

Development and Evaluation of Polyherbal Formulations for Hepatoprotective Activity

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ABSTRACT

Background: Liver is one of the most important human organs and is a sensitive target for chemicals that modulate biotransformation. The present study aimed to develop a polyherbal formulation in the form of both capsules (solid dosage form) and suspensions (liquid dosage form) with the combination of powders of *Ricinus communis* leaves, *Allium sativum* cloves, and *Piper nigrum* seeds and evaluated for hepatoprotective activity. **Methods:** Three batches of each formulation (CF1, CF2, CF3 for capsules and SF1, SF2, SF3 for suspensions) were developed by varying starch (as binder) concentration for granulation in capsules and by varying concentrations of sodium CMC (carboxy methyl cellulose) a thickening agent in suspension formulations. These were initially evaluated for *in-vitro* parameters. Then calculated OD (overall desirability) factor for both formulations based on good flow properties and pourable viscosity of capsules and suspensions, respectively. **Results:** It was found that CF2 and SF1 formulations were selected as the best. Hence, these two formulations were considered for further pharmacological evaluation by

ex-vivo studies using rat liver slices with CCl₄ triggered lipid peroxidation assay and reduced glutathione assay, which revealed that the CF2 and SF1 formulations effectively prevented lipid peroxidation and considerably increased the glutathione levels. **Conclusion:** The present study demonstrated that the polyherbal formulation in both dosage forms could be used for the hepatoprotective activity to treat liver toxicities for adults (capsules) and for pediatric and geriatric (Suspension) patients.

Keywords: *Ricinus communis*, *Allium sativum*, *Piper nigrum*, Hepatoprotective activity, Capsules, Suspension.

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INTRODUCTION

The liver is the most significant gland in the human body that controls the majority of chemical levels in the blood and excretes bile which aids in removing toxic materials from the liver. As it is the primary site for metabolizing harmful substances and medications, it is incredibly vulnerable to various infections and injuries. Hepatotoxicity is a major public health issue in most countries. Chemicals that cause damage to the liver are called hepatotoxins. Carbon tetrachloride, Thioacetamide, Paracetamol, chemotherapeutic drugs, microbes, prolonged alcohol intake, viruses (Hepatitis A, Hepatitis B, Hepatitis C, and Hepatitis D),¹ Obesity, genetic defects (Haemochromatosis), etc., can induce hepatotoxicity. The control of these liver diseases is a strenuous job due to the high cost, and additional side effects of currently available modern medications have not been able to provide a satisfying solution for liver problems. As a result, alternative medications for treating liver illnesses must be sought to replace the currently utilized drugs, which are of dubious efficacy and safety.

In ancient systems of medicine, particularly in ayurveda, many medicinal plants have been used to treat liver problems, as these are significant sources of hepatoprotective drugs.² Hepatoprotective action has been claimed for various plants and polyherbal preparations.³ The polyherbal formulations in the present study include *Ricinus communis*, *Allium sativum*, and *Piper nigrum*.

Ricinus communis has both traditional and medicinal value, which helps to maintain a healthy life. This can be used as a purging agent, laxative,

fertilizer, insecticide, etc.⁴ as the plant possess beneficial effects such as antioxidant, antibacterial, antifungal, anti-inflammatory, antidiabetic, hepatoprotective,^{5,6} antihistaminic, antinociceptive, antiasthmatic, antiulcer, immunomodulatory, antifertility, central analgesic activity, bone regeneration activity, lipolytic, wound healing, insecticidal, larvicidal and many other medicinal properties.⁷

Allium sativum is well known for its medicinal properties from ancient times, and recent research has also annexed various pharmacological activities.⁸ The health benefits of garlic are accounted for due to organo-sulfur compounds such as allicin, diallyl disulfide, diallyl trisulfide, and S-allyl cysteine. *Allium sativum* has numerous therapeutic roles such as antimicrobial, antioxidant, antibiotic, antiviral, antitumor, anti-inflammatory, antigenotoxic and, hepatoprotective.⁹⁻¹¹

