Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Bioavailability and Stability Enhancement of Natural Isoflavones of *Saraca asoca* by Cryogenic Encapsulation Technique

Mojeer Hasan, Bibhu Prasad Panda

Microbial and Pharmaceutical Biotechnology Laboratory, Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India

Submitted: 15-09-2018 Revised: 02-11-2018 Published: 17-01-2019

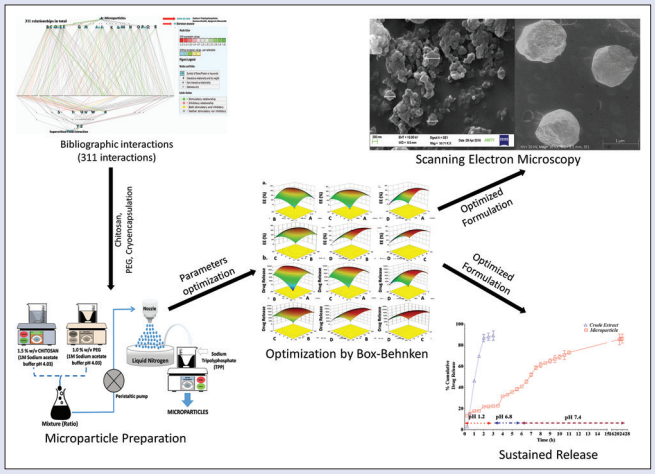
ABSTRACT

Background: The detailed study to overcome the limitations related to oral drug release and better encapsulation efficiency of the *Saraca asoca* extract has not been reported, despite having so many uses as sacred as well as a good medicinal plant. **Objective:** The aim of the present study was to fabricate a *S. asoca* extract-based chitosan microspheres conferring sustained released to enhance its medicinal use. **Materials and Methods:** A data mining online tool was used to check the interactions between the polymer used and formulation properties to analyze the relationships between them. The method uses a formulation of chitosan: polyethylene glycol (PEG) (2.1:0.9), drug (18.13%), sodium tripolyphosphate (6.55%), and crosslinking time (34.44 min) with a very good encapsulation efficiency and drug release. **Results:** The optimized formulation have showed an average particle size of 1157 nm, encapsulation efficiency of 73%, and drug release of 1052 mg GAE. *In vitro* release studies have shown a sustained release profile with maximum release till 24 h in pH 7.4 phosphate-buffered saline. The flowability of the optimized microparticles has shown a better Carr's index of 19.90 ± 0.21% and Hausner ratio of 1.24 ± 0.03. *In vitro* antioxidant activity of crude drug and microparticles has IC_{50} of 250.8 µg/mL and 188.2 µg/mL, respectively, showing an increase in the activity. **Conclusion:** Chitosan-based *S. asoca* microspheres with enhanced encapsulation efficiency, *in vitro* drug release as well as improved *in vitro* antioxidant activity could be attained by chitosan-PEG coated microparticles.

Key words: Box-Behnken, Chilibot, chitosan, microparticles, response surface methodology, *Saraca asoca*

SUMMARY

- A Chitosan-based microparticle was prepared by a novel cryoencapsulation process
- A good encapsulation as well as release was obtained after a successful prediction of hypothesis from an online data mining tool.



Abbreviations used: PEG: Poly ethylene glycol; TPP: Sodium tripolyphosphate; GAE: Gallic acid equivalent; DSC: Differential scanning calorimetry.

Correspondence:

Dr. Bibhu Prasad Panda,
Microbial and Pharmaceutical Biotechnology Laboratory, Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi - 110 062, India.
E-mail: bibhu_panda31@rediffmail.com
DOI: 10.4103/pm.pm_468_18

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Saraca asoca (Roxb.) De Wilde is a sacred as well as most broadly used Indian traditional medicinal system plant of Caesalpiniaceae family.[1,2] It is commonly known as "Sita Ashok, Ashok or Anganapriya" having numerous preparations for the different formulations in the Ayurveda as well as Unani mode of traditional therapy, most notably in herbal formulations named aristas, bhasmas, garitas, and prasad.[3] The bark of *S. asoca* has several therapeutic benefits such as in menorrhagia, cancer, leukorrhea, uterine disorders, diarrhea, dysentery, vibriocidal, molluscicidal, antimicrobial, and osteoporosis.[4,5] Despite having so much of pharmaceutical importance, the use of the *S. asoca* extracts is unpractical and challenging because of its endangered stature and limited availability with only in Western Ghats of Maharashtra, Goa, Karnataka, Kerala, Tamil Nadu, and Eastern Ghats of Odisha and Meghalaya, that too in mere patches.[6] Designing a suitable delivery system for the perfect delivery of the active constituents of *S. asoca* is important as it can increase the bioavailability and stability of phytoconstituents.[7] It will lessen the consumption of the plant, which is getting extinct because of unrestrained mowing in the wild conserved areas and also because of constricted or no new plantation.[1] A report of the survey done by FRLHT (Bengaluru) during 2007–2009 in the Western and Eastern Ghats of India, expected that the formulations of *S. asoca* being marketed, either have other plants adulterated or completely lacking the extracts because of its limited availability.[8,9]

A drug to be able to deliver orally, controlled drug release is important, as it can forecast a specific site of action, for which several drug-polymer

Cite this article as: Hasan M, Panda BP. Bioavailability and stability enhancement of natural isoflavones of *Saraca asoca* by cryogenic encapsulation technique. Phcog Mag 2018;14:S619-29.

combinations are already well-established.[10] As like, Chitosan-based hydrogel system, which have been used in many pharmaceutical preparations, its pH sensitivity provides the capability of altering the release pattern during the variations.[11] The gastrointestinal tract is one such place where pH varies from extremely acidic–to–slightly alkaline, together with salts and various digestive enzymes. A hydrophilic polysaccharide of 2-amino-2-deoxy-D-glucopyranose and β-[1-4]-linked 2-acetamido-2-deoxy-D-glucopyranose; has several advantages such as nontoxic, biocompatible, biodegradable, cheap, and easily available.[12] Besides this, it has found to be improving the adsorption of the drugs across paracellular route through its mucoadhesive and permeation augmenting properties.[13,14] A long passage in the gastrointestinal tract of 3–16 h makes the time controlled release a necessity and a limitation to the chitosan-based drug delivery system.[10] Hence, polyelectrolytic complexes with several anionic polymers, such as cellulose, carrageenan, pectin, sodium alginate, and polymethacrylic acid-g-ethylene glycol. have attracted much attention.[15] Chitosan alone is too delicate for the harsh acidic condition of gastric region hence, ionic crosslinking with several oppositely charged chemicals such as glutaraldehyde, ethylene glycol, and polyethylene glycol (PEG) are used.[16]

PEG is an FDA approved polymer with similar benefits as like chitosan; biocompatible, good water solubility and is used for several pharmaceutical preparations to enhance the drug loading and to prolonged the drug release period.[17] Despite this, the use of PEG in high concentration is toxic and as an alternative, a nontoxic crosslinking agent sodium tripolyphosphate (TPP) is used which has found to be increasing drug loading capacity and have maintained sustained release.[18] It is because of the ionotropic crosslinking between the TPP molecules which are multivalent negatively charged particles and oppositely charged amino (-NH_3+) groups of chitosan.[10-19]

To increase the loading and release of *S. acosa* extract further, the crosslinking time is a vital factor but hampers the drug loading capability as it leaks out after some time in the aqueous phase while fashioning the microparticles. The PEG has found to be playing a role in it. Hence, it has been chosen to form an enteric coated microparticles for delayed release.

The limitation related to this formulation was the encapsulation efficiency of the drug because of phytomolecules in the extract. To overcome this, a new exemplary was obligatory which can escalate the contact time between the polymer and the drug so that the crosslinking possibly will be finished efficiently as well as the degradation caused by high temperature while spray drying could be omitted.[20] Hence, in the present approach to increase the contact time of crosslinker with the polymer, cooling of the mixture was used. The liquid nitrogen freezes the mixture up to a subzero level and due to the icing of the mixture, an increase in the entrapment was attained.

