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Optimization of fermentation variables for Ayurvedic formulation, *Drakshasava* by Response Surface Methodology and its marker based validation studies

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The concept of fermentation has been part of various Ayurvedic formulations since ages. *Drakshasava* is one of the self fermented *Ayurvedic* medicine containing *Vitis vinifera* L. as major ingredient. However, lack of modern technology and long processing time of these classical formulations may interfere with growing needs of industries. To cope up with the current health care standards, optimization of existing *Ayurvedic* formulations with multi-marker approach is required. A statistical model has been developed to optimise the fermentation process of *Drakshasava* (without *prakshepa dravya*). The selected fermentation process parameters i.e., pH, incubation temperature and fermentation time were optimized by BBD of Response Surface Methodology. The combination of parameters was selected to achieve a required alcohol percentage. The method was validated by HPTLC with respect to non established biomarker piperine, a bioavailability enhancer required to prevent early metabolism of resveratrol (therapeutic active constituent of *Drakshasava*). The quadratic model was found significant with F and P value, 2.20 and 2.302, respectively. The optimum conditions obtained in the batch fermentation process were incubation temperature (30.59°C); pH (4.84) and fermentation period of 9 days with predicted concentration of 9.82% alcohol. The method was validated in accordance to ICH guidelines and found statistically reproducible and selective for the quantitative estimation of piperine in *Drakshasava*. The in-house fermented samples were found to have significant increase in piperine concentration (2.42 µg/mL), compared to decoction (0.11 µg/mL). The optimized method reduced the manufacturing time without interefering the Pharmacopoeial limits. It leads to standardization, better quality and consistency of *Drakshasava*.

Keywords: Chromatography, Drakshasava, Response Surface Methodology, *Vitis vinifera*

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Revival of interest in herbs and their use in healthcare has been seen from last few decades[1]. Today, plant derived medicines are becoming prime as well as alternative source of pharmaco-therapeutics over allopathic treatment. Ayurveda is one of the oldest traditional systems of medicine (India) along with Traditional Chinese medicine TCM (China) and Kampo (Japan) using plants as raw material[2]. It is based on fundamental principles and practices derived from "traditional scripts including *Charaka Samhita, Shushruta Samhita, Kashyapa Samhita, Sharangadhara Samhita, Chakradatta, Ashtanga Hridaya Gada Nigraha, Bhaishajya Ratnavali*, *Yogaratnakara* Ayurvedic Formulary of India (AFI) etc.[3-7]. In Ayurvedic system, extraction of active constituents are carried by different manufacturing processes and formulated to various dosage forms[8]. Among those, '*Sandhana kalpana*' is a distinctive dosage form involving production of acidic and self fermented alcoholic formulations[9]. It Includes both *asava* (fresh herbal juices) and *aristha* (herbal decoction) where self-generated ethyl alcohol, limits up to 10%[10]. The required yeast inoculums for fermentation, comes from *Woodfordia fruticosa* (Dhataki) flowers, an herbal source. Most of these preparations contain additional herbs (usually spices) added in very small quantity to improve assimilation. The fermentation used to take place in a closed vessel, which is to be kept undisturbed for a month[11,12]. The quality of medicated wines thus formed depends on various variables including temperature, pH, time, type of raw drug, inoculum and followed methodology[10]. However, the basic principles of fermentation had remained fundamentally similar from those used in

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traditional formulations compared to other medicines. The studies are required to preserve and improve this ancient knowledge for betterment of society following the classical indications with compliance of Good Manufacturing Practices (GMP). Thus, there is a need to build a relation between ancient and modern science (including use of advanced analytical and biotechnological techniques) to improve the methodology and validate the effectiveness of Ayurveda basedmedicines[13]. To cope up with growing needs of herbal industries for health care drugs, standardization and optimization of existing classical formulations with multi-marker approach is required.

Drakshasava is a classical fermented formulation of *Ayurveda*, of which *Vitis vinifera* L. (grapes) is a major ingredient[14] along with *prakshepa dravyas* (spices added in small quantity). This grape-vine preparation is well known drug in market for blood, cardiac and various inflammatory diseases[14]. Various preparations of grapes including wines, juices and dried fruits have characteristic phenolic profiles providing exceptional health benefits including cardio protective, anti-inflammatory, anti-carcinogenic, antimicrobial and antioxidant properties[15]. In the present research, the classical method to prepare an *Ayurvedic* formulation containing *Vitis vinifera* L. (*Drakshasava*) has been improvised using modern biotechnological practices and its impact has been studied. The method is optimized by response surface methodology (RSM), taking consideration of exact temperature, pH and duration of fermentation to prepare the modified *Drakshasva* (without *prakshepa dravyas*).

