 10% led to decrease in yield. This might be due to insufficiency of the hydrotrope to extract the optimum amount of BSG. Thus, 3 parameters were found to influence the extraction process significantly: concentration of hydrotrope, temperature, and solid loading. Thus, these 3 parameters were considered for RSM based modelling and optimization.

Amount of metabolites in each optimization step and experimental runs according to RSM model was analysed by High performance thin layer chromatography. The HPTLC profiles and the resulting chromatogram are given in Fig. 2. The HPTLC method was developed and validated for effective quantification of BSG in each sample to determine its purity obtained during precipitation process. The mobile phase was methanol: chloroform, 1:10 (%, v/v) which resulted in a sharp significantly resolved peak at R_f values of 0.23 for BSG. Peaks of BSG from different process samples were identified by comparing their spots at their respective R_f = 0.23 values with those obtained by chromatography of the standards under the same conditions as given in Fig. 2. The comparison of lane 1 -3 depicts an increase in the amount of BSG, with other impurities minimum in precipitate obtained by acetone than water as illustrated by the peaks. Lanes 4 confirms the R_f values of BSG which corresponded with the values of those samples from experiments.

Using the Box-Behnken experimental design, the second-order polynomial quadratic response equation (Eqn. 1) was used to establish a mutual link between the response dependent variables and independent parameters. The link based on the coded factors was established according to the equation below.

$$Y_1 = 9.07 + 0.1X_1 + 0.56\ X_2 + 0.012\ X_3 + 0.58\ X_1\ X_2 + 0.075\ X_1\ X_3 - 0.5\ X_2X_3 - 0.48\ X_1^2 - 1.31\ X_2^2 - 0.46\ X_3^2 \text{ ------ (2)}$$

The Box-Behnken matrix and experimental results for hydrotropic extraction and yield of BSG are summarized in Table S1†. Analysis of variance (ANOVA) was used to assess the statistical significance of quadratic model. The results of ANOVA for amount BSG are depicted in Table S2 and S3† whose statistical observations demonstrates that the regression model has a high coefficient of determination (R^2 = 0.998). Also, the R^2 adj (0.994) value explains significance of the model. There seems no significant difference between R^2 and R^2adj values which is desirable for the model. In addition, low Coefficient of variation was observed (0.88) which is usually desired indicating good reliability for the experiments carried out in the process. In the present study, F-values are greater and P values are much lesser as depicted in Table S2†, implies that most of the coefficients obtained are significant in the model.

The coefficients and standard error are depicted in Table S3†. The corresponding F-values for coefficients indicate that the Temperature (X_2) produces the largest effect in extracting BSG in the process (F-value: 523.71, P<0.0001). It was followed by Concentration of hydrotrope (X_1) (F-value: 16.55, P<0.0001). Solid loading (X_3) had the least effect. The results of the lack of fit test for the models are depicted in Table S4† describing the variation in the data around the fitted model. In present case, the F-value for lack of fit test is 1.75 and is not significant implying that the models sufficiently describe the obtained data. A three-dimensional response surface and contour graph were plotted based on obtained model equation to evaluate the interaction among the operational factors and to determine the optimum values of each parameter. The effects of influential parameters on yield of BSG are shown in Figure 3. The yield of metabolites increases with increase in factor levels up to moderate level (0) and then decrease. For example, in Fig 3, interaction between Concentration of hydrotrope (A) and Temperature (B) is plotted where, yield of metabolites increases as A increases from 1-1.5 mol and then decreases when it extends to 2 mol. Similarly, as Temperature (B) reaches 45°C, the yield reaches maximum and then decreases. This phenomenon is seen in all the surfaces drawn based on interaction effects of different factors.