MATERIALS AND METHODS

Material and instruments

Authenticated AR roots (Letter no. NISCAIR/RHMD/Consult/2014/2366-146) were powdered and sieved through 40/60 nos. (250–400 μm particle size) mesh size and extracted with aqueous, ethanolic (25 δ) and aqueous ethanolic (50 δ) solvent. Standard molecules of Catechin, Trans-Chalcone, and Quercetin were purchased from Sigma Aldrich, New Delhi, and SD Fine Chemical Limited, New Delhi, respectively. Cellulase enzyme was purchased from Link Biotech, New Delhi. The Analytical Technologies Limited, India high-performance liquid chromatography (HPLC) system equipped with LiChroCART® 250 reverse phase C-18 column of 250 mm × 4.6 mm diameter (5 μm) from Merck, India with the binary pump system (P3000A) and the ultraviolet detector (UV3000) was used in the study. Precoated silica gel 60 F 254 high-performance thin-layer chromatography (HPTLC) plates (20.0 cm × 20.0 cm) were purchased from Merck, India.

Extraction, high-performance liquid chromatography, and high-performance thin-layer chromatography analysis of *Saraca asoca* bark extract

Different extracts of pretreated powdered *S. asoca* bark were prepared by combining the different physical and chemical extractions. Out of different extracts the best extract was carefully chosen on the basis of HPL chromatogram, total phenolic content, total flavonoid content, and total antioxidant activity (Data not shown). The 25 δ ethanol (100% v/v) mediated soxhlet extract was found be best on the basis of optimum number of compounds along with the *in vitro* activity.[21,22]

In HPLC, Li-Chrospher® 100 RP-18e column (250 mm × 4 mm, 5 μM) was used for analysis, the mobile phase was water (A) and 0.02% trifluro acetic acid in acetonitrile (B) with linear gradient elution (A-80% (5 min)→20%–40% (8 min)→40%–50% (12 min)→50%–40% (17 min)→40%–20% (21 min)) at a flow rate of 1 ml/min. Detection was carried out at λ_{max} 280 nm.[23] HPTLC was run on precoated silica gel 60F 254 HPTLC plates (20.0 cm × 10.0 cm). Methanolic solutions of standard compounds (quercetin, trans-chalcone, and catechin) and samples of known concentrations were applied to the plate positioned (x = 10 mm and y = 10 mm) of 6 mm bandwidth using a CAMAG Linomat 5 automated TLC applicator with the nitrogen flow of 120 nl/s from the plunger. The mobile phase was Toluene: Ethyl Acetate: Formic Acid: Methyl alcohol (6:6:1.6:0.4 v/v/v/v) and detection was carried out by using the CAMAG TLC scanner 3 equipped with WINCATS software (CAMAG) at the λ_{max} 280 nm.[24]

Microencapsulation of *Saraca asoca* extracts

Microencapsulation of *S. asoca* extract obtained by different extraction was done in chitosan (SRL chem, New Delhi) and PEG (SDFCL, New Delhi) in a different ratio to get maximum encapsulation efficiency and drug release by Box Behnken design in Design expert 9.0 from Stat-ease Inc. USA [Figure 1]. The summary association based functional relationship between selected polymers, crosslinking agent and *S. asoca* extracts was identified by a text mining tool Chilibot. It helps to identify the relationship between the keywords searched in PubMed abstracts. The ranking of the important words on the basis of occurrence in the literature was used to draw a hypothesis between the key terms.[25] The selected four variables and three levels were chitosan level (A), drug concentration (B), sodium tri-polyphosphate (C), and crosslinking time (D). The variables and levels tested were established with the -1, 0 and +1 level of analysis. Chitosan solution (1.5% w/v) was made in 1M Acetic acid and sodium acetate buffer (pH 4.03). PEG-4000 solution was made in the same buffer (1% w/v). Different ratio of these two solutions was optimized by Box-Behnken design to get optimum encapsulation efficiency of the extracts. Different concentration of extract was added to the mixture and stirred at 300 RPM for 30 min. This mixture was later poured into liquid nitrogen dropwise with the help of peristaltic pump (1 ml/min flow rate) and stirred continuously while pouring. After the small spheres of the mixtures are formed, the sodium tripolyphosphate solution of different concentration was added to it and kept under constant stirring for the crosslinking at 300 rpm for different time intervals to get optimum crosslinking time. Finally, the beads were freeze-dried at −42°C for 24 h after filtering and washing with deionized water. The response surface methodology using three level design having −1, 0 and +1 levels was selected to understand the influence of independent variables, chitosan and PEG ratio (A), drug

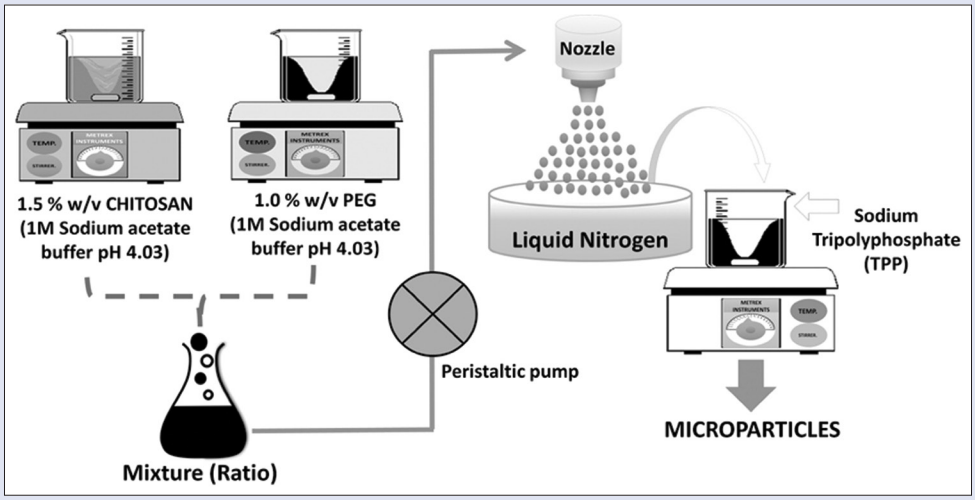


Figure 1: Schematic representation of the system used to produce the chitosan microparticles

concentration (B), TPP conc. (C) and crosslinking time (D) on playing the role on the dependent variables, encapsulation efficiency, and drug release. For the estimation of a statistical perspective, 29 runs including 24 factorial and 5 zero point of the Box-Behnken model to evaluate the error were performed in triplicates. For the study Chitosan-PEG ratio (1:1, 2:1, 3:1), drug concentration (5, 10, 20% v/v), TPP concentration (5, 7.5, 10% w/v) and crosslinking time (30, 60, 90 min) were used as different levels (−1, 0 and +1) of Box-Behnken design, respectively.

Particle size distribution

The particle size distribution of chitosan microspheres after drying was measured by laser diffractometry. Freeze-dried microspheres were re-dispersed in distilled water and sized by laser diffractometry using LS 230 Coulter (Coulter Co., USA).

Measurement of encapsulation efficiency

The actual amount of extract loaded in the chitosan microspheres was determined as follows. Accurately weighed (0.1 g) blank and extract-loaded chitosan microspheres were immersed into 2 ml of Phosphate-buffered saline (PBS) buffer (pH 7.4) and sonicated for 15 min with 100% amplitude and 10/05 s on/off cycles to rupture the microspheres completely. The amount of drug released was calculated by interpolation from a calibration curve containing gallic acid standards as the total phenolic content of the microspheres and was analyzed by folin reagent to check the encapsulation efficiency. A cumulative correction was made for each of the previously removed samples, to determine the total amount of drug release. The release experiments were done in triplicate.

In vitro release studies

Two hundred milligrams of microspheres was packed in charcoal free filter paper and suspended in 0.1N HCl (pH 1.2) for 2 h and stirred at 100 rpm continuously. To simulate the gastric pH condition of pH 1.2, 0.1 N HCl was selected and to mimic the duodenal, jejunum and ileum conditions the pH 6.8 and pH 7.4 phosphate buffers were used, respectively.[11] Sample (0.5 ml) was taken from it after 30 min interval and volume was adjusted to its original level after each reading. PBS buffer (pH 6.8) was used for the suspension of this bag after 2 h of suspension in 0.1N HCl and samples were collected after every 30 min. Later, PBS (pH 7.4) was replaced in the suspension and samples were drawn after 1 h interval for 50 h. The same volume of fresh PBS buffer was added into the release medium to keep the volume constant. A regression coefficient (R^2) based, best-fit model was designated for optimized formulation by placing the drug release data into zero-order, first-order, Hixson-Crowell, Higuchi, and Korsmeyer-Peppas release kinetics models.[26]

Flowing properties and swelling study

The bulk (BV) and tapped (TV) volume were determined in a 25 mL glass graduated cylinder by taking 1 g of each sample and poured through a funnel into the cylinder. The tap density and bulk density were calculated by direct observation of the cylinder, Hausner ratio and Carr's index (CI) were calculated later from this.[27] Two buffer dissolution systems (0.1 M HCl, pH 1.2 and phosphate buffer saline, pH 7.4) were used to check the swelling behavior of the CS/PEG beads (100 mg) by submerging them in the individual solutions. The swollen beads were later weighed after specific time intervals for up to 24 h. The swelling behavior of the microspheres was analyzed by weighing them after blotting on the filter paper to remove the surface bound water. The ratio between the initial weight (W_0) and after a definite time interval (W_t) was calculated by taking an average of at least three experiments according to the following equation.[11]

$$S = (W_t - W_0)/W_0 \times 100\%$$

Where, W_0 is the initial and W_t is weight after time 't' of the vacuum-dried beads.

