RESEARCH PAPER

Statistical Optimization Applying Box Behenkent Design to Kumaryasava Formulation for it's in Vivo Antioxidant and Ccl4 Induced Hepatoprotective Activity

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Received: 14th Aug, 2025; Revised: 16th Sep 2025; Accepted: 15th Nov, 2025; Available Online: 30th Nov, 2025

ABSTRACT

Objective: The study was aimed at exploring the development of polyherbal formulation of *aloe vera* as Kumaryasava using statistical approach and evaluates there*in vitro* antioxidant, *in vivo* hepatoprotective activity.

Method: The Kumaryasava formulation was statistically optimized using Box-Behenken Design (BBD). The effect of independent variables sweetening agent, fermentation duration and fermentation temperature was analyzed on response alcohol content (%) generated determined by gas chromatography. Further, the formulation was investigated for its physicochemical parameters, *in vitro* and *in vivo* studies.

Result:*In vitro* antioxidantscreening was performed by DDPH scavenging assay designated that the polyherbal formulation has 82.70 ± 0.98 % potential to deactivate free radicals than standard ascorbic acid.Liver enzyme levels including glutathione, catalase, and superoxide dismutase were shown to be enhanced in wistar rats that underwent *in vivo* hepatoprotective activity in response to CCl4-induced liver damage. Additionally, the histological examinations showed that the modified formulation significantly improved liver tissues compared to the standard.

Conclusion: Hence, the Kumaryasava formulation being an effective antioxidant that indicates elevating effect on hepatic enzyme and has potential in managing liver disorders.

Keywords: *Kumaryasava, Madhu, Antioxidant, Aloevera, Hepatoprotective, Polyphenols.*

How to cite this article: Gaikwad P; Patil SS, Statistical Optimization Applying Box Behenkent Design to Kumaryasava Formulation for it's In Vivo Antioxidant and Ccl4 Induced Hepatoprotective Activity. Int J Drug Deliv Technol. 2025;15(4): 1762-1771, DOI: 10.25258/ijddt.15.4.27

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

The medicinal plants use for therapeutic determinations represents a worldly tradition in diverse cultures[1]. In India as well as western country the use of herbal medicine is increased. Herbal medicines have gained significant use in various health practices, including traditional medicine systems like Chinese medicine, Ayurveda, Unani, Naturopathy, Osteopathy, and Homeopathy[2]. Kumaryasava (KS) is a unique and valuable ayurvedic polyherbal formulation in Indian ayurvedic formulary, containing aloe vera as the key ingredient and self-fermented galenical containing 40-50 crude drugs[3]. KS contains phytochemicals such as saponins, phenolic compounds, tannins, and anthraquinone glycosides, which contribute to various therapeutic uses[4]. KS formulations are widely used over the counter for treating urinary disorders, digestive system, liver disorders, and cough. However, there is limited modern scientific data available for KS in managing liver ailments and high daily doses are required[5]. Aloe vera has excellent antioxidant effects due to its polyphenol content, which prevents oxidative stress and degenerative conditions[6]. Efforts are being made to evaluate the exact efficacy of an herb for its pharmacological activity. Limited data is available for developing KS with statistical optimization and for managing hepatic disorders. This research aims to prepare statistically optimized Kumaryasava, a polyherbal formulation comprising aloe vera, madhu, and jaggery with effective reduced doses for its antioxidant and hepatoprotective benefits [2].

MATERIALS AND METHODS

Procurement and authentication of raw materials:

Aloe leaves of *Aloe barbadensis* were collected from medicinal garden of Ashokrao Mane College of Pharmacy Peth-Vadgaon. A raw drug was collected from S. G. Phyto Pharma Pvt. Ltd, Kolhapur. Raw drugs identification and authentication has been done in the research Laboratory S. G. Phyto Pharma Pvt. Ltd, Kolhapur. Kumaryasva was prepared as per the reference of Yoga ratnakara Utrardha Gulma rogadhikar.

Methods

*In silico*molecular docking study

For simulated screening, docking, and post-screening, try out iGEMDOCK, a freeware program with an interactive graphical user interface. There is a requirement to filter the substances related to their antioxidant and hepatoprotective characteristics because the commercial Kumaryasava formulation has over fifteen compounds. To further understand the antioxidant and hepatoprotective properties

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of the herbs haritaki and honey, a molecular docking research was conducted. The docking parameters were specified by choosing accurate docking, an extremely precise and slow docking method, with an 800-person population size, 80 generations, and 10 solutions. The best-docked postures underwent post-docking evaluations utilizing the features interaction profile, interaction analysis, and interaction profile cluster.

Preparation of Raw drugs:

Formulation of Kumaryasava was carried out comprising the composition for formulation batch with minimum selective ingredients as mentioned in Table 1. Collected leaves of *Aloevera* were cleaned and washed. The lateral spins of leaves were cut and divided equally into two parts before cutting leaves in pieces. The green rind portion of the leaves was scrapped out; inner gel portion further exposed to grinding[7]. Drugs (2 to14) mentioned in Table 1 were appropriately shade dried, separately powdered in a mixer for essential size, packed and sealed individually airtight containers. All the containers were labeled accurately and stored in a cool place in closed room.