The RSM is a statistical, interactive multi variable tool for studying combined effects of various factors at a time and useful over classical method with single factorial approach[16]. It is used to study the operational variables for experimental design, model development, test variable and conditions optimization. The methodology has been popularly used for optimization of process parameters for extraction[17,18], fermentation[19-21], drug delivery systems[22,23], analytical techniques[24] etc, which lead to enhancement of vegetable oil bioconversion[25], alcohol, biomass and enzyme production in different studies[26-28]. It involves various experimental designs of which Box–Behnken design (BBD)[29-32] is most popularly used, as it is flexible, more convenient and less expensive to run, compared to others[33]. It is a independent quadratic design, rotatable or almost rotatable, involving combinations treated at the midpoints of the edges and at the center of the process space[22].

The major aim of the research is to achieve better uniformity and reliability in manufacturing of classical fermented formulations. To the best of our expertise, this is a foremost attempt in optimizing *Drakshasava* using BBD of RSM the generated alcohol is thus considered as the key response for optimization. Further the standardization with non reported biomarker, piperine has been accomplished through validated High performance Thin Layer Chromatography (HPTLC) for the first time. The decoction and in-house *Drakshasava* samples (without *prakshepa dravya*) were quantitatively standardized and their comparative studies were carried out.

Material and Methods

Plant materials and formulations

All the raw materials for *Drakshasava* preparation were procured from the local departmental store and unjha pharmacy, rohinimarket, Delhi (Table 1). They were authenticated by Prof Vidhu Aeri at Dept of Pharmacognosy and Phytochemistry, Jamia Hamdard, New Delhi. Materials and deposited for further reference (Ref no. PP-V0117).

Standards and chemicals

The analytical grade organic solvents from Merk (Darmstadt, Germany) were used. CAMAG Linomat V applicator was used for HPTLC analysis having win CATS software (CAMAG, Muttenz, Switzerland). Aluminum backed silica gel 60 F_{254} HPTLC Plates from Merk. Marker compounds (piperine), of 98% purity was purchased from Natural Remedies Pvt. Ltd., Banglore, India.

Experimental Design

Production of medicated wine

The in-house *Drakshasava* was developed with certain modifications with reference to AFI (ii). The dried grapes were soaked in water over night. The grapes were grinded and further boiled to form

Table 1 — Composition of *Drakshasava* formulation **(AFI)**

S.no	Biological name	Quantity
1	*Vitisvinifera L.*(dried fruit)	4.8 kg
2	Water	49.152L to 12.288L
3	Sarkara API	4.8 kg
4	Honey API	4.8 kg
5	*Woodfordia fruticosa* (flowers)	336g

decoction (1/4). Then decoction was filtered using muslin cloth. After addition of *Sarkara* and honey to the decoction, it was stirred properly until homogeneous solution was obtained. Again filteration was carried out along with the addition of *Woodfordia fruticosa* L. flowers. The Table 1 describes the composition of Drakshasava prepared. The decoction samples were placed in incubator for fermentation at 30±2°C using sterile air tight containers. The study was carried out during the year 2017-2019.

Single factorial experiments

Based on previous 15 day studies on fermentation process of *Drakshasva*[34], single factorial experiments were conducted to assess the effect of certain parameter, on ethanol production. The experimental work was strictly carried out based on the BBD matrix.

Optimization of ethanol fermentation

The three level BBD (RSM) was choose to optimize the ethanol production in *Drakshasva* (without *prakshepa drvya*) using Design Expert software (Version 12.0.4.0). The design consisted of twelve factorial and five centre point replicates. A second order quadratic model was used to speculate the optimal point by correlating independent variables and its response. The three independent variables i.e., incubation temperature, medium pH and fermentation time (days) were selected as basic parameters for the study. They are assigned as X_1, X_2, X_3, respectively, further coded as +1,0 and -1at three different levels shown in Table 2. Table 3 predicts 17 runs of BBD experimental planning. The quadratic equation for three factors as follows:

$$Y = \beta0 + \beta1X_1 + \beta2X_2 + \beta3X_3 + \beta12\ X_1\ X_2 + \beta13\ X_1\ X_3 + \beta23\ X_2\ X_3 + \beta11X^2_1 + \beta22X^2_2 + \beta33X^2_3$$

Where, Y = predicted response

βo = Model constant
β1, β2, β3 = Linear coefficients
X1, X2, X3 = Independent variables
β12, β13, β23 = Cross product coefficient
β11, β22, β33 = Quadratic coefficient.