Differential scanning calorimetric, Fourier-transform infrared spectroscopy and scanning electron microscopy

Shimadzu calorimeter (Model differential scanning calorimetric [DSC]-50, Japan) with the heating cycle of 0°C–250°C was used for DSC analysis. Constant flow (10 mL/min) of Nitrogen was passed into the samples (1–2 mg), with heating rate of 5°C/min. The empty pan was used as blank and maximum positions of melting endotherms were taken as designated melting points.[28] To approve the synthesis of various microparticles, infrared (IR) spectra were recorded on a Shimadzu FT-IR 8400S IR spectrophotometer using the attenuated total reflection accessory a resolution limit of 16 cm^{-1}, omitting the need of KBr to prepare the sample.[29] The surface morphology for the shape and size of the lyophilized *S. asoca* extract loaded microparticles was visualized under scanning electron microscope (Zeiss Gemini 5 1530 FEG). The lyophilized particles were sputter-coated with gold in inert gas condition (argon).[29]

Measurement of antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl method

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability of *S. asoca* extract and encapsulated microcapsule was estimated. Both the *S. asoca* extract (125 µL) and encapsulated microcapsule (125 µL) were mixed with 125 µL of 250 mM DPPH solution, and the mixture was shaken vigorously and sonicated, followed by incubation for 30 min at 28˚C. The mobile phase was a mixture of methanol and water (80:20, v/v) with a flow rate of 1 ml/min equipped with Li-Chrospher® 100 RP-18e column (250 mm × 4 mm, 5 µM) for HPLC analysis. Detection was carried out at λ_{max} 517 nm by measuring the decrease in the concentration of remaining DPPH by using the calibration curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) as the standard. The antioxidant activity was measured in µg TROLOX equivalent/g of sample and was measured by the following equation.[30]

Radical Scavenging = $(A_{control}-A_{sample})/A_{control}$

RESULTS AND DISCUSSION

Extraction and analysis of *Saraca asoca* bark extract

Alcoholic (25 δ) soxhlet was selected on the basis of extractive value (9.46% w/w), total flavonoid content (8.6 µg/mg/g), total phenolic content (4.45 mg GAE/g^{-1}), and total antioxidant activity (80.91% µM TROLOX equivalent). The fingerprinting analysis of the sample by HPLC has shown the presence of six phenolic groups, isoflavones, hydroxycinnamic acid, flavones, catechins, and chalcone [Figure 2a]. The HPTLC chromatogram [Figure 2b] has also confirmed the presence of Ferulic acid (R_f = 0.79), chalcone (R_f = 0.88), and catechin (R_f = 0.67) by the comparison with known standards.

Preparation of microparticles

Production of microparticles was done by the ionic gelation of the chitosan and PEG polymers with TPP. The analysis of variance (ANOVA) was calculated for each of the independent variable and signal to noise ratio was determined with a desirable ratio of >4. The aim of the optimization was to maximize the encapsulation efficiency of the drug as well as to maintain the controlled release of the same in a pH-dependent manner.

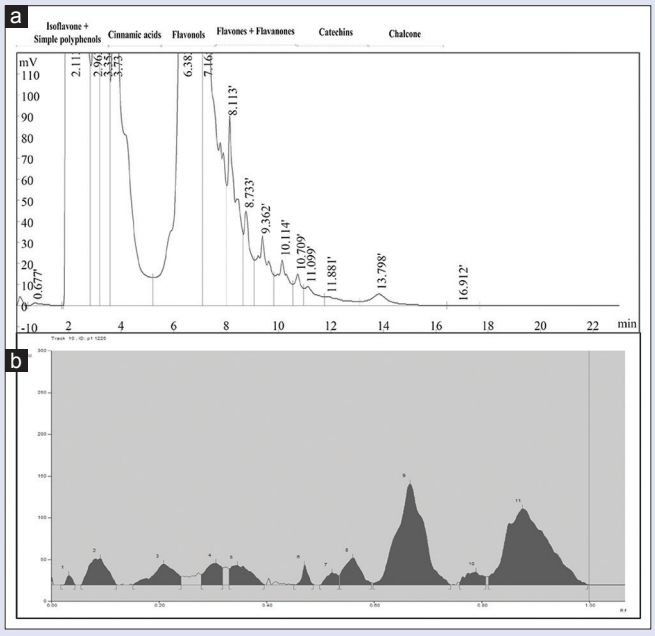


Figure 2: (a) High-performance liquid chromatogram. (b) High-performance thin layer chromatogram of *Saraca asoca* extract

As a complementary approach, a network analysis was done by using the Chilibot (http://www.chilibot.net/) on 31 keywords related to microparticles synthesis in the PubMed abstracts. A network between nanoformulation, microparticles construction, response surface methodology, polymers, drug release, encapsulation efficiency, polyphenols, and *S. asoca* plant was constructed [Figure 3a]. In the graph, only the strong interactive relationships (excluding the noninteractive and abstract only co-occurrence) were selected for observation. The stimulatory and/or inhibitory types of interactions, together with the weight was designed in the graphs on the basis of number of abstracts available in PubMed, confirming the relationships. No direct relationship was reported for the *S. asoca*, sustained release and the polymers used in the study, on the basis of the hypothesis generated in the Chilibot [Figure 3b].

Model fitting for encapsulation efficiency (EE % mgGAE)

The evaluation of the fitted model with true response surface was evaluated by observing the ANOVA and lack-of-fit test. The encapsulation efficiency ranged from 2.85% to 73.78%, which was calculated by analyzing the total phenolic content of the ruptured beads [Table 1]. The maximum yield was obtained with levels of independent variables A = 0, B = 0, C = 0, and D = −1. The mathematical multi-regression analysis to the independent variables and dependent variables has given the following second-order polynomial equation:

$$EE\ (\%) = +66.05 + 6.11A + 6.64B - 0.40C - 23.88D + 1.71AB - 5.92AC - 6.50AD + 2.82BC - 4.18BD + 0.62CD - 23.72A^2 - 11.38B^2 - 13.04C^2 - 16.50D^2$$

The ANOVA for the dependent variable was highly statistically significant ($P < 0.01$) and lack of fit was not statistically significant ($P > 0.05$), verifying the adequacy of the statistical model. The adequate precision was 74.24, which was higher than 4 and proves the validation of the design to be able to navigate between the indicated space. The high degree of correlation was obtained with adjusted $R^2 = 0.9962$, leading to a reasonably close value of predictive and observed runs together with the very low coefficient of variance% (3.40). The three-dimensional response surface plots have shown the relation between the independent variables. It is clear from the above polynomial equation that on increasing the TPP concentration, the EE increases. The increased chitosan to PEG ratio and drug concentration increases the EE by enhancing the available drug to bind with the polymer. The crosslinking time has shown a significant decrease in EE while increasing the time, due to the release of the drug because of the excessive swelling of the water-soluble PEG [Figure 4a].