An alkaloid, piperine is a crucial prime component of *Piper nigrum* attributed to its medicinal significance. Black pepper is used in traditional Indian medicine, and is also a commonly used spice in daily food routine, which exerts different biological activities like antimicrobial, antitumor, anti-inflammatory, antioxidant, anti-larvicidal, anti-obesity, antidiabetic, hepatoprotective, neuroprotective etc.¹²⁻¹⁴ It also helps to increase the bioavailability of drugs and nutrients in the body.

Based on the above brief literature survey, the present study focused on developing and evaluating the polyherbal formulations containing *Ricinus communis*, *Allium sativum*, and *Piper nigrum* for hepatoprotective activity.

MATERIALS AND METHODS

Collection of Herbal Powders

For the preparation of polyherbal powder, *Ricinus communis* leaves were collected from the plant, *Piper nigrum* seeds were purchased from the local market, and *Allium sativum clove* powder was purchased from Amazon Pvt. Ltd. The collected *R. communis* leaves were washed with water to remove dust particles, shaded at room temperature for one week, and powdered with a mixer grinder. *P. nigrum* seeds were also powdered using a mixer grinder.

Phytochemical Screening

The powders of *R. communis* leaves, *A. sativum* cloves, *P. nigrum* seeds were subjected to phytochemical screening for the presence of phytosterols (Salkowski's test), alkaloids (Wagner's test), glycosides (Keller - Kiliani test), flavonoids (Shinoda test), and carbohydrates (Molisch's test).^{15,16}

Formulation of Polyherbal Capsules

Preparation of Granules: Granules were prepared by using the wet granulation method. A coherent mass was made by mixing dried powders of the three components (*R. communis* leaves, *A. sativum* dried cloves, *P. nigrum* seeds) with lactose and a binding agent (starch) with a composition shown in Table 1. The powder mixture thus prepared was run through #20 to form granules. Later, the granules were spread out gently and dried below 60°C. Then, the weight of dried granules was measured and recorded, and the uniform sized granules were collected by passing the dry granules through #22, positioned on top of size #44. The capsules were filled uniformly with granules by adding other ingredients such as talc and magnesium stearate¹⁷ and were further evaluated for flow properties.

Filling of Capsules: The prepared uniform sized granules were packed into a hard gelatin (size 00) using hand operated capsule filling machine such that each capsule contained 600 mg of granules. Polyherbal capsules containing granules made with 10%, 12%, and 15% of starch were labeled as CF1, CF2, and CF3, respectively (Table 1).

Evaluation of Granules: The prepared granules are evaluated for the following parameters¹⁸

- **Angle of repose:** The flow characteristics of granules were measured by the angle of repose. Angle of repose is the maximum angle between the surface of a pile of powder and the horizontal plane. It was determined by the fixed funnel method using the formula. $\tan \theta = h/r$, where, h = height of the pile, r = radius of the pile, θ = angle of repose.
- **Bulk density:** Definite quantity of granules was placed in a 100ml measuring cylinder and the volume occupied by the granules was noted without tapping. The bulk density was calculated as the ratio of weight to volume.
Bulk density = weight of the granules ÷ volume of the granules
- **Tapped density:** It was determined by taking the granules into a graduated 100ml measuring cylinder, and the volume occupied was noted after tapping (x100). The tapped density was calculated by the formula.
Tapped density = weight of the granules ÷ volume after tapping
- **Compressibility index (or) Carr's index (C%):** It is a simple, fast, and popular method of predicting powder flow characteristics and is indirectly related to the relative flow rate, cohesiveness, and particle size. The compressibility index value of granules was computed according to the equation.
Compressibility index = (tapped density- bulk density) ÷ tapped density ×100

Table 1: Composition and results of evaluation tests of polyherbal capsules.