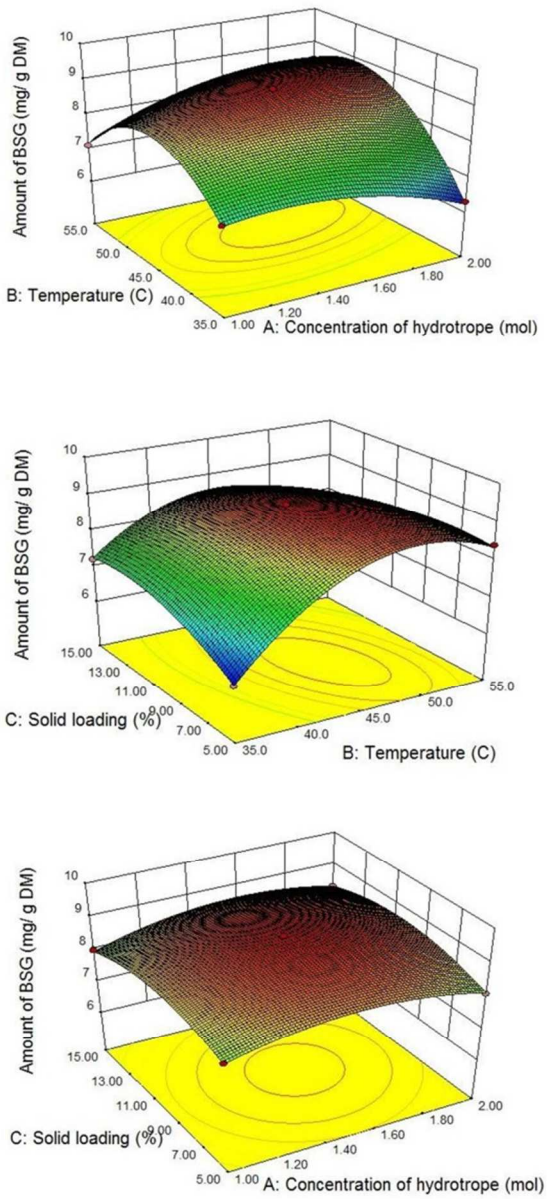

Fig. 3 Response surface plots for yield of BSG showing interaction of different process parameters

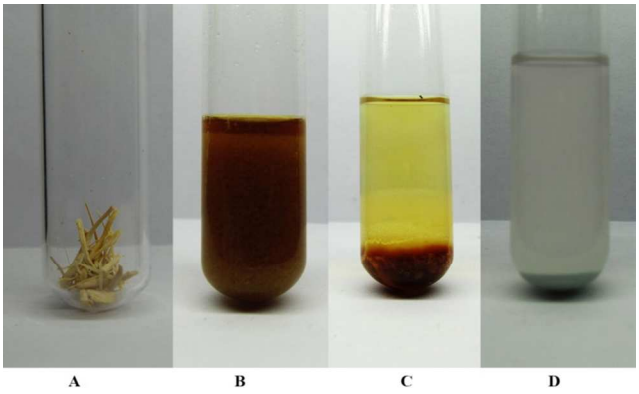

Fig 4 (A) Stem and roots of *D. bipinnata*, (B) Methanolic Extract of *D. bipinnata*, (C) Hydrotropic precipitate using water containing (BSG=34% pure) (D) Hydrotropic Precipitate using acetone (BSG=99.6% pure).

Table 1 Yield and Purity of BSG from different methods

Methodology	Yield (%)	Purity (%)*	Amount of BSG** (%)	Increase in Yield of BSG (%)
Conventional isolation	0.18	99	0.17	-
HE (Water)	1.04	34	0.35	48
Optimized through one variable at a time (HE)	1.8	34	0.61	70
Optimized through RSM (HE)	2.1	34	0.72	75
SAHP (Acetone)	0.8	99.6	0.8	77.5
SAHP (Acetone) optimized through RSM.	0.92	99.6	0.92	80.4

HE – Hydrotropic extraction using water, SAHP- Solvent assisted hydrotropic precipitation. *Calculated through HPTLC, Purity = 95% was considered as pure BSG.**Amount of BSG present in crude extract/precipitate calculated through HPTLC.

Usually numerical optimization method is used for optimization in which a desirable value for each input factor and response can be selected[21]. Using these conditions, the maximum achieved amount of BSG was 9.14 mg/g DM at 1.97 mol of Na-PTS, 49.5°C and 9.73% of solid loading at 1000 rpm agitation for 3 h. This result indicates an acceptable fit among the obtained data and the desirability of the model at all points. An additional experiment was carried out to confirm the amount of BSG yielded at optimized conditions which was 9.14 mg/g DM. This was in accordance to predicted value of 9.2 mg/g DM as in Table S5†. The final yield of 0.92% which clearly substantiates a significant increase compared to initial yields of 0.18% as given in Table 1. The final purity of BSG through optimized SAHP is 99.6%. The colour and texture of precipitates from different processes are shown in Fig. 4. The optimized yields depict an increase of 80.4% in total recovery of BSG, further emphasizing the potential for development and optimization of hydrotropic extraction procedure taking into account the process economics.

Conclusions

In the present study, a novel investigation of solvents in hydrotropic extraction of β-Sitosterol-D-glucopyranoside (BSG) for the first time from *D. bipinnata* was optimized by the Box-Behnken experimental design and response surface methodology based model fitting and optimization in a batch mode 'green' extraction process. Analyses of the response surfaces were carried out as a function of concentration of hydrotrope (X_1), temperature (X_2), solid loading (X_3) and for the resulting model, ANOVA demonstrated a high correlation coefficient (R^2=0.998) indicating a good fit between the second order regression model and the experimental observations. Optimal conditions obtained through RSM, which yielded a maximized amount of BSG (9.14 mg/g of Dry plant material) included 1.97 mol of Sodium p-toluenesulphonate (Na-PTS), 49.5°C and 9.73% of solid loading at 1000 rpm agitation for 3 h. Thus it is illustrated that the standard experimental design and RSM based optimization was an efficient strategy for optimizing the operational parameters towards maximizing the recovery of BSG depicting an increase of 80.4 %. The operational parameters optimized elucidate the lowest cost needed in extraction process thus, providing an efficient, rapid and cost-effective method for isolation and scale up of BSG from *D. bipinnata.*

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Notes and references

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†Electronic Supplementary Information (ESI) available: Description of experimental procedure, Statistical data of RSM, Solubility studies, NMR spectra, HPLC chromatogram, Classical optimization observations. See DOI: 10.1039/b000000x/

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