Model fitting for drug release (mgGAE)

Cumulative drug release plays a very important role in determining the quality of the microparticles as it ensures the controlled release of the entrapped drug. The drug release was estimated by evaluating the total phenolic content of the released buffer. The polynomial equation for the drug release of the second order mathematical relation of the independent variables was expressed with the following equations:

$$Drug\ release\ (mgGAE) = +945.58 + 109.32A + 109.02B - 14.14C - 264.02D - 131.54AB - 102.41AC - 81.57AD + 7.03BC - 40.62BD - 36.24\ CD - 368.39A^2 - 133.25\ B^2 - 116.48\ C^2 - 240.07\ D^2$$

The range varied from 55.58 mg GAE to 985.42 mg GAE of the tests performed in the Box-Behnken model [Table 1]. The maximum release was obtained for the run having levels of independent variables

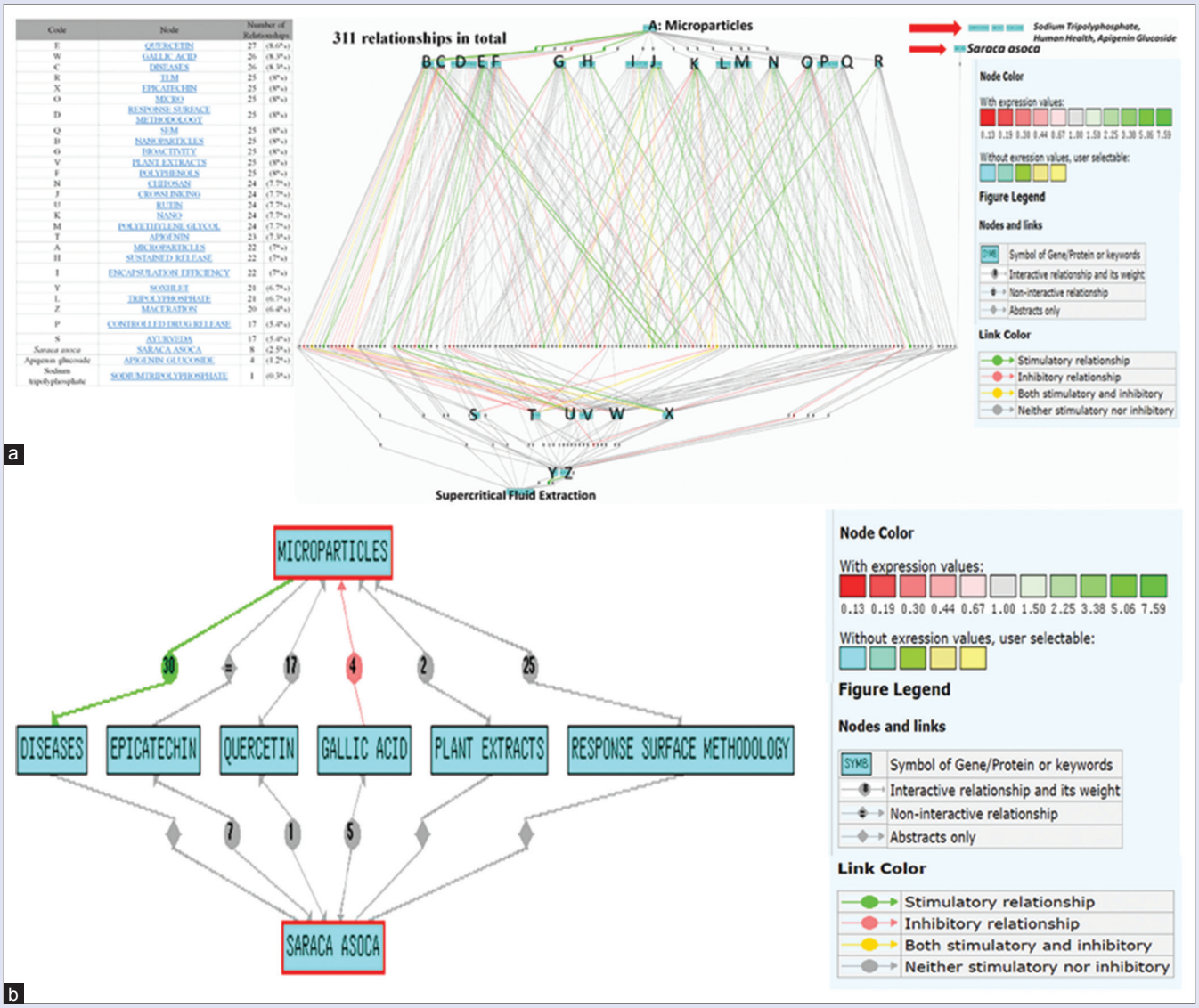


Figure 3: (a) Chilibot analysis on 31 parameters discovered from multifaceted interaction studies. Chilibot (http://www.chilibot.net/) searched the PubMed abstracts and constructs a content-rich relationship networks among the research of formulations, drug loading, drug release or natural polymers. A Chilibot query using 31 keywords was selected from Nano and microparticles fabricating studies. No relationship was reported for *Saraca asoca* and chosen polymers. We further filtered the graph by displaying only strong (weight >5%) interactive relationships (i.e., excluding noninteractive relationship and abstract co-occurrence only). The resulting graph, containing 31 query terms, the isolated *Saraca asoca was* shown in the above right corner. It was included in the Figure, but due to the existence of noninteractive relationship or abstract co-occurrence only relationship (not shown) between them and some query parameters. It was displayed as isolated units because there was no interactive relationship connecting it to desired parameters. (b) Chilibot analysis on the keywords have produced a hypothesis on the basis of no interactive relationship connecting it to other parameters searched throughout the PubMed abstracts and constructs content-rich relationship networks among parameters

A = 0, B = +1, C = 0 and D = −1. The ANOVA was found to be significant ($P < 0.01$) and lack of fit was not significant ($P > 0.05$), concluding the linearity and adequacy of the model. The signal to noise ratio was 79.85 with an adjusted $R^2 = 0.9968$, showing the model to be highly suited and correlative to the predictive and actual data. The relation between independent variables was drawn from the three-dimensional graphs between the factors. The drug release was found to be increasing on giving the increased amount of chitosan-PEG ratio and TPP concentration by aggregating the drug concentration available for entrapment. The increase in crosslinking time has lowered the drug release efficiency by decreasing the amount of entrapped drug in the particles due to excessive swelling of water-soluble PEG and chitosan while crosslinking. Drug concentration was found to be enhancing the drug release capability of the particles as the amount of binded drug was more on the surface of the microparticles [Figure 4b].

Validation of the best-fitted model

The optimal conditions selected after the fitting of the models were considered to be maximizing the drug loading and drug release of the particles. The best condition to be validated by the graphical optimization of Design expert 9.0. The predictive and experimental values of the overlay predictive graph were found to be in the range of the standard deviation of the determinations. The standard deviation of encapsulation efficiency and drug release were found to be 1.33 and 16.37 with the experiments run in triplicates. A two-sided validation have found these to be in the 95% of confidence interval showing the validation of

Table 1: Effects of variables at three levels on the encapsulation efficiency of polyphenols and drug release on *Saraca asoca* extract