Names	CF1	CF2	CF3
Ingredients in composition			
<i>R. communis</i> dried leaf powder (mg.)	200	200	200
<i>A. sativum</i> dried clove powder (mg.)	200	200	200
<i>P. nigrum</i> seed powder (mg.)	150	150	150
Lactose mono hydrate (mg.)	30	30	30
Starch paste (%)	10	12	15
Talc (mg.)	10	10	10
Magnesium stearate (mg.)	10	10	10
Total weight (mg.)	600	600	600
Parameters evaluated			
Bulk density (g/ml) (Mean ±SD)	0.287 ±0.083	0.321±0.020	0.329 ±0.060
Tapped density (g/ml) (Mean ±SD)	0.359 ± .020	0.364±0.030	0.395 ±0.03
Hausner's ratio (Mean ±SD)	1.25 ± 0.64	1.13 ±0.15	1.20 ±0.81
Carr's index (%) (Mean ±SD)	20.0± 0.55	11.8± 0.47	16.7 ± 0.25
Angle of repose θ (Mean ±SD)	31.87± 0.49	25.19 ±0.64	32.40±0.30
Average Weight (Mean ±SD)	595± 0.15	597± 0.52	608± 0.57
Weight variation (Mean ±SD)	0.21± 0.056	0.17± 0.065	0.25± 0.05
OD value	0.6	0.8	0.7

n=3 and values are given in mean±SD

- **Hausner's ratio:** Hausner's ratio of granules was determined by comparing the tapped density to the bulk density using equation.
Hausner's Ratio = Tapped density/Bulk density

Evaluation of Capsules

Weight Variation Test: In this test, 20 polyherbal capsules were randomly selected from each formulation, weighed individually, and calculated their average weights. For all the capsules, the difference between each tablet's weight and the average weight was noted. Then percentage variation of each capsule from the average weight of tablet was calculated. The capsules meet the pharmacopeia specifications, when not more than 2 capsules are outside the percentage limit and if no capsule differs by more than 2 times the percentage limits.

$$\% \text{ Weight variation} = \frac{\text{Weight individual} - \text{Weight average}}{\text{Weight individual}} \times 100$$

Preparation of polyherbal suspension

R. communis leaves powder, *A. sativum* clove powder, and *P. nigrum* seeds powder were properly mixed using a mortar and pestle. After that, Sodium Carboxy Methyl Cellulose (CMC) solution (Table 2) was added to the prepared powder mixture to form a suspension. Followed by this, also added stevia powder, Amaranth color and peppermint oil. Finally, the volume was made to 15ml with purified water, as shown in Table 2.

Table 2: Composition and results of evaluation tests of polyherbal suspensions.

Names	SF1	SF2	SF3
Ingredients in composition			
<i>R. communis</i> dried leaf powder (mg.)	200	200	200
<i>A. Sativum</i> dried clove powder (mg.)	200	200	200
<i>P Nigrum</i> seed powder (mg.)	150	150	150
Sodium CMC (%)	0.1	0.15	0.2
Sodium benzoate(mg.)	10	10	10
Stevia powder(mg.)	5	5	5
Peppermint oil(ml.)	0.2	0.2	0.2
Amaranth solution	Quantity sufficient	Quantity sufficient	Quantity sufficient
Purified water	up to 15 ml	up to 15 ml	up to 15 ml
Parameters evaluated			
Particle size (microns) (Mean ±SD)	0.287 ±0.083	0.321 ±0.020	0.329 ±0.060
pH (Mean ±SD)	0.359 ± .020	0.364 ±0.030	0.395 ±0.03
Viscosity (cps) (Mean ±SD)	1.25 ± 0.64	1.13 ±0.15	1.20 ±0.81
OD value	20.0± 0.55	11.8± 0.47	16.7 ± 0.25

n=3 and values are given in mean±SD

Evaluation of Polyherbal Suspension

- **Sedimentation Volume:** Sedimentation volume was recorded from the ratio of the sediment's ultimate volume (Vu) to the total suspension's initial volume (Vo). It was measured by keeping a fixed suspension volume in a measuring cylinder in an undisturbed state for a certain period and calculating sedimentation volume (F) % in different time intervals to plot sedimentation rate.