Table 1 Ingredients used for control and formulation batch Kumaryasva

Sr. No.	Ingredient	Kumaryasava (Dhootpapeshwar Marketed formulation)	Kumaryasava formulation batch	Quantity (Kg)
1	Kumari rasa			4l
2	Haritaki			1 kg
3	Dhataki			256 gm
4	Jatiphala			16 gm
5	Lavanga		-	16 gm
6	Kankola		-	16 gm
7	Jatamansi			16 gm
8	Kababka		-	16 gm
9	Chvya		-	16 gm
10	Eranda		-	16 gm
11	Jatipatra		-	16 gm
12	Karkatashrngi		-	16 gm
13	Bibhitaka		-	16 gm
14	Pushkarmula		-	16 gm
15	Tamra bhasma		-	16 gm
16	Lauha bhasma		-	8 gm
17	Guda			1.2 kg
18	Madhu			1 kg
19	Water			4l

Preparation of Swarasa:

The fresh leaves were washed thoroughly to make it devoid of dust and mud. Further, the leaves were scrapped and grinded in mixer for 8-12 min to obtain thick mucilage that stained to get Swarasa[8].

Preparation of Kwatha:

The Haritaki fruit coarse powder was soaked in water overnight. Kwatha was prepared by reduction of water to 1/4th volume and further it was strained through double layered cotton cloth.

Prakshep Dravya:

The dried ingredients (Prakshep drvya) pulverized (Table 1) to form coarse powder for preparation of formulation[9].

Sandhana Dravya (Fermenting agent):

Dhataki flower buds,Jaggery and honey were used as fermenting agent. The guda and madhu was mixed properly in Sandhana drvya and Prakshepa dravyas and set asidefor fermentation in BOD incubator for 21 days at a temp of 30 °C.[10]

3. Optimization and statistical analysis of formulation:

Standardized ayurvedic preparations are crucial for therapeutic efficacy, and a statistical approach is used to ensure maximum efficacy. The Box Behenken Design (BBD) approach was used to examine the effect of three independent variables: sweetening agent concentration, fermentation duration, and fermentation temperature, and the possible combinations of Kumaryasava formulations[11].

Table 2 Composition of formulation batches acoording to CCD

Formulation code	Factor 1 A: Sweetening agent (% w/v)	Factor 2 B:Fermentation duration (Days)	Factor 3 C:Fermentation Temperature °C	Response Alcohol content (% v/v)
K1	35	30	35	1.94± 0.65
K2	45	30	35	1.54±0.58
K3	45	20	25	1.83±0.0.98
K4	45	20	45	1.34±0.85
K5	40	20	35	2.17±0.17
K6	40	10	25	2.08±1.23
K7	35	10	35	1.98±0.54

K8	35	20	25	1.97±1.63
K9	40	30	45	1.33±1.25
K10	40	30	25	2.21±0.31
K11	35	20	45	1.46±1.78
K12	45	10	35	1.85±0.92
K13	40	10	45	2.01±0.25

All of these elements were tested to see how they affected the formulation's reaction alcohol content. Limits for KS formulations according to quality control factors are most affected by their varying ethanol contents. This study set out to quantify the ethanol concentration of a formulation using the gas chromatography method in order to determine its time-dependent limit.Initially the K1 to K13 batches was prepared by following the composition mentioned in method of preparation for 200 ml. After applying BBD according to the observed results for alcohol generated KS formulation batch K5, K6 and K13 was further formulated in 1000 ml quantity and evaluated for the alcohol content for the recognition of final optimized formulation.

The data acquired from experimentation with total thirteen formulation batches generated (K1 to K13) was examined statistically using Design-Expert 11 software (Stat-Ease Inc., USA) and Graph Pad Prism version 8.0 (San Diego, CA, USA). The composition of formulation batches and composition applying BBD illustated in Table 2.

Extraction of aloin from Kumaryasava

The aloin was extracted from prepared Kumaryasava and further confirmed through spectroscopic investigation. After cooling, diluting with water, and an evaporation on a steam bath, approximately 100 ml of the optimal Kumaryasava formulation was obtained. Afterwards, chloroform was used for extraction, and the resulting extract was then dried at ambient temperature under decreased pressure.The presence of aloin in formulation was determined by HPLC analysis and compared with standard extract of aloin[4].

Characterization of polyherbal formulation:

Aloin confirmation by HPLC analysis

Aloin was found in the chloroform extract, according to HPLC research. To achieve a concentration of 100 µg/ml, the standard solution was made by adding 10 mg aloin to methanol. To make the concentration 100 µg/ml, 10 mg of the sample was added to methanol to create the sample solution. Using a PDA detector, the material was scanned between the 200-400 nm wavelength range. The suitable intensity of aloin was considered while selecting a wavelength for examination, and 250 nm was chosen. The experiment was conducted using a Phenomenex Kinetex XB-C18 column (150 x 4.6 mm, 5 µ), with a temperature of 30°C, a flow rate of 1 ml/min, and an injection volume of 10 ml. The run time was 10 minutes, and the mobile phase was a mixture of 50:50 methanol and water.