Run order	Chitosan level (w/v)	Drug concentration (w/v)	TPP concentration (w/v)	Crosslinking (mins)	*Saraca asoca*			
					Encapsulation efficiency (%)		Total drug release (mg GAE)	
					Actual value	Predicted value	Actual value	Predicted value
1	1 (−1)	15 (0)	7.5 (0)	30 (−1)	35.2	37.1	410.7	410.3
2	1 (−1)	20 (+1)	7.5 (0)	60 (0)	29.4	29.8	565.2	575.2
3	1 (−1)	15 (0)	5 (−1)	60 (0)	18.6	17.7	251.3	263.1
4	1 (−1)	10 (−1)	7.5 (0)	60 (0)	20.1	19.9	118.1	94.1
5	1 (−1)	15 (0)	10 (+1)	60 (0)	29.3	28.7	426.7	439.7
6	1 (−1)	15 (0)	7.5 (0)	90 (+1)	2.9	2.3	55.6	45.4
7	2 (0)	15 (0)	10 (+1)	30 (−1)	59.2	59.4	880.9	875.2
8	2 (0)	10 (−1)	7.5 (0)	30 (−1)	51.9	51.2	677.4	686.6
9	2 (0)	15 (0)	5 (−1)	30 (−1)	61.6	61.4	840.9	830.9
10	2 (0)	20 (+1)	7.5 (0)	30 (−1)	73.8	72.9	985.4	985.9
11	2 (0)	15 (0)	7.5 (0)	60 (0)	66.1	66.0	933.5	945.6
12	2 (0)	15 (0)	7.5 (0)	60 (0)	66.9	66.0	927.1	945.6
13	2 (0)	15 (0)	7.5 (0)	60 (0)	64.8	66.0	946.4	945.6
14	2 (0)	20 (+1)	5 (−1)	60 (0)	46.1	45.8	823.8	812.0
15	2 (0)	10 (−1)	10 (+1)	60 (0)	32.3	31.8	557.7	565.7
16	2 (0)	15 (0)	7.5 (0)	60 (0)	65.2	66.0	959.2	945.6
17	2 (0)	10 (−1)	5 (−1)	60 (0)	38.1	38.2	594.6	608.0
18	2 (0)	20 (+1)	10 (+1)	60 (0)	51.6	50.7	815.0	797.8
19	2 (0)	15 (0)	7.5 (0)	60 (0)	67.4	66.0	961.8	945.6
20	2 (0)	15 (0)	10 (+1)	90 (+1)	11.8	12.8	274.0	274.6
21	2 (0)	10 (−1)	7.5 (0)	90 (+1)	11.0	11.8	227.1	239.8
22	2 (0)	20 (+1)	7.5 (0)	90 (+1)	16.1	16.7	372.7	376.6
23	2 (0)	15 (0)	5 (−1)	90 (+1)	11.8	12.4	379.0	375.4
24	3 (+1)	15 (0)	7.5 (0)	30 (−1)	62.6	62.3	785.7	792.0
25	3 (+1)	10 (−1)	7.5 (0)	60 (0)	28.3	28.7	595.1	575.8
26	3 (+1)	20 (+1)	7.5 (0)	60 (0)	44.3	45.4	516.0	530.7
27	3 (+1)	15 (0)	5 (−1)	60 (0)	41.1	41.7	686.3	686.6
28	3 (+1)	15 (0)	10 (+1)	60 (0)	28.2	29.1	452.1	453.5
29	3 (+1)	15 (0)	7.5 (0)	90 (+1)	4.2	1.6	104.3	100.8

TPP: Tripolyphosphate; GAE: Gallic acid equivalent

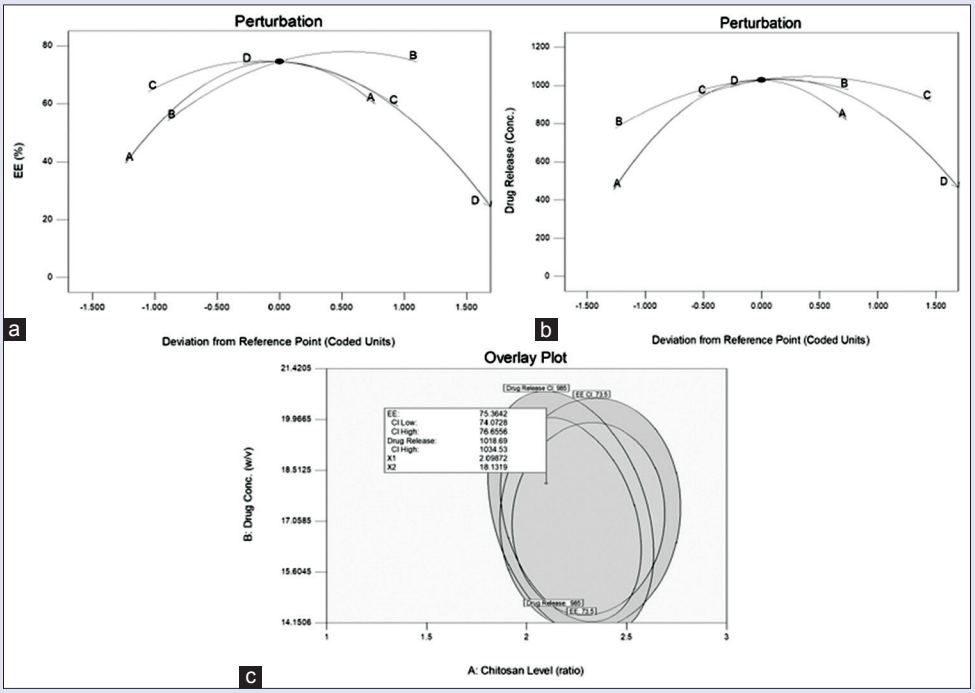


Figure 4: Perturbation plot showing the effect of all parameter on (a) Encapsulation efficiency (%) (b) drug release (µg GAE g_{beads}^{-1}) (c) overlay plot of optimized values showing best response at optimized factors, where X1: Chitosan level, X2: Drug conc. of *Saraca acosa* microparticles A, B, C and D represents Chitosan: Polyethylene glycol ratio, Drug Conc.(w/v), sodium tripolyphosphate conc. (%) and crosslinking time (mins.) respectively

the model. The optimized level after the validation were selected with the levels of independent variables A = 2.1, B = 18.13, C = 6.55, and D = 34.44 [Figure 4c].

Flowability and swelling behavior

The quality control of dried microparticles is performed observing the powder flowability.[31] The crude extract of *S. asoca* is very sticky in nature and was checked for its flowability and a CI of 28.07 ± 0.12 and Hausner ratio (HR) of 1.39 ± 0.02 was observed, which shows its poor fluidic nature.[31,32] The fluidic properties of empty formulation have shown a CI (%) of 25.35 ± 1.39 with HR of 1.34 ± 0.02, which corresponds with a good flowability in comparison to crude extracts. The drug-loaded microparticles have shown a better CI of 19.90 ± 0.21% and HR of 1.24 ± 0.03 [Table 2]. As it is clear that due to the high bulk density of the loaded microparticles it can be store and maintained in small space. The HR and CI determine the compressibility of the microparticles as well. The microparticles prepared by chitosan-PEG crosslinking were better than the crude extract in storage and processing conditions. Higher the HR and CI values more cohesive and sticky the product would be. The swelling behavior of the microparticles in pH 7.4 has shown the less amount of swelling (21%) after 25 min of immersion; contrary pH 1.2 indicated the swelling of 85%. The microparticles in pH 7.4 have shown no erosion till 24 h, because of the deprotonation of the amino groups in chitosan reducing the electrostatic repulsion between the polymeric chains by forming the hydrogen bonds. Whereas, due to acidic condition the solubility of PEG and protonation of chitosan amino groups have led to the erosion of microparticles, similar to earlier reported works.[10-12]

Particle size distribution and Poly Despersity Index (PDI) of sample

Zeta sizer (Malvern, USA) was used to check the particle size and polydispersity index of the microparticles. *S. asoca* validation microparticles have PDI of 1.00 and size was observed to be 1157 nm.

In vitro release of encapsulated particles

In vitro release of the microencapsulated particles was done in three buffers (0.1 M HCL (pH 1.2) and Phosphate-buffer saline (pH 6.8 and 7.4). Maximum release was observed of 86.3% for *S. asoca*, respectively, at 24 h [Figure 5]. Approximately 38.34% drug was released, respectively, at HCl (pH 1.2) and PBS (pH 6.8). The percentage release in difference in different media suggests the release to be in pH-dependent manner. With the increase in the pH of the release medium, there was an increase in the drug release. Chitosan in the acidic medium can swell very efficiently due to the electrostatic repulsion and solvation of the ionic groups of the chain. This movement of the ions generates a gap between the molecules and facilitates the penetration of water molecule between the networks.[11] In microparticles, the drug has binded with the protonated amino group of chitosan due to which, the large-sized phytomolecule forms a denser crosslinked bead and prevents the entry of water molecules leading to less release in low pH.[33] The mechanism of drug release was derived from the drug released data of optimized formulation as shown in Figure 4. The Higuchi model of drug release kinetics with first order release having R^2 = 0.9662 and R^2 = 0.9853 was found to be best fitted for the model [Figure 6]. The first-order release model describes the porous matrix based dissolution of the drug and the dependency of the release behavior on the amount of drug entrapped. The *n*-value model was further evaluated to establish the mechanism of release pattern. To find the exponent of *n* the portion of the release curve, where the ratio between the slope at time "*t*" and at end time is <0.6 should be used. If the *n* is ≤0.45 it corresponds to fickian diffusion and if it is >0.45 but <0.89, it describes nonfickian transport for spherical particles.[34,35] The slope obtained by the ratio between the fraction of drug release and the square root of time predicts the n value. With the *n* = 0.2020 for the microparticles, the diffusion behavior was found to be following the Fickian diffusion mechanism.