$$F = 100Vu / Vo$$

- **pH:** All the formulations pH was determined by using pH meter. The pH meter was calibrated with standard pH4 and pH7 buffer solutions before each use. The pH of formulation was calculated 30ml into a clean beaker.
- **Particle Size:** The particle size of the suspension was determined by taking the sample suspension after shaking the bottle using the microscopy method with eyepiece and stage micrometer. Then the particle sizedistribution was plotted by taking particle size on X- axis and percentage of particles on Y- axis.
- **Viscosity:** The viscosity of the formulations was determined using Brookfield Digital Viscometer (Model: LV DV-E). The viscosity of the formulations was determined using Brookfield Digital Viscometer (Model: LV DV-E). The fixed volume of the formulation was taken in a beaker and maintained at room temperature. Viscosities were determined at 20 rpm in triplicate with spindle no. T.F-96 and recorded the mean viscosity for three formulations.

Calculation of Overall Desirability or Desirability Function

The Overall Desirability (OD) or Desirability Function (DF) was used to select the best desired polyherbal formulation by combining all the responses to get desired characteristics. The best formulation should have the desired characteristics. These include high flow properties for

capsule, small particle size, viscosity and high sedimentation volume for suspension. The individual desirability of each formulation was calculated using the following method.^{19,20}

The desirability function for low particle size was calculated using the equation 1: $ID_1 = Y_{max} - Y_i / Y_{max} - Y_{target}$ (1) $ID_1 = 1$ for $Y_i = Y_{target}$, where ID_1 is the individual desirability of particle size. The desirability function for high flow properties, viscosity and sedimentation volume was calculated using the equation 2: $ID_2, ID_3 \& ID_4 = Y_i - Y_{min} / Y_{target} - Y_{min}$(2) $ID_2, ID_3 \& ID_4 = 1$ for $Y_i = Y_{target}$, where $ID_2, ID_3 \& ID_4$ are the individual desirabilities of flow properties, viscosity, and sedimentation volume, respectively. The overall desirability values (OD) for each formulation were calculated from the individual desirability values by using the equation 3: $OD = (ID_1 ID_2 ID_3 \dots ID_n)^{1/n}$(3), where n = number of desirable responses of the experiment.

Ex vivo studies

Ex vivo studies²¹ were conducted to determine the hepatoprotective activity of prepared formulations using liver slices as per protocol shown in Table 3.

Liver Homogenate Preparation: After immediate dissection of the anesthetized and decapitated male Wistar rat, the fresh liver was collected, rinsed with phosphate buffer saline with pH 7.4 and transferred to a sterile vessel containing PBS, which was sliced into required groups with a sterile scalpel. 1g of liver tissue was added to 5 ml. of buffer solution. Likewise, six separate groups were formed, each containing 1 gm of liver tissue. In all groups except the control group. CCl_4 was added and placed in an incubator for 60 min. The samples were then homogenized in a boiling tube with a homogenizer. And the homogenate was centrifuged at 5000 rpm for 30 min. The supernatant obtained was used to estimate lipid peroxidation and reduced glutathione assays.