Physicochemical investigation

A number of physicochemical characteristics were used to screen the KS formulations for asava quality and purity, including viscosity, specific gravity, total solid content, total reducing sugars, non-reducing sugars, methanol, and total phenolic content. The specific gravity and gas chromatography analyses determined the alcohol concentration[12,13].

Gas chromatography for determination of alcohol content

Preparation of standard curve

2.5 ml absolute ethanol standard (99.9%) was added in 25 ml volumetric flask and required volume was made by Tetrahydrofuran (THF). (Labelled as SSS-I) (Concentration of ethanol = 100 ppm). Further the solution of concentration 10 ppm to 50 ppm was prepared from the stock.

Preparation of sample solution

The sample solutions were prepared by pipetting 1 ml each in 10 ml volumetric flask and 5 ml of THF followed by sonicationfor 2 min. and finally maintain the volume by dilution with THF.

DPPH scavenging assay:

Ability of developed formulation to scavenge DPPH free radical was conducted on optimized formulation by considerably modifying the procedure reported in the literature. 100 µL of optimized K1 formulation wascollected in the microliter plate. Further, 0.1% methanolic DPPH of 100 µL was added and incubated for 30 minutes in dark condition. In addition, the color change from purple to yellow was identified as a strong positive and pale pink as a faint positive, based on eye inspection. Elisa plate reader readings at 630 nm were recorded. [14]

Radical scavenging was determined using formula:

DPPH radical scavenging activity (%) = [(Ac - As) / (Ac)] x 100…Eq. 1

Where, Ac - absorbance of control solution, and As - absorbance of sample solution.

***In vivo*antioxidant and Hepatoprotective activity:**

The Wistar rats weighing 150 g to 200 g were used for the purpose of *in vivo* antioxidant activityand CCl_4 induced hepatotoxicity[15]. All experimental animals and activity were performed at Biocyte institute of research and development, Sangli. (Approval number: IAEC/Sangli/2020-21/13). Housed at a comfortable 20°C, the animals were provided with a balanced meal and plenty of water. Before the research, the rats were kept in a controlled environment for one week to help them adjust to the new environment. Wistar rats weighing 180-200 grams were used to conduct the *in vivo* hepatoprotective activities. The following is a rundown of the four groups into which twelve wistar rats were randomly assigned:

Group 1: Normal - rats feed with distilled water for 21 days.

Group 2: Positive Control -50 mg/kg bw of vitamin C for a period of 21days.

Group 3: Toxicant - CCl_4 in olive oil in 1:1 ratioat dose 1.0 mL/Kg/dayfor 10 days followed by optimized Kumarysava for next 12 days.

Group 4: Test I - Administer Test I (Ratio 1.5ml optimized formulation and 1.5 ml DW) for 21 days.
Group 5: Test formulation II (marketed KS formulation 5 ml/kg/day)
On 21st day, all treated animals were anesthetized by administration of diethyl ether for the collection of blood samples by retro-orbital puncture of animals for the hematological and biochemical analysis (SGOT, SGPT and Bilirubin). After sacrifice of animals the liver of every animal was dissected; washed with ice-cold saline, slapped dry and record the weight. To get 10% (w/v) homogenate, it was further homogenized in Tris HCl buffer (pH 7.4). After homogenate was achieved, it was centrifuged for 15 minutes at 4000 rpm and 4 °C. A variety of parameters, including malondialdehyde (MDA), reduced glutathione (GSH), lipid peroxidation (LPO), and catalase (CAT), were measured in the centrifuge's clear byproduct.The 10% formalin was used for fixation of dissected liver tissue and further for histopathological screening[16,17].

Determination of Superoxide dismutase (SOD) enzyme activity

The study's supernatant was extracted from around 1 ml of prepared tissue homogenate by centrifuging it at 5000 rpm.According to the following protocol solutions were added and recorded the absorbance at 420 nm continually for about 4 minutes immediately after the addition of pyrogallol. Subsequently the first absorbance was noted at the end of 1.30 minute after addition of solution no.3 and thereafter, then record result exactly after one minute each time up to 3.30 minute. Determine the change between the optical densities obtained at 1.30 and 3.30 minutes[18].