Table 2: Fluidic properties of *Saraca asoca* microcapsules prepared by freeze drying

Samples	BV (mm)[a]	TV (mm)[a]	HR[a]	CI (%)[a]	Flowability
Control	1.97±0.06	1.47±0.02	1.34±0.02	25.35±1.39	Passable
Microparticles	2.16±0.05	1.73±0.03	1.24±0.03	19.90±0.21	Passable
Extract	2.28±0.04	1.64±0.03	1.39±0.02	28.07±0.12	Poor

[a]Mean±SD based on triplicate runs. BV: Bulk volume; TV: Final tap volume; HR: Hausner ratio; CI: Carr's Index; SD: Standard deviation

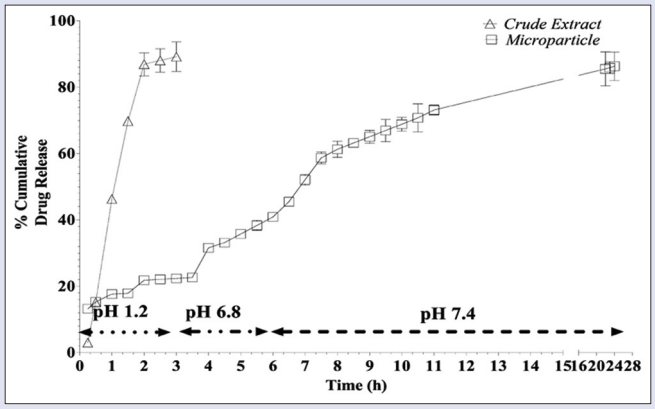


Figure 5: Percentage release graph for the selected *Saraca asoca* microparticles where; (a) Crude *Saraca asoca* extract. (b) Chitosan: Polyethylene glycol (2.1:0.9), Drug (18.13%), sodium tripolyphosphate (6.55%) and crosslinking time (34.44 min)

Fourier transform infrared spectroscopy

The IR spectra of the empty particle, *S. asoca* extract and particle loaded with the extract is given in Figure 7. The IR spectrum of the extract reveals distinct bands at 3254 besides 1616 cm^{-1} and 1090 as well as 890 cm^{-1} showing the presence of aromatic ring stretch in addition out of plane bends. The characteristic peaks have confirmed the presence of polyphenolic groups in the extracts. The empty particle crosslinked with TPP have shown the peaks at 3846–3620 cm^{-1}, 3148–3039 cm^{-1}, 2886 cm^{-1}, 2357 cm^{-1}, 1769–1701 cm^{-1}, 1559–1485 cm^{-1}, 1239 cm^{-1}, and 770–669 cm^{-1}. The peaks at 1701 cm^{-1} of C = O bond of primary amide group of chitin was shifted from its primary location of 1652 cm^{-1} instigating the ionic interaction between the Chitosan and TPP. A peak at 1559 cm^{-1} of N-H bending was also observed in the same. The peaks at 1239 cm^{-1}, 2886 cm^{-1} and 3620 cm^{-1} were consistent with the distinctive peak of PEG which is characteristic to the bending vibration of C-O, C-H and O-H bonded to N-H, respectively. The outcome of this study was that drug-loaded microparticles follow a very orderly disintegration pattern when compared to either pure extract or the blank particles, which confirms by the not altered drug-loaded matrix. It further indicates that loaded drug and wall material has not undergone any chemical change while producing microparticles.

Differential scanning colorimetry

For the analysis of the physical state of the drug inside the microparticles the DSC was performed on the validated microparticles and crude extract of *S. asoca*. *S. asoca* extract has shown a peak at 80°C [Figure 8a] and empty microparticles have shown three peaks at 59°C, 101°C and

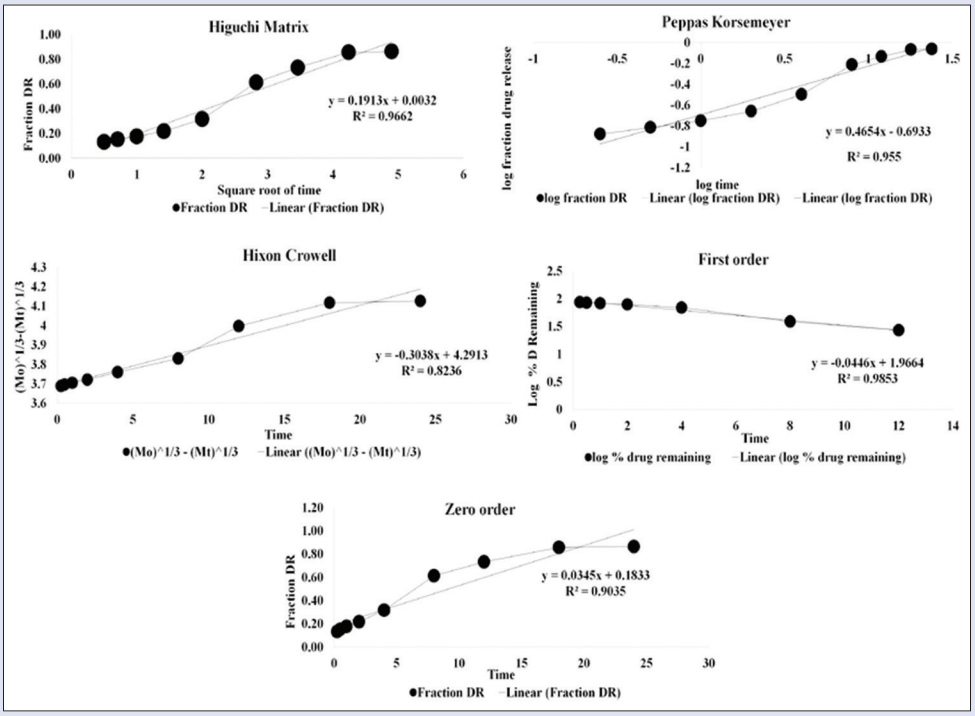


Figure 6: *In vitro* release kinetic models of *Saraca asoca* extract loaded microparticles of Chitosan

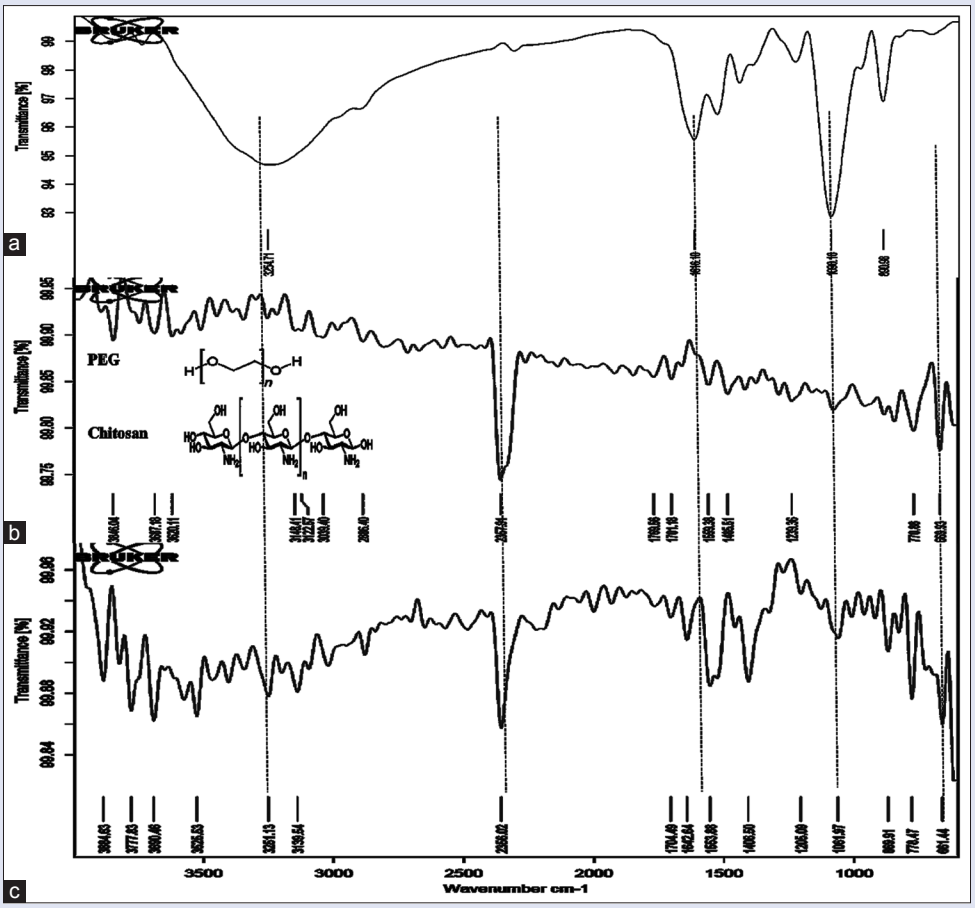


Figure 7: Fourier-transform infrared spectroscopy spectrum of (a) *Saraca asoca* extract, (b) Empty microparticle (c) *Saraca asoca* extract loaded microparticles