Lipid Peroxidation Assay: Lipid peroxidation²² was estimated calorimetrically by measuring Malondialdehyde (MDA). 0.5 ml of tissue homogenate was treated with 0.5 ml carbon tetrachloride (CCl_4) and different concentrations of formulations (Table 4), i.e., standard (Liv 52), capsule formulation, suspension formulation along with 2 ml of 1:1:1 ratio thiobarbituric acid (TBA 0.6%), trichloroacetic acid (TCA 1.5%), hydrochloric acid (HCl 0.25N) reagents were added and placed in a water bath at 85°C for 30 min and cooled. The optical density of the pink color solution was measured at 535nm, and the control group samples were prepared similarly without CCl_4 . The MDA formed was calculated using the molar extraction coefficient of thiobarbituric acid reactive substances (TBARS: 1.56×10^5 mol/cm). The product of Lipid peroxidation (LPO) was expressed as the number of moles of MDA

Table 3: Protocol for ex-vivo studies

S. No	Group	Purpose	treatment
1	I	Normal Control	Not infected
2	II	Disease control	Infected by treating with CCl_4
3	III	Standard (reference group)	Infected with CCl_4 and treated with standard (Liv52) formulation at different concentrations
4	IV	Test group -1	Infected with CCl_4 and treated with capsule formulation at different concentrations
5	V	Test group-2	Infected with CCl_4 and treated with suspension formulation at different concentrations

Table 4: The results of ex-vivo studies.

Group No.	Group name	Concentration (µg/ml)	LPO (TBARS) (nM/mg of tissue)	GSH (µg of GSH in mg of wet tissue)
I	Normal control	-	0.244 ± 0.07	17.1 ± 0.72
II	Disease control	-	0.452 ± 0.07	8.234 ± 0.74
III	Standard formulation	100	0.247 ± 0.04	10.512 ± 0.77
		200	0.234 ± 0.02	10.784 ± 0.78
		400	0.231 ± 0.07	12.832 ± 0.7
		800	0.158 ± 0.07	15.944 ± 0.72
		1600	0.105 ± 0.01	16.952 ± 0.71
IV	Capsule formulation	100	0.234 ± 0.06	9.72 ± 0.28
		200	0.166 ± 0.01	9.232 ± 0.70
		400	0.141 ± 0.04	10.432 ± 0.70
		800	0.128 ± 0.04	11.184 ± 0.77
		1600	0.116 ± 0.03	14.416 ± 0.28
V	Suspension formulation	100	0.273 ± 0.03	10.512 ± 0.70
		200	0.266 ± 0.07	10.716 ± 0.82
		400	0.218 ± 0.01	12.352 ± 0.77
		800	0.213 ± 0.0701	12.624 ± 0.70
		1600	0.209 ± 0.056	13.36 ± 0.28

n=3 and values are given in mean±SD

formed per gram of tissue. The percentage oxidation inhibition was calculated from the following formula.

$$\% \text{ Inhibition} = \frac{\text{control absorbance} - \text{test absorbance}}{\text{control absorbance}} \times 100$$

Reduced Glutathione Assay: 100 mg of liver tissue was homogenized in 1ml of 0.01 mM phosphate buffer. Then, 0.5 ml of the homogenate was mixed with 1.5 ml of distilled water, 0.5 ml of respective formulation and 1.5 ml of 0.2 M Tris buffer. 0.1 ml of 0.01 M Ellman's reagent and mixture was made to 10 ml by the addition of absolute methanol. The tubes were shaken intermittently for 10-15 min and then centrifuged at 3000 rpm for 15 min (REMI centrifuge).²³ The absorbance was recorded within 5 min of the addition of 5,5- dithio-bis-(2-nitrobenzoic acid) DTNB at 412 nm against reagent blank with no homogenate by UV spectrophotometer (SHIMADZU). For control, the same procedure was followed but used std. glutathione solution (50 µg/ml) instead of the formulation. The amount of GSH in the tissue was calculated from the following equation: % of reduced glutathione (ΔA) = $(A_0 - A_1)/A_0 \times 100$, (A_0 – absorbance of the control, A_1 – absorbance of the sample) Concentration of reduced glutathione = $\Delta A \times 1.36 \times 10^4$.