Table 3 SOD enzyme action

Reagent	Blank	Test
Tris buffer	3.0	2.95
Supernatant	--	0.05
Working Pyrogallol reagent	0.3	0.3

The SOD activity can be determined using formula,

SOD (units/mg proteins) = A-B/A X40 mg of protein …Eq. 2

A- The variance between optical densities of control, B- The variance between optical densities of test

Malondialdehyde (MDA) Level

Using a modified version of Satoh's approach, we measured the serum MDA levels. Two hundred microliters of serum samples were mixed with three hundred microliters of a solution containing trichloroacetic acid (TCA), thiobarbituric acid (TBA), and hydrochloric acid (HCl). The exact proportions of TCA(15%), TBA(0.375%), and HCl (1 M) are used to make the TCA-TBA-HCl solution. After 10 minutes in a boiling water bath, the solution was cooled and 500 µl of 1 M NaOH was added to prevent white precipitate. Using a molar extinction value of 1.55 x 105 M^{-1} cm^{-1}, the concentration of thiobarbituric acid reactive substances (TBARS) was determined as nmol/ml of serum, and the solution's absorbance was recorded at 535 nm.

Statistical Analysis

Values were expressed as mean ± standard deviation and were considered significant when $P < 0.05$. The study's results were statistically evaluated using one-way ANOVA followed by Tukey's test.

RESULT AND DISCUSSION

***In Silico* molecular docking**

II] Molecular docking study of *Terminalia chebula* (Haritaki)

Chebulinic acid is docked with the binding pocket of Protein kinase (PDB ID – 1gzo) with binding affinity (-9.2 Kcal/mol). It formed conventional hydrogen bonds with ARG A:176, LYS A:285, ARG A:208, PRO B:210, TYR A: 231. Moreover pi-alkyl bond with LYS A:216, and also exhibit van der Waals interactions with TYR A :177, TYR A :178, ALA A :214, GLU A :230, ALA A :232, THR A:174, ASN A:233.

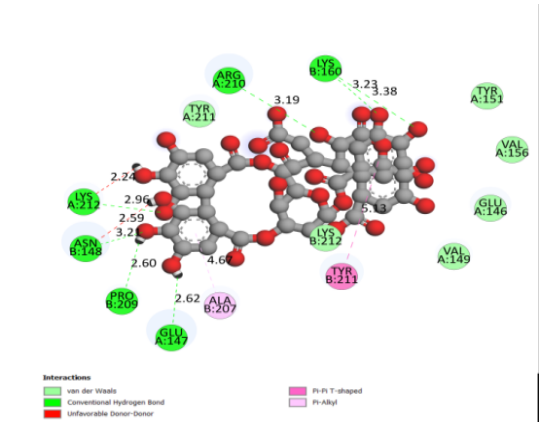

Fig.1. Molecular docking of chebulinic acid with the binding pocket of Protein kinase

Quercetin (PubChem ID- 5280343) is docked by binding pocket of AKT Serine / Threonine kinase 1 (AKT 1) (PDB ID – 6s9x) with binding affinity (-9.7 Kcal/mol) [10]. It formed conventional hydrogen bonds with GLN A;79, SER A:205, TYR A:272, THR A:211. Moreover Pi- alkyl interaction with LEU A:264, LEU A:210 and also exhibit van der Waals interactions with TYR A :263, VAL A :271, LYS A:268, AG A:206, THR A:291, ILE A:290, ASP A:292, ARG A:27, ASN A:54.

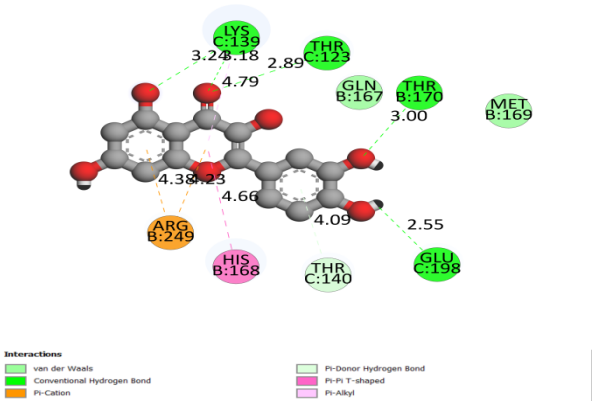

Fig.2. Molecular docking of Quercetin with the binding pocket of AKT Serine / Threonine kinase 1 (AKT 1)

The aforementioned results for molecular docking study revealed that the affinity of honey,aloevera, and haritaki for the antioxidant and hepatic benifits. Hence, for the development of Kumaryasava formulation honey was selected as sweetening agent and haritaki affinity for hepatocellular carcinoma proteins was used for preparation of KS that will have benefits against hepatic alignments.

Optimization of Formulation

The formulation was optimized statistically using BBD to evaluate the impact of independent variable on response. The effect of independent variables was studied on the response for alcohol content (%) generated.