Determination of ethyl alcohol (distillation method)

Out of each prepared sample 25 mL was diluted with 150 mL of water in a distillation flask. About 90 mL of the distillate was collected into a 100 mL volumetric flask with adjustment of temperature up to 24.9ºC to 25.1ºC. The volume was make up and its specific gravity was determined. Through relative density table, alcohol content was analyzed as given in USP/NF[35].

Table 2 — Independent process variables with coded level

Process variable	LEVEL		
	-1	0	+1
Fermentation Time (Days)	6	9	12
Temperature (°C)	20	30	40
pH	3	5	7

Table 3 — Box-Behnken experimental design and response outcome

Run	A: Fermentation Time (Days)	B: Incubation Temperature (°C)	C:pH	Alcohol % (v/v)	
				Experimental	Predicted
1	9	40	7	4.12	4.08
2	6	40	5	4.67	4.70
3	9	30	5	9.82	9.80
4	9	30	5	9.78	9.80
5	9	30	5	9.85	9.80
6	9	40	3	4.48	4.08
7	9	20	3	4.38	4.46
8	6	30	3	3.82	4.41
9	9	30	5	9.77	9.80
10	12	30	7	2.88	2.88
11	9	30	5	9.78	9.80
12	12	20	5	4.11	4.08
13	6	20	5	3.8	3.76
14	12	40	5	4.49	4.52
15	12	30	3	4.61	4.60
16	9	20	7	2.73	2.75
17	6	30	7	3.5	3.51

HPTLC analysis

Preparation of test sample

50 mL each of decoction and fermented sample were dried to remove alcohol completely using water bath. Then, volume was made up by addition of 50 mL water. The samples were further subjected to successive solvent extraction, using n- hexane [50*3 mL], chloroform [50*3 mL] and ethyl acetate (EA) [50*3 mL] respectively. The, EA samples were evaporated till dryness and further reconstituted with methanol (AFI, Part-I). 10 mg/mL of each sample was prepared of which 2 µL was used for for HPTLC analysis.

Preparation of stock solution (reference) and calibration curves

Stock solutions (1 mg/mL) of piperine were prepared with methanol by dilution method. To detect the linearity, calibration curves were plotted. TLC plates were spotted with 2 µL piperine of each concentration range to get final concentration 20-400 ng/spot. The densitometry scanning was performed. Through calibration plots, their presence in samples were detected and quantified

HPTLC instrumentation

Both the samples and standards were applied on pre coated 60 F 254 silica gel plates with a dimension of 20 cm × 10 cm on CAMAG HPTLC System (Merck, Darmstadt, Germany) equipped with a 100 µL sample syringe with a Linomat V applicator under a flow of N_2 gas. Samples (2 µL) were applied as 6 mm wide bands and 13.2 mm distance was kept in between each band. The TLC development was carried out in linear ascending manner in CAMAG glass twin trough chamber of 20×10 cm after saturation. The mobile phase was selected using a polarity Vario System and optimized to Toluene: Ethyl Acetate: Formic acid: Methanol (6:5.5:0.8:0.4). The saturation time of 20 min were optimized for a good resolution with 22 min (85%) total run time at room temperature (27±2°C), 50±4% relative humidity respectively. The developed plates were dried and further scanned at different lamda max varying from 254 to 366nm using a spectro-densitometer (Scanner 3, CAMAG) having win CATS planar chromatography manager software (Version 1.30, CAMAG). The plates were developed upto 85% with slit dimension of 5*0.45 mm. Quantification of piperine in the samples was performed using the peak area with linear regression. The developed quantitative HPTLC validation method was evaluated for specificity, linearity, recovery, precision, sensitivity, robustness and accuracy studies as per ICH guidelines[36].