RESULTS

Phytochemical Screening

The qualitative analysis of Phytochemical constituents in individual powders of *R. communis* leaves, *A. sativum* cloves, *P. nigrum* seeds, and the combination of three powders proved to contain Phytosterols, Alkaloids, Flavonoids, Glycosides, and carbohydrates.

In vitro Evaluation of Polyherbal Formulations

Capsule Formulations

The flow properties and weight variation of granules were assessed and the results are tabulated in Table 1. CF2 formulation showed good flow

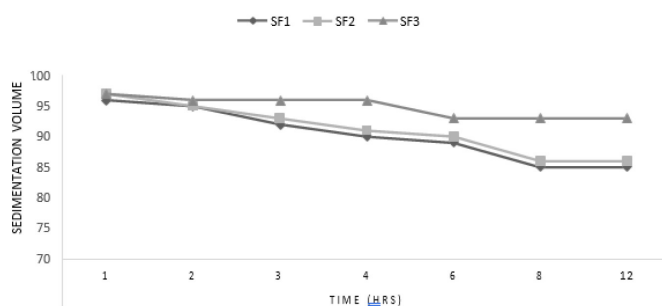


Figure 1: Sedimentation rate of suspension formulations.

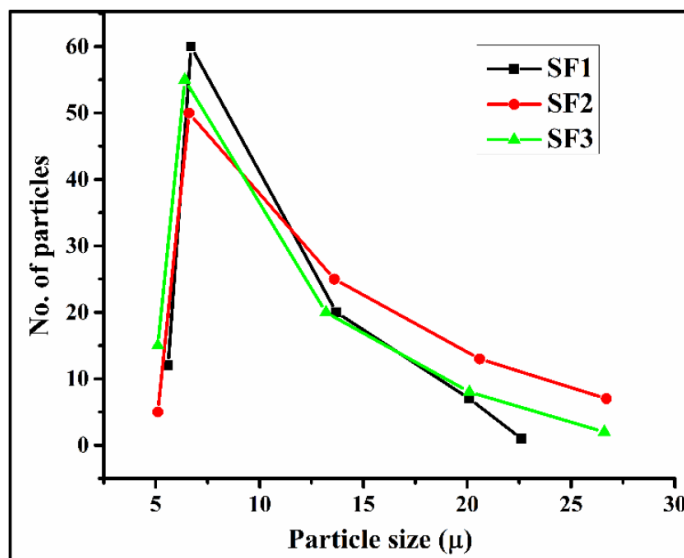


Figure 2: Particle size distribution of suspensions.

properties, whereas the remaining two formulations showed fair flow properties. The weight variation of all three batches was within limits.

Suspension Formulations

The polyherbal suspensions were evaluated for the parameters like particle size, pH, and viscosity, and the results are given in Table 2. The sedimentation rate and particle size distribution of suspensions are shown in Figure 1 and Figure 2. The pH of all the prepared formulations was between 4.2 and 4.75, and the viscosity was between 390 cps and 1,152 cps. The mean particle size of suspensions ranged from 13 to 14.5 micrometers. The sedimentation volume F% was in the range of 85 to 93.

Ex vivo Studies

The results of the Lipid Peroxidation assay and reduced glutathione assay are given in Table 4.

DISCUSSION

The two polyherbal formulations (capsules, suspension) were developed with the combination of powders of *RC* leaves, *AS* cloves, and *PN* seeds for hepatoprotective action as these already proved to possess good antioxidant activity individually.²⁴⁻²⁶ The plants were also claimed to use in liver diseases. Hence, the present study initiated the development of one solid dosage form (capsules) for adults and one liquid dosage form (suspension) for easy administration to pediatric and geriatric patients. In the development of these two dosage forms, three batches of each

dosage form were developed and evaluated by *in vitro* studies to select the best formulation by calculating OD/DF value which was further assessed by *ex vivo* studies for hepatoprotective activity.