Response 1: Alcohol content (% v/v)

Alcohol content (%)= +2.17 -0.0988A -0.1125B -0.2438C -0.0675AB +0.0050AC 0.2025BC -0.3000A^2- 0.0425B^2 -0.2200C^2 ….Eq.3

The data was most adequately described by a quadric model, which yielded a significant result (F=25.55, P=0.0500). Here, the significant model terms A, B, C, BC, A^2, and C^2 have an accuracy of 15.271, which is sufficient to navigate the design space. The alcohol concentration (%) is influenced by the sweetener, the period of fermentation, and the fermentation temperature[19]. With a 45 percent concentration of sweetener, the alcohol percentage was lower than with a 35 percent or 40 percent concentration. Due to its role as a nutritive meal to enhance microbial development, glucose affects the pace of fermentation depending on its kind and composition. Three hundred and sixty-six percent of the sugars in Sandhana kalpana are fermentable, say Acharya Charaka and Sharangadhara. Only 40% of the sweet ingredients are introduced at the start of the simple and early fermentation process; the remaining sweet ingredients are added after fermentation has started[20]. Seasonality plays a role in the duration required for fermentation to finish. The literature states that fermentation takes 6 days in the summer and autumn, 10 days in the winter, and 8 days in the rainy and spring seasons. It only takes 7–10 days in hot tropical climates and 30 days in cold climates. Given that fermentation time greatly affects alcohol yield, the proposed recipe aimed for a fermentation time of about 30 days in order to achieve a sufficiently high alcohol content [21]. The fermentation process of asavarista formulations is influenced by temperature.The fermentation temperature at 35°C and 45 °C resulted in decreased alcohol generated due to killing of yeast cells at high temperature that hampered fermentation process. On contrary at 25°C yielded desired alcohol content due to balanced pH, lower acid value and viable yeast cells[22]. Hence, the data generated from BBD revealed that these independent variables statistically optimized to generate the significant quantity of alcohol content in formulation. Aforementioned results revealed, that formulation K10 with alcohol content generated 1.94 % v/v with concentration of sweetening agent 40% w/v and duration of fermentation 30 days at 25°C was considered as optimized formulation and utilized for further analysis. The graphical presentation showing effect of independent variable on response illustrated in Fig. 3.The correlations between actual values versus the predicted values for different variables are plotted and depicted in Fig. 3. Without interaction and with a significance level of $p < 0.05$, the computed findings of the ANOVA research of the applicable model for sweetening agent, fermentation period, and fermentation temperature are suitable. Table 4 provides a quick summary of the optimization data that was further assessed using ANOVA and retrieved from Design Expert®.

Table 4 ANOVA parameters for quadric model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1.11	9	0.1229	25.55	0.0110
A-Sweetening agent concentration	0.0780	1	0.0780	16.22	0.0275
B-Fermentation duration	0.1013	1	0.1013	21.06	0.0194
C-Fermentation Temperature	0.4753	1	0.4753	98.85	0.0022
AB	0.0182	1	0.0182	3.79	0.1467
AC	0.0001	1	0.0001	0.0208	0.8945
BC	0.1640	1	0.1640	34.11	0.0100
A^2	0.2057	1	0.2057	42.78	0.0073
B^2	0.0041	1	0.0041	0.8586	0.4225
C^2	0.1106	1	0.1106	23.01	0.0172
Residual	0.0144	3	0.0048		
Correlation Total	1.12	12			

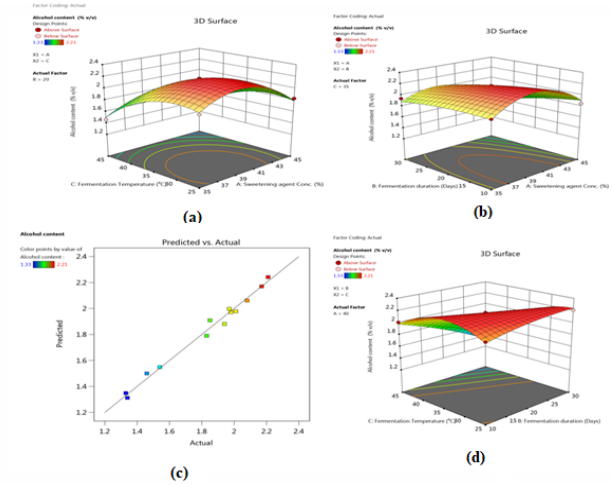

Fig. 3. 3D plot showing effect of independent variable on alcohol content and plot of actual versus predicted values

Aloin confirmation by HPLC analysis

The HPLC analysis of chloroform extracted fraction has recorded for standard aolin extract and chloroform extract from optimized KS formulation. The aloin is one of the major constituent of aloes hence the standard aloin extract was utilized for the comparative analysis. The extracted chloroform fraction has showed the HPLC profile containing two peaks with retention time 5.51 and 6.53 min respectively corresponding to the characteristic peaks indicating presence of quinones as shown in Fig4 and Fig. 5. The HPLC retention peak of standard aloin recoded at 5.56 and 6.43 that was in correlation with sample extracted aloin. The aforementioned results confirmed the aloin presence in optimized KS formulation that contributes to antioxidant property and hepatoprotective benefits.