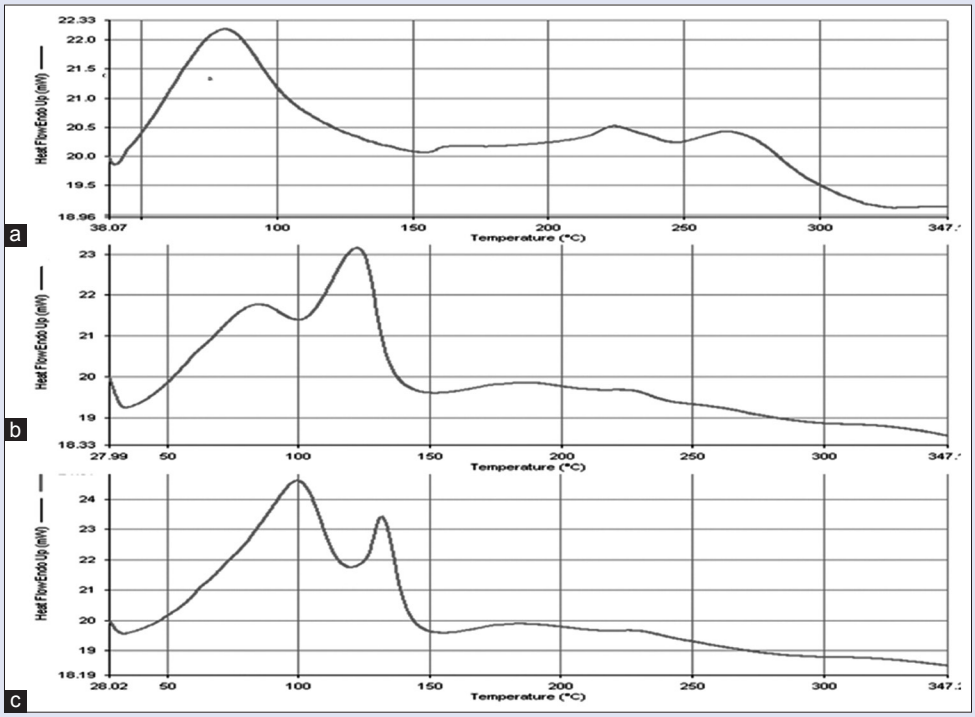


Figure 8: Differential scanning calorimetric thermograms of; (a) *Saraca asoca* extract. (b) Empty particle with Chitosan: Polyethylene glycol (2:1), drug (0%), sodium tripolyphosphate (5%) and crosslinking time (60 min). (c) Chitosan: Polyethylene glycol (2.1:0.9), Drug (18.13%), sodium tripolyphosphate (6.55%) and crosslinking time (34.44 min)

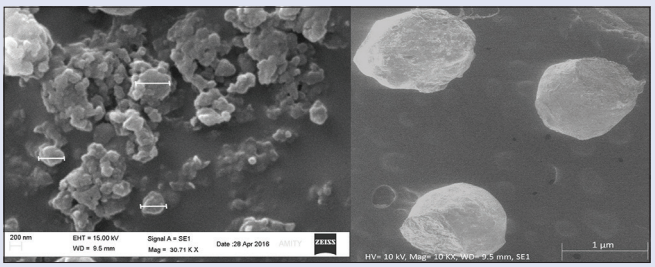


Figure 9: Scanning electron microscopy of *Saraca asoca* microparticle (Chitosan: Polyethylene glycol (2.1:0.9), Drug (18.13%), sodium tripolyphosphate (6.55%) and crosslinking time (34.44 min)

195°C [Figure 8b]. Validation sample (Chitosan: PEG [2.1:0.9], drug [18.13%], TPP [6.55%] and crosslinking time [34.44 min]) have shown a very less peak at 80°C showing crosslinking of the drug with the polymer [Figure 8c]. And due to which the polymer and drug mixture have shown a shift in the peak of 123°C. These results suggested that a binded form of drug existed in the microparticles and the physical state influences the release kinetics as well as the morphology of the microparticles. The melting points of drug or the polymers in the microparticles indicate that there were no chemical interactions with the polymers which were found similar by Fourier-transform IR spectroscopy studies.

Scanning electron microscopy of particles

Surface morphology and particle distribution were checked by scanning electron microscopy of microparticles. The lump of particles was observed due to free drying of the particles before performing the microscopy. *S. asoca* validation was found to be having particle size of 1.15 µm [Figure 9], and smooth surface was also observed for the same.

Anti-oxidant activity

Due to DPPH paramagnetic nature, it gives a strong signal at 517 nm, which decreases from purple to yellow by the scavenging of free radicals available in it by the antioxidant molecules. This yellow color gets interference from the colored substance present in plant extracts, as like chlorophyll, xanthophyll, pigments, etc. The HPLC-based DPPH radical scavenging activity is better in comparison to spectroscopic methods due to the colored nature of herbal extracts, which hinders with the absorbance. The standard plot was generated and a linearity was derived between 31.25 and 250 µg/mL of TROLOX, with a R^2 = 0.9918 and R_t = 10.10 min. The IC_{50} was derived by plotting a non-linear regression between the $\log_{10}$ of concentration versus % inhibition and it was found to be 28.87 µg/mL. Similarly, the IC_{50} of crude drug and microparticles was generated for the concentration range of 31.25–1000 µg/mL and the IC50 value was found to 250.8 µg/mL of crude and 188.2 µg/mL, respectively [Figure 10]. An IC_{50} is a concentration at which 50% DPPH remains unused or the sample was able to scavenge up to 50% of DPPH only. Lower value denotes the stronger activity, which suggests the sustained release of the sample with microencapsulation and due to the protection of drug by the polymer.

CONCLUSION

Due to the sacred nature and scarcity of the availability of the *S. asoca* it is very important to maintain its usefulness as a medicinal boon. The controlled release of the extract will enhance the chances of controlled use as well as a benefit for the medicinal use. The finding in data mining by Chilibot suggests the novelty of the method and the possible importance of the sustained release in the human health and preservation of the plant. An optimum formulation was designed by statistical optimization using Box-Behnken design. The enhanced encapsulation was achieved by incorporating a novel method of chilling by liquid nitrogen which

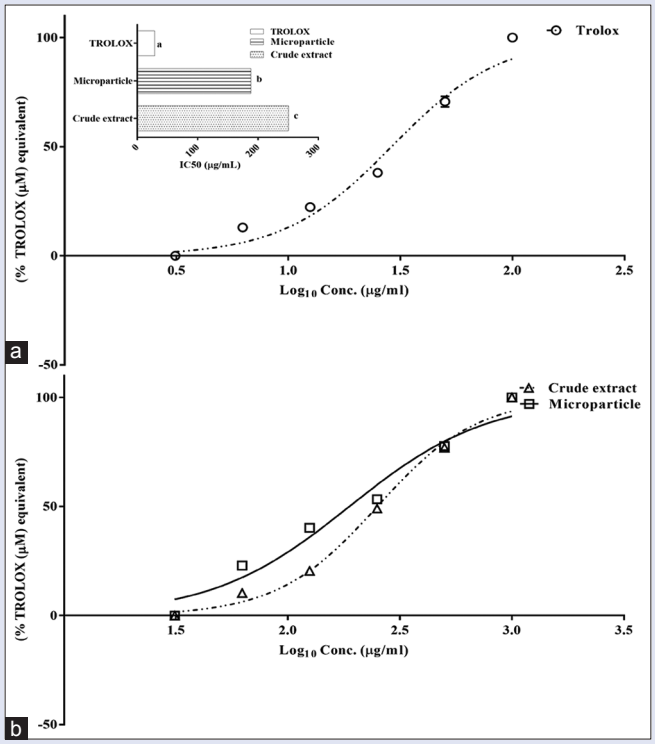


Figure 10: 1,1-Diphenyl-2-picrylhydrazyl free radical scavenging activity of (a). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid standard (3.12-100 µg/ml) (b). *Saraca asoca* extract and Microparticles (Chitosan: Polyethylene glycol [2.1:0.9], Drug [18.13%], sodium tripolyphosphate [6.9%] and crosslinking time [34.44 min]). Box-plot showing mean values of the IC_{50} values with each different alphabet showing statistical difference at $P \leq 0.05$

reduces the further use of toxic crosslinking agents and decreases the health hazard issues. The method uses a formulation of chitosan: PEG (2.1:0.9), drug (18.13%), TPP (6.55%) and crosslinking time (34.44 min) with a very good encapsulation efficiency and drug release. The controlled release of the drug was obtained by the formulation with a sustained release up to 24 h. The increase in antioxidant activity of the drug was obtained due to its use as the microparticles which shows the benefits of using the formulation in oral doses. From above, it can be inferred that *S. asoca* extract loaded microparticles have definitely increased the bioavailability and stability of the drug.