Results and Discussion

Fermentation process

The fermented formulations scripted in *Ayurveda* (arishtas and asavas) are considered as self generators of ethanol. They involve a gradient of rising alcohol leading to better extraction and biochemical transformation of phyto-constituents present in it[12]. These dosage forms shows better absorption, longer shelf life and high therapeutic efficacy, in comparison to other *Ayurvedic* medicines. Since a lot of fermented products including wines (grapes, rice, molasses etc.) are being processed worldwide with use of external sugar, yeast and modern biotechnologies; the *Sandhana kalpana* formulations does not requireany external source for carrying out fermentation. However, these formulations faces the constraints of long time process and lacks proper standardization and validation studies.

Drakshasava is one of the asava of grapes which uses *Woodfordia* flowers as yeast source and widely used for its high range inflammatory properties. Earlier studies on *Drakshasava* has came out with modified method of its preparation using modern technique. Since *Sandhana Kalpana* has limitation of 5-10% alcohol, the present studies are based on optimizing the fermentation process parameters of *Drakshasava* (without *prakshepa dravya*) by RSM.

Single factorial experiments (SFE)

To evaluate the effect of acertain selected parameter on alcoholpercentage, SFE were carried out.The ranges evaluated for different parameters are described in Table 3.

Optimization of extraction parameters by BBD

The statistical BBD proves to be an effective tool for optimizing *Drakshasava* formulation. Depending on the determined ranges, further optimization was carried out by appointing multiple regression analysis on the experimental data. Both the response variable and tested variables were associated by the second order polynomial equation as follows:

$$\text{Alcohol } \% = 9.80 + 0.0375*A + 0.3425*B - 0.5075*C - 0.1225*AB - 0.3525*AC + 0.3225*BC - 2.88*A^2 - 2.65*B^2 - 3.22*C^2$$

To determine the goodness of the model, summary of analysis of variance (ANOVA) for the fitted

quadratic polynomial model was used (Table 4) with coefficient of variation (CV) 0.734% and adjusted R^2 0.999. Since being the measure of standard deviation (0.0417), expressed as a percentage of the mean (5.68), so the lesser the CV value, reproducibility will be better[37]. The fitted model was found highly significant with R^2_{adj}=0.9998 which was close to R^2_{pred}=0.9990. Basically signal to noise ratio of greater than 4 is desirable, to navigate the design space. Thus reliability of the present model with respect to experimental values was confirmed by the CV (0.734%) and adequate precision (220.02), which in turns represent the ratio. The goodness of the model was determined through lack of fit test, with F (2.20) and p-value (2.302) respectively, being non significant. The p value of less than 0.1 proves that each coefficientis significant in the developed model, making it suitable for response prediction.

Interactions between the variable and the response were studied through three dimensional (3-D) response surface plots and contour plots respectively (Fig. 1). Among the selected variables, incubation temperature is effectual at higher levels while pH is effectual at lower level for better response. It takes initial 4-5 days to start fermentation (lag phase) i.e., alcohol production and then it increases with time (log phase) till it enters stationary phase with no further rise.

Incubation temperature has astounding impact on the formulation. At constant temperature, alcohol content increases with increase in pH with respect to time simultaneously, to a certain limit then decreases with further increase in pH. However similar results are noted in case of constant pH. Since *Sandhana Kalpana* preparation allows upto 10% permissible limit of alcohol, fermentation time is essential to be optimized, keeping temperature and pH constant. So all the selected parameters, pH, temperature and fermentation time proved to be rate limiting factors for alcohol production for Ayurvedic fermented formulation.

Model validation

According to BBD Model, the selected parameters i.e., temperature 30.59°C, pH 4.84 and fermentation time 9.03 (approx 9 days) have been recognized as best for the production of alcohol in modified *Drakshasva*. The alcohol production is estimated as 9.82% v/v, with desirability and standard error of 0.997 and mean 0.0862 respectively.

Marker based quantification

The piprine is one of the important alkaloid known for its pungency and bioavailability enhancer properties[38]. It is found commonly in most of all the plants used in traditional medicines and also as a spice world wide[39]. The concept of bio-enhancement originated in Ayurveda, is used since centuries to prepare formulations using crude drugs namely *Piper longum* Linn., *Zingiber officinale* Rosc., *Glycyrhhiza glabra* L. and others[40]. Bioenhancers act by either reducingthe therapeutic dose of co-administered drug, thus lowering toxicity and side effects, or enhancing its efficacy by reducing the resistance thus decreasing the raw material cost of manufacturing. The most of the classical formulations are rich in phenolics, known for their antioxidant and anti-inflammatory diseases. Since these phenols are water soluble, they are unable to cross the lipid membranes; hence less biovailable in therapeutic doses, so absorption boosters are required to enhance the