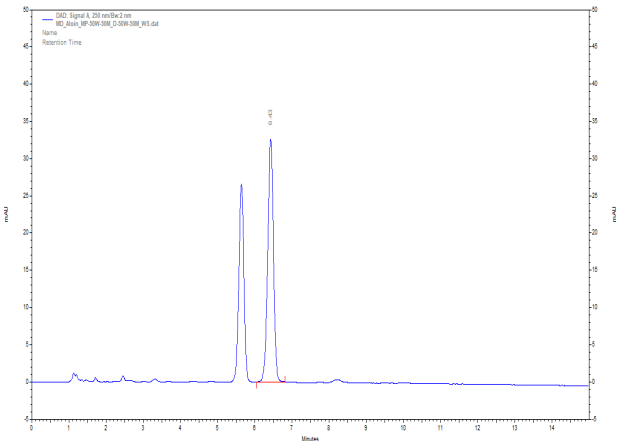

Fig. 4 HPLC profile of standard aloin extract

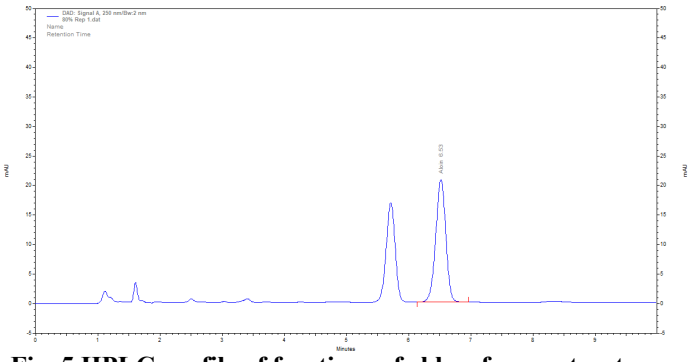

Fig. 5 HPLC profile of fractions of chloroform extract from Kumaryasava formulation

Physicochemical investigation

The optimized formulation of KS was evaluated by various parameters for quality and purity of Asavas. It includes physicochemical properties like pH, viscosity, specific gravity, total solid content, total reducing sugars, non-reducing sugars, and total phenolic content[23]. These preliminary studies are considered as desired for the standardization of Asavas. The results of organoleptic properties are briefed in Table 5 and that of standardization parameters in Table 6. According to the recorded results it can be revealed that the developed formulation exhibited the standard limit of API specifications that assures the purity of product.

Table 5 Organoleptic properties of polyherbal formulation

Sr. No.	Parameters	Kumaryasava (Fd)
1	Colour	Light brown
2	Odour	Aromatic
3	Taste	Bitter

Table 6 Standardization parameters of polyherbal formulation

Sr. No.	Parameters	Specification as per API	Values ± SD* (Marketed formulation)	Values ± SD* (K10 Formulation)
1.	pH	3.40-4.20	3.62 ± 0.017	3.62 ± 0.017
2.	Specific gravity	1.01-1.10	1.1243 ± 0.014	1.09 ± 0.02
3.	Total solid content(% w/v)	NLT 13	34.68 ± 1.990	33.10 ± 0.37
4.	Total alcohol content(% w/v)	5-10	7.03 ± 0.8141	6.18 ± 0.12
5.	Reducing sugars (% w/v)	NLT 7.5	9.07±0.11	10.22±0.16
6.	Non- reducing sugars(% w/v)	NMT 0.80	0.27 ± 0.010	0.69 ± 0.17

7.	Total phenolic content (% w/v)	-	0.63 ± 0.001	0.77 ± 0.03

Determination of alcohol content by gas chromatography

The standard curve of absolute ethanol performed by gas chromatography has recorded the linearity within concentration range 10 ppm to 50 ppm with equation mentioned in concentration (ppm) plotted against area depicted in Fig. 6. The alcohol content generated in all formulation K1-K13 was estimated through gas chromatography[24]. The formulations K1-K13 have recorded the alcohol content (% v/v) in the range 1.33± 1.25 to 2.21± 0.31. The optimized formulation K1 has recorded area 472467 with alcohol generated 1.94± 0.65 as shown in Fig.7. Further formulation K10 prepared in bulk 1000 ml has yielded alcohol content 6.18±0.12% v/v that is in accordance with the marketed formulation.

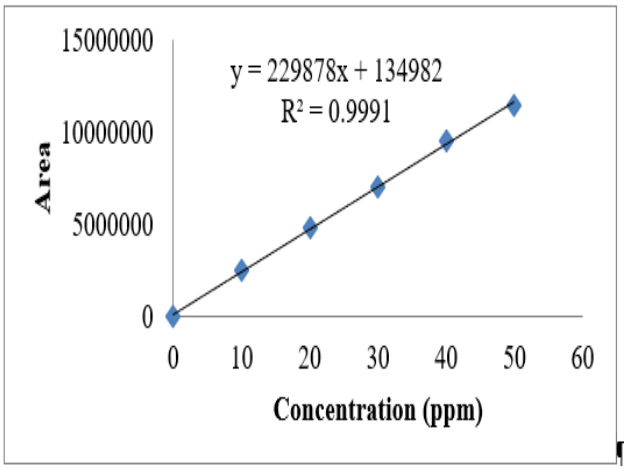

Fig. 6 Standard curve of concentration of ethanol (ppm) against area obtained by gas chromatography