Three batches of capsules (CF1-CF3) were prepared by filling granules made by wet granulation method with varying concentrations (10%, 12%, 15%) of starch as a binder (Table 1). The granules were evaluated for their flow properties like bulk density, tapped density, angle of repose, Carr's index, Hausner's ratio, and capsules for their weight variation. It was observed that among the three formulation batches, the formulations CF1 and CF3 showed fair flow properties as per Hausner ratio (1.25, 1.20), Carr's index (20, 16.7), and angle of repose (31.87, 32.4), but CF2 formulation demonstrated good flow characteristics with Hausner's ratio of 1.13, Carr's index 11.8 and angle of repose 25.19 (Table 1). So, the CF2 formulation was considered as the best capsule formulation due to its high OD value. The weight variation of the three formulations was within limits.

Three formulation batches of suspension (SF1-SF3) were prepared with varying concentrations of sodium CMC (0.1%, 0.15%, 0.2%), a thickening agent (Table 2), and were evaluated for sedimentation volume and rate, viscosity, pH, particle size and particle size distribution.

The pH of formulations was within the range of 4.2 to 4.5. The mean particle size was less for the SF1 formulation though there was no significant difference in particle size and its distribution (PSD) (Figure 2) among all the three suspension formulations. There was also no significant difference in sedimentation volume F (%) among the three formulations. The viscosity of the suspension formulation was significantly increased with an increase in the concentration of sodium CMC. The viscosity of the formulation reduces the sedimentation volume and assures the uniform distribution of active ingredients in all doses; eventually, it should be easily pourable to withdraw the dose from the container. Hence, SF1 was selected as the best formulation due to its easy pourability compared to SF2 and SF3 in spite of their high viscosity and OD value.

Then *ex vivo* studies were conducted with CF2 and SF1 formulations to confirm their hepato- protective activity using CCl_4 induced model. It has been extensively utilized in animal models to induce liver injury caused by reactive oxygen species (ROS), similar to hepatotoxicity in humans. The harmful effects of CCl_4 are due to the free radical generation. Unless neutralized by the radical scavengers, the formed peroxy radicals absorb hydrogen atoms from the other lipid molecules, further the lipid peroxidation (LPO) process. *Ex vivo* (liver tissue) tests were intended to assess the harmful effects of carbon tetrachloride using a biochemical parameter indicative of oxidative stress and their amelioration by different formulations (i.e., capsule and suspension) formulations compared to standard formulation) (Table 4).

CCl_4 triggered lipid peroxidation in CCl_4 -induced rat liver slices with a TBR concentration of 0.4522 mm/mg, confirmed the hepatotoxicity induction. CF2 and SF1 formulations showed substantial results compared to the standard formulation in reducing TBR levels. In comparison, the CF2 formulation effectively prevented the lipid peroxidation on par with the standard formulation than the SF1 formulation may be due to more contact time with tissue in solid form (Table 4).

CCl_4 reduced the glutathione levels in the liver slices produced with 8.234g/ml, indicating the generation of hepatotoxicity. CF2 and SF1 formulations performed almost similarly to elevate the reduced glutathione levels but were not equal to the standard formulation.

In both the assays of *ex-vivo* studies, it was found that the reduction in TBAR levels and rise in GSH levels in liver tissue were increased with increase in concentrations of standard, capsule, and suspension formulations. It was also observed that there was no significant

difference ($P \leq 0.1$) in the results of *ex-vivo* studies conducted by standard, capsule, and suspension formulations (Table 4) indicating that both the formulations have shown hepatoprotective activity.

CONCLUSION

The present study developed a polyherbal formulation in the form of both capsules (solid dosage form) and suspensions (liquid dosage form) with the combination of powders of *Ricinus communis* leaves, *Allium sativum* cloves, and *Piper nigrum* Seeds for hepatoprotective activity successfully to administer to adults, pediatric and geriatric patients easily. Further, its activity may be confirmed by *in vivo* studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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