Table 4 — ANOVA for the Quadratic model

Source	Sum of Squares	df	Mean Square	f-value	p-value
Model	124.76	9	13.86	7953.92	<0.0001
A-Fermentation time	0.0113	1	0.0113	6.45	0.0386
B-Temperature	0.9385	1	0.9385	538.45	<0.0001
C-pH	2.06	1	2.06	1182.23	<0.0001
AB	0.0600	1	0.0600	34.44	0.0006
AC	0.4970	1	0.4970	285.18	<0.0001
BC	0.4160	1	0.4160	238.70	<0.0001
A^2	34.89	1	34.89	20020.85	<0.0001
B^2	29.65	1	29.65	17013.54	<0.0001
C^2	43.62	1	43.62	25029.32	<0.0001
Residual	0.0122	7	0.0017		
Lack of Fit	0.0076	3	0.0025	2.20	0.2302
Pure Error	0.0046	4	0.0012		

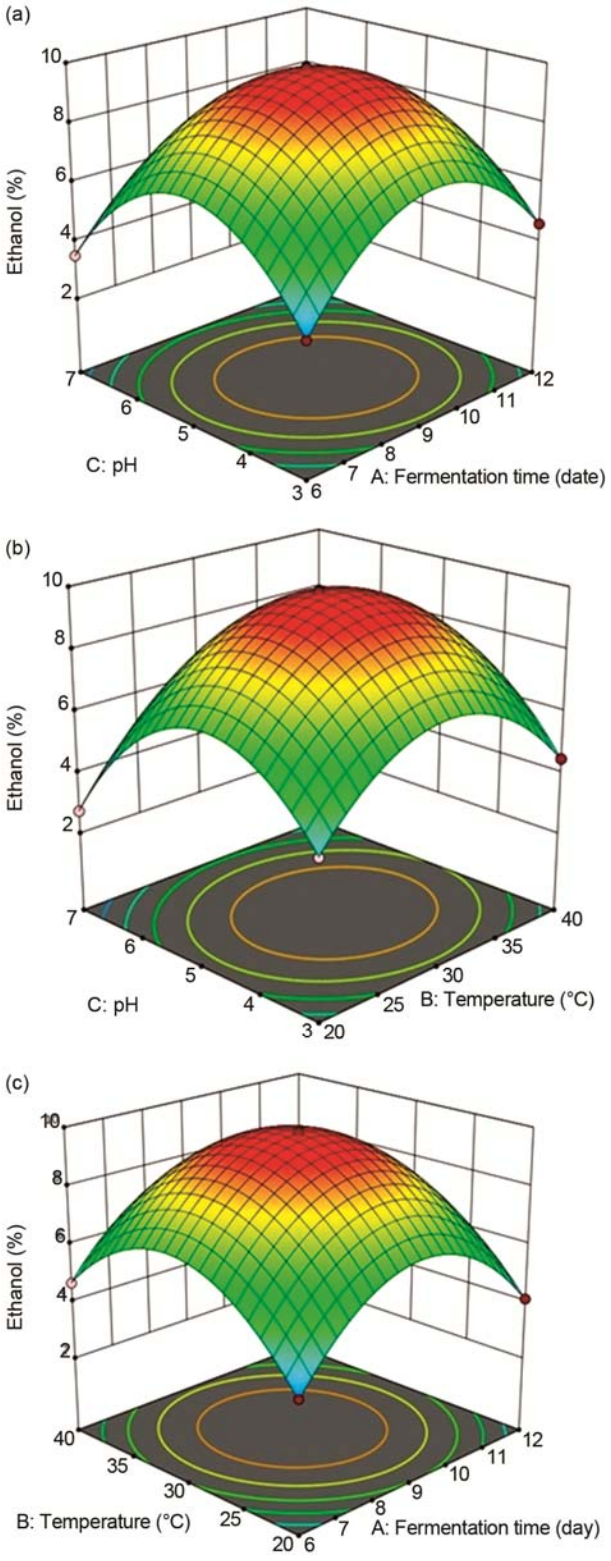

Fig. 1 — 3D Response surface plots showing influence of two variables on alcohol production in Drakshasava while keeping third variable constant. A. Impact of fermentation time and medium pH; B. influence of temperature and pH; C. influence of fermentation time and pH on alcohol percentage