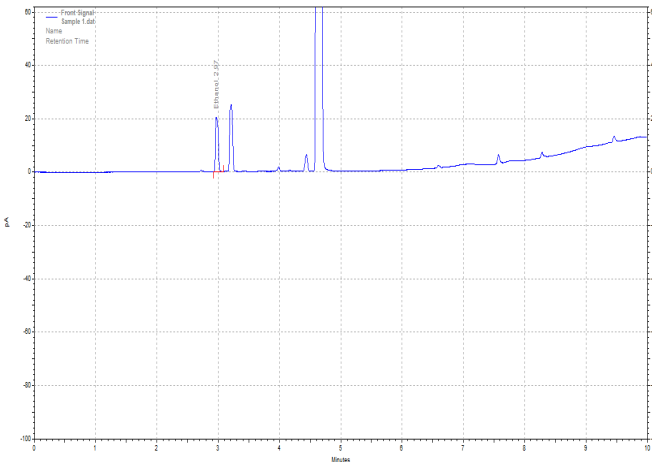

Fig. 7 Gas chromatogram of alcohol content (% v/v) of optimized K1 formulation

DPPH Scavenging Assay

An evaluation of in-vitro antioxidant activity of optimized formulation wasperformed using DPPH scavenging assay. The prepared formulations and ascorbic acid solution observed to decrease the concentration of DPPH significantly from the concentration 100 µL/mL to 1000 µL/mL. The optimized formulation exhibited good scavenging activity 82.70 ± 0.98 % than standard ascorbic acid. The antioxidant activity of developed formulation was due to the presence of polyphenolic compounds in the *aloe*.

***In vivo*Hepatoprotective Activity**

The findings of the animal's body weight and liver weight after 21 days were not significantly different, as shown in Table 7. The formulation's anti-inflammatory effects may explain the treatment group's little increase in body weight and decrease in liver weight[25].The LFT test like SGOT, SGPT and bilirubin was performed on the blood serum sample, the results revealed that the SGOT level was slight increase in test formulation as compared to control group as mentioned in Table 8. However, SGPT level of test formulation II (Cd controlled batch) has been increased but standard ascorbic acid and test formulation I (optimized K10 formulation) has been decreased and restored. The bilirubin test indicated that there was no change in the treatment group as well as in standard group[26].In the enzymatic assay the increase levels of enzymes indicate the membrane damage along with lipid peroxidation due to oxidative stress depicted Fig. 9.

Table 7 Effect of formulation on the body weight and liver weight

Sr. no.	Treatment Group	Treatment	Body Weight in gm		Liver Weight in gm
			1st Day	21st Day	
1	Normal	Normal Saline	187.0±8.18	191.0±1.54	5.133±0.20
2	Positive Control	Ascorbic Acid 50 mg/kg	195.3±11.68	205.7±12.26	5.83±0.20
3	Toxicant group	CCl4 in olive oil in 1:1	197.3±9.68	212.23±1.30	5.96±2.56
4	Test I	Test formulation I	198.3±10.41	210.3±0.37	5.733±0.41
	Test II	Test Formulation II	200.0±13.23	213.0±1.79	5.767±0.40

Table 8 Effect of optimized formulation on tissue antioxidant enzyme

Sr. No.	Treatment Group	Treatment	GSH	LPO	SOD
1	Normal	Normal Saline	0.61±0.043	0.10±0.009	10.00±1.00
2	Positive Control	Ascorbic Acid 25mg/kg	0.93±0.049	0.33±0.07	11.33±2.08
3	Toxicant group	CCl4 in olive oil in 1:1	101.30±0.69	0.89±0.46	19.54±1.23
4	Test I	Test formulation I (Optimized K1 formulation)	0.69±0.030	0.50±0.01	10.33±2.51
5	Test II	Test Formulation II (Cd controlled batch)	0.43±0.14	0.35±0.005	18.67±1.52

In vivo study revealed that the optimized formulation exhibited significant reversal in SGOT, SGPT, and bilirubin whereas toxicant group exhibited significantly elevated levels of SGOT, SGPT and bilirubin with P< 0.001 vs. control. The liver tissue of each treatment groups was evaluated for histopathological performance, which recorded toxicant group with a maximum percentile of cellular necrosis and vacuolization. However, the treatment group exhibited the comparatively less hepatic damage with miniature vacuolization and maintaining normal liver tissue architect ureas shown in Fig. 8[27]. The formulation lowers the increased levels of these enzymes representing a reversal of CCl_4 induced liver toxicity illustrated in Table 9.The presence of polyphenol and madhu has contributed for hepatoprotective activity of formulation. However, the antioxidant benefits of formulation enhance the hepatoprotection, liver necrosis and liver cirrhosis.