Acknowledgment

The author would like to thank the university grant commission, Ministry of Human Resource and Development, India for providing the Basic Scientific Research grant fellowship to carry out the research work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Sadhu SK, Khatun A, Phattanawasin P, Ohtsuki T, Ishibashi M. Lignan glycosides and flavonoids from *Saraca asoca* with antioxidant activity. J Nat Med 2007;61:480-2.
2. Pradhan P, Joseph L, Gupta V, Chulet R, Arya H, Verma R, *et al. Saraca asoca* (Ashoka): A review. J Chem Pharm Res 2009;1:62-71.
3. Middelkoop TB, Labadie RP. The action of *Saraca asoca* roxb. De Wilde Bark on the PGH2 synthetase enzyme complex of the sheep vesicular gland. Z Naturforsch C 1985;40:523-6.
4. Thakur RS, Pawar RS, Ahirwar B. Evaluation of *Saraca indica* for the management of dexamethasone-induced osteoporosis. J Acute Med 2016;6:7-10.
5. Jain P, Nale A, Dabur R. Antimicrobial metabolites from *Saraca asoca* impairs the membrane transport system and quorum-sensing system in *Pseudomonas aeruginosa*. Arch Microbiol 2018;200:237-53.
6. Begum SN, Ravikumar K, Ved DK. 'Asoka' an important medicinal plant, its market scenario and conservation measures in India. Curr Sci 2014;1:26-8.
7. Parthasarathi S, Ezhilarasi PN, Jena BS, Anandharamakrishnan C. A comparative study on conventional and microwave-assisted extraction for microencapsulation of *Garcinia* fruit extract. Food Bioprod Process 2013;91:103-10.
8. Hiremath K. Recent Advances in Environmental Science. New Delhi: Discovery Publishing House; 2003.
9. Hiremath MB, Gopinath S. *Saraca asoka*. Curr Sci 2005;88:1359.
10. Lin WC, Yu DG, Yang MC. PH-sensitive polyelectrolyte complex gel microspheres composed of chitosan/sodium tripolyphosphate/dextran sulfate: Swelling kinetics and drug delivery properties. Colloids Surf B Biointerfaces 2005;44:143-51.
11. Buranachai T, Praphairaksit N, Muangsin N. Chitosan/polyethylene glycol beads crosslinked with tripolyphosphate and glutaraldehyde for gastrointestinal drug delivery. AAPS PharmSciTech 2010;11:1128-37.
12. El-Gibaly I. Development and *in vitro* evaluation of novel floating chitosan microcapsules for oral use: Comparison with non-floating chitosan microspheres. Int J Pharm 2002;249:7-21.
13. Hejazi R, Amiji M. Chitosan-based gastrointestinal delivery systems. J Control Release 2003;89:151-65.
14. Müller C, Rahmat D, Sarti F, Leithner K, Bernkop-Schnürch A. Immobilization of 2-mercaptoethylamine on oxidized chitosan: A substantially mucoadhesive and permeation enhancing polymer. J Mater Chem 2012;22:3899-908.
15. Tigli Aydin RS, Pulat M. 5-Fluorouracil encapsulated chitosan nanoparticles for ph-stimulated drug delivery: Evaluation of controlled release kinetics. J Nanomater 2012;2012:1-10.
16. Wang LY, Gu YH, Zhou QZ, Ma GH, Wan YH, Su ZG, *et al.* Preparation and characterization of uniform-sized chitosan microspheres containing insulin by membrane emulsification and a two-step solidification process. Colloids Surf B Biointerfaces 2006;50:126-35.
17. Prego C, Torres D, Fernandez-Megia E, Novoa-Carballal R, Quiñoá E, Alonso MJ, *et al.* Chitosan-PEG nanocapsules as new carriers for oral peptide delivery. Effect of chitosan pegylation degree. J Control Release 2006;111:299-308.
18. Vasconcellos FC, Goulart GA, Beppu MM. Production and characterization of chitosan microparticles containing papain for controlled release applications. Powder Technol 2011;205:65-70.
19. Fernandes SC, de Oliveira IR, Fatibello-Filho O, Spinelli A, Vieira IC. Biosensor based on laccase immobilized on microspheres of chitosan crosslinked with tripolyphosphate. Sens Actuators B Chem 2008;133:202-7.
20. Celik M, Wendel SC. Spray drying and pharmaceutical applications. In: Handbook of Pharmaceutical Granulation Technology. North Carolina, 2005;195:129-55.
21. Prakongpan S, Nagai T. Solubility of acetaminophen in cosolvents. Chem Pharm Bull 1984;32:340-3.
22. Song FL, Gan RY, Zhang Y, Xiao Q, Kuang L, Li HB, *et al.* Total phenolic contents and antioxidant capacities of selected Chinese medicinal plants. Int J Mol Sci 2010;11:2362-72.
23. Sakakibara H, Honda Y, Nakagawa S, Ashida H, Kanazawa K. Simultaneous determination of all polyphenols in vegetables, fruits, and teas. J Agric Food Chem 2003;51:571-81.
24. Saha J, Mukherjee S, Gupta K, Gupta B. High-performance thin-layer chromatographic analysis of antioxidants present in different parts of *Saraca asoca* (Roxb.) de Wilde. J Pharm Res 2013;7:798-803.
25. Chen H, Sharp BM. Content-rich biological network constructed by mining pubMed abstracts. BMC Bioinformatics 2004;5:147.
26. Dash S, Murthy PN, Nath L, Chowdhury P. Kinetic modeling on drug release from controlled drug delivery systems. Acta Pol Pharm 2010;67:217-23.
27. Quispe-Condori S, Saldana MD, Temelli F. Microencapsulation of flax oil with zein using spray and freeze drying. LWT Food Sci Technol 2011;44:1880-7.
28. Qi L, Xu Z, Jiang X, Hu C, Zou X. Preparation and antibacterial activity of chitosan nanoparticles. Carbohydr Res 2004;339:2693-700.
29. Saharan V, Mehrotra A, Khatik R, Rawal P, Sharma SS, Pal A, *et al.* Synthesis of chitosan

based nanoparticles and their *in vitro* evaluation against phytopathogenic fungi. Int J Biol Macromol 2013;62:677-83.

30. Chandrasekar D, Madhusudhana K, Ramakrishna S, Diwan PV. Determination of DPPH free radical scavenging activity by reversed-phase HPLC: A sensitive screening method for polyherbal formulations. J Pharm Biomed Anal 2006;40:460-4.
31. Onwulata C. Encapsulated and Powdered Foods. Pennsylvania: CRC Press; 2005.
32. Fuchs M, Turchiuli C, Bohin M, Cuvelier ME, Ordonnaud C, Peyrat-Maillard MN, *et al.* Encapsulation of oil in powder using spray drying and fluidised bed agglomeration. J Food Eng 2006;75:27-35.
33. Shu XZ, Zhu KJ. The influence of multivalent phosphate structure on the properties of ionically cross-linked chitosan films for controlled drug release. Eur J Pharm Biopharm 2002;54:235-43.
34. Ritger PL, Peppas NA. A simple equation for description of solute release II. Fickian and anomalous release from swellable devices. J Control Release 1987;5:37-42.
35. Ritger PL, Peppas NA. A simple equation for description of solute release I. Fickian and non-fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs. J Control Release 1987;5:23-36.