intestinal absorption. As *Drakshasava* too rich in phenolics, is reported to contain various active constituents, such asgallic acid, catechin, rutin, quercetin, kaempferol, caffeic acid[34] and stilbenes (resveratrol and pterostilbene), respectively[41]. Out of which, resveratrol was found responsible for its major therapeutic efficacy and has become major area of research these days[42-45]. These are phytoalexins, produced by plant in response of stress or fungal infection[46]. However, the poor bioavailability of resveratrol in humans, being rapidily metabolized is a major drawback[47,48]. As per literature, various studies have been conducted to improve bioactivity of resveratrol in humans using piperine as potent bioenhancer in combination[49,50]. As per *Ayurvedic Pharmacopoeia of India* (API), *Drakshasava* too contains *Piper longum* and *Piper cubeba* (both rich in piperine) as *prakshepa dravya*, in very minute quantity, may predicts presence of piperine in the formulation, butthere was no report on standardisation of *Drakshasva* (with or without *prakshepa dravya*) with piperine as biomarker so far.

The present research envisaged on determination of piperine in *Drakshasava* (without *prakshepa dravya*) and its quantification by validated HPTLC. The best peak profiling was determined at 340 nm. The Figure 2 represents HPTLC chromatogram of piperine standard, decoction and the fermented sample (modified *Drakshasava*), respectively. Both the sample shows presence of piperine and resulted in significant increase in piperine concentration post fermentation (2.42 ug/mL) compared to decoction (0.11 ug/mL).

Validation

Specificity

The bands for piperine in the samples were compared with reference standards with respect to R_f value. The compounds peak purity was estimated by comparative analysis of the UV spectra at 3 different levels, i.e., peak (start, apex and end positions) respectively.

Linearity and calibration curves

The calibration curves were plotted, with a concentration range of 20-400 ng/spot for piperine with regression coefficients (R^2) and the samples were quantified using calibration curves (Table 5).

Precision

The repeated scanning of the spot of piperine was done at same concentration (six times). The repeatability of the method was determined through

Table 5 — Method validation parameters for the quantification of piperine

Parameters	Piperine standard
Rf	0.58
slope	23.83
intercept	2345
Linearity range	20-400 ng/ spot
Regression equation	y = 23.83x + 2345.
Correlation coefficient, r	0.992
LOD (ng)	43.94
LOQ (ng)	146.4
Specificity	Specific
Robustness	Robust

intra-day and inter-day precision studies (5 days) at three concentration levels 100, 200 and 300 ng/spot for piperine (Table 6).

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were obtained by spotting blank methanol (signal-to-noise ratio). It was based on standard deviation (SD) of the response and the slope (S) of the calibration curve. The LOD and LOQ were considered as 3: 1 and 10: 1 respectively (Table 5).

Robustness

Robustness is a estimation of the reliability of the technique to remain invariable by slight alterations in