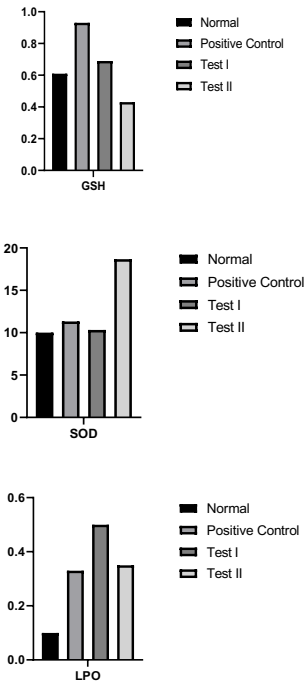

Fig. 8 Effect of optimized formulation on tissue antioxidant enzyme (a) GSH (b)SOD (c) LPO

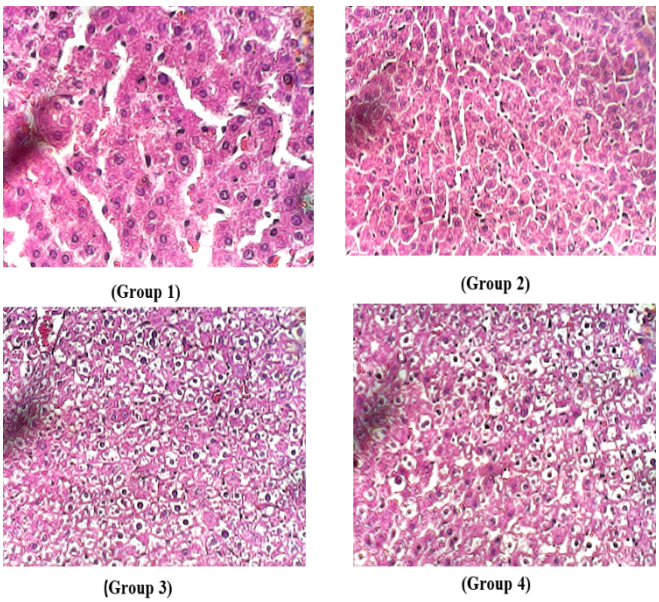

Fig. 9 Histopathological evaluation of liver tissues: Group 1 – Control group with normal hepatic tissue architecture, Group 2- Positive Control treated with Vit. C showing normal histological structure, Group 3 – Optimized Kumaryasava formulation showing normal histology of liver tissues, Group 4 – Toxicant group hepatocellular necrosis with high vacuolization

Table 9 Effect of formulation on rat liver enzymes

Sr. no.	Treatment Group	Treatment	SGOT (IU/L)	SGPT (IU/L)	Bilirubin (IU/L)	MDA (mol/mg)
1	Normal	Normal Saline	129.7±2.509	83.33±2.371	0.00±0.011	1.92±1.03
2	Positive Control	Ascorbic Acid 25mg/kg	120.3±3.56	73.33±3.22	0.00±0.0057	3.02±0.98
3	Toxicant group	CCl4 in olive oil in 1:1	168.23±0.68	120.32±4.06	1.20±0.23	7.63±0.32
4	Test I	Test formulation I	138.3±1.28	64.67±1.512	0.01±0.0057	3.21±0.74
5	Test II	Test Formulation II	139.0±0.937	77.67±2.110	0.00±0.0057	4.36±1.65

According to the reported literature for optimum therapeutic efficacy, approximately 60 ml KS formulation has toadministerdaily orally. However, the equivalent therapeutic effectiveness was perceived with optimized KS formulation (15 ml) in the *in vivo* screening on wistar rats that revealed the developed optimized KS formulation has potential to reduce the high dose of KS to approximately one-fourth of the traditional dose. Hence the developed KS formulation has desired significance for dose reduction with therapeutic efficacy for hepatoprotection thereby enhancing the patient compliance.

CONCLUSION

The polyherbal Kumaryasava formulation comprising *aloevera*, madhu and jaggery was statistically optimized using BBD design has demonstrated the efficacy for antioxidant and hepatoprotection. The sweetening agent, fermentation temperature and fermentation duration has confirmed desired impact on alcohol generation. The optimized formulation has significant physicochemical parameters within pharmacopeia standards. The *in vitro*DPPH scavenging assay of developed formulation has explored superior antioxidant activity due to the polyphenols content in *aloevera*.The *in vivo study* revealed the hepatoprotective benefits of developed KS polyherbal formulation in CCl_4 induced hepatotoxicity. The histopathological evaluation of liver tissues indicated hepatoprotective potential of the formulation.This effect may be due to its anti-oxidative,anti-inflammatory, immunomodulating as well as restorative effects of the *aloevera* in optimized KS formulation and thus can be an effective treatment for hepatotoxicity. Further, the hematological biochemical analysis has confirmed the formulation potential to reduce the enzymatic oxidative stress. The overall outcome of the investigation advocated that anti-oxidative, anti-inflammatory; immunomodulating as well as restorative effects of the *aloevera* in optimized KS formulation judiciously contribute in treatment for hepatotoxicity.

CONFLICT OF INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

This research Approval number IAEC/Sangli/2020-21/13) performed at Biocyte institute of research and development, Sangli..

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