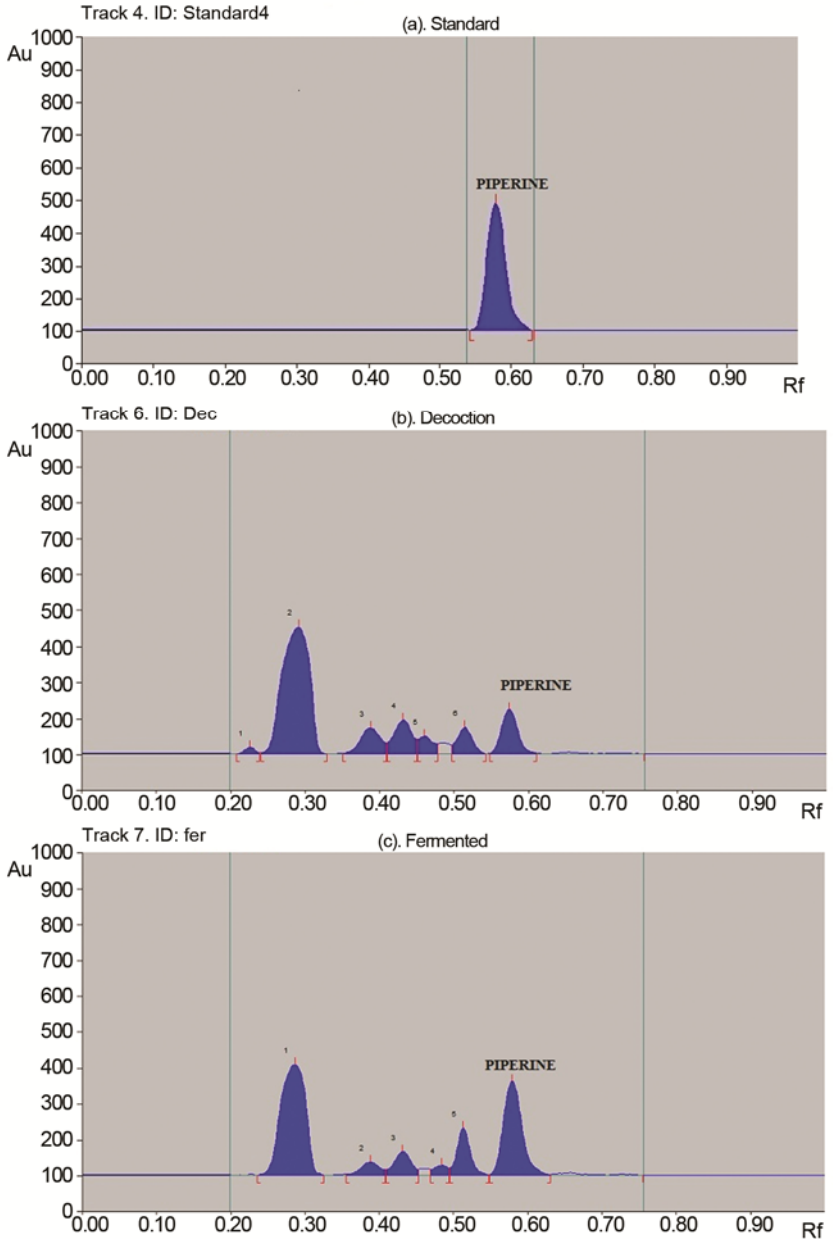

Fig. 2 — HPTL Chromatogram of piperine a. standard, b. decoction sample, c. fermented sample (optimized *Drakshasava*)

Table 7 — Recovery studies of piperine

Sample	Amount present in sample of 1 mg/mL (µg)	Amount added (µg)	Theoritical value	Amount found (Observed Value)	Recovery	Average recovery (%)
Pre fermented sample (Decoction)	0.11	0.10	0.21	0.208±1.43	0.99	97.66
	0.11	0.15	0.26	0.252±1.38	0.969	
	0.11	0.20	0.31	0.301±1.50	0.97	
Post fermented Sample (optimized *Drakshasava*)	2.42	2.0	4.42	4.213±1.18	0.952	91.2
	2.42	2.5	4.92	4.165±1.44	0.845	
	2.42	3.0	5.42	5.10±1.38	0.94	

Table 6 — Precision (intraday and inter day) study of piperine

Concentration (ng/band)	Intraday		Inter day	
	%RSD	Mean RSD	% RSD	Mean RSD
100 (n=6)	0.13	0.12	0.17	0.16
200	0.17		0.23	
300	0.07		0.09	

the experimental parameters. The studied parameters included mobile phase ratio; time interval between drying and scanning; time interval between spotting and development for a particular concentration of piperine. The method was found significantly robust.

Accuracy

The percentage recoveries of piperine in samples were calculated by standard addition method. Three sets of the standard were taken and spiking was performed with pre-quantified sample (80, 100 and 120%). The peak area was noted and further percentage recoveries were calculated (Table 7).

The proposed HPTLC method was found simple, reproducible, specific, precise and accurate.

Conclusion

The *Ayurvedic* formulations has been in use since ages, but its time taking manufacturing process and lack of proper standardization methods, makes it difficult for pharmaceutical industries to process. So, novel modified methods (within API acceptance limits) with use of advanced techniques may be introduced to enhance its acceptance worldwide. Implementation of optimized formulation strategies and validated marker based standardization may fulfill the regulatory requirement leading to better quality, consistency and safety of herbal preparations.

The developed BBD method of Response Surface Methodology was found efficient for optimizing fermentation parameters of *Drakshasava*, an Ayurvedic formulation within API limits. The new validated method of piperine quantification shows positive effect of fermentation in enhancing the biomarker concentration. The method was found simple, precise, specific, sensitive, robust and easy to use. All the statistical reports prove that method is repeatable and selective for further analysis.

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Conflict of Interest

Authors declare no conflict of interests

Authors' Contributions

The first author has carried out the research work under the guidance of VD.

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