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A novel multiherbal formulation alleviate Carbon Tetrachloride (CCl_4) induced serum and tissue protein alterations in experimental murine model

Soumendra Darbar[1], Srimoyee Saha[1] and Atiskumar Chattopadhyay[2*]

[1]Research and Development Division, Dey's Medical Stores (Mfg.) Ltd., 62, Bondel Road, Kolkata-700019, West Bengal, India
[2]Department of Allied Health Science, Brainware University, 398, Ramkrishnapur Rd, Barasat, Kolkata, West Bengal 700125

E-mail: anwesh.chattopadhyay@gmail.com/ dac.ah@brainwareuniversity.ac.in
[*]**Address for Correspondence**
Dr. Atiskumar Chattopadhyay, M.Sc., M.Tech, M.Phil, Ph.D
Professor
Department of Allied Health Science
Brainware University
398, Ramkrishnapur Rd,
Near Jagadighata Market,
Barasat, Kolkata,
West Bengal 700125
Contact No.: 0919433144548
E-mail: anwesh.chattopadhyay@gmail.com / dac.ah@brainwareuniversity.ac.in

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ABSTRACT

The proteins are essential for normal physiological functioning and maintaining the homeostasis. To know about general health the serum albumin, serum globulin and albumin-to-globulin ratio (AGR) are considered as the good parameters. These markers also help to assess the immune functioning and (AGR) is a prognostic factor for many medical conditions. To understand the effects of a novel multiherbal formulation (AKSS16-LIV01) prepared on this aspect of the health experiments are done with CCl4 treatment and administration of the formulation. This CCl4 is also considered as an environmental toxicant which also broadens the implications of this study. From results of the experiments, it has been seen that administration of the formulation significantly lowered elevated albumin and globulin levels and normalised the albumin-to-globulin ratio. It can be inferred from the study that the formulation has potent effects on maintaining the levels of the serum marker proteins, which implicates the healing effects of the formulation. It can be due to synergistic effects of the molecules present in the constituents of the formulation.

KEYWORDS: Multiherbal formulation, albumin, globulin, albumin-to-globulin ratio (AGR), CCl4

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1. INTRODUCTION

The body needs protein to function and survive. Alteration of serum total protein leads to various complications and sometimes damage vital organs [1]. The major components of the serum protein are albumin and globulin, represents the nutritional status of the body which maintain the colloidal osmotic pressure in blood [2-4]. These two proteins also maintain body's immune function, which prevent infection [5]. Scientific literature revelled that albumin-to-globulin ratio (AGR) is a prognostic factor of various diseases and medical complications [6,7]. Clinical study indicate that decline albumin levels showed poor nutrition status, sometimes very fatal to survive [8,9].

Carbon tetrachloride (CCl_4) is a major industrial pollutant associated with production of free radicals which creates various organ dysfunction like liver and kidney [10]. It is established that metabolic activation of CCl_4 by cytochrome P450 produced trichloromethyl radical ($\cdot CCl_3$) and peroxy trichloromethyl radical ($\cdot OOCCl_3$) which initiates lipid peroxidation, responsible for membrane disruption leads to liver and kidney injury [11]. Long-time exposure of CCl_4 alter the normal protein level in the body which creates various type of organ dysfunctions [12]. Animal study showed that administration of CCl_4 decrease normal food and water intake produce nutrition deficiency syndrome [13].

Different allopathic medications are used to treat hepatotoxicity; among these are cholestyramine, ursodeoxycholic acid, spironolactone, loratadine, vasopressin, and others. Some of these medications have side effects that include constipation, diarrhoea, flatulence, abdominal pain, in extreme cases, they may even cause encephalopathy [14-20]. In ethnomedical practices and traditional medical systems in India, Africa, and other places, a number of medicinal plants and their formulations are widely used as more reliable therapeutics for liver disorders to evade side effects caused by the modern hepatoprotective drugs in allopathic medical practices [21, 22].The phytochemical components found in these medications, such as phenol, coumarins, monoterpenes, glycosides, flavonoids, alkaloids, and xanthenes, are significant contributors to their hepatoprotective effects [23]. Four billion people, or around 80% of the world's population, live in underdeveloped nations where they rely on herbal medicines to cure a variety of illnesses. In recent years, the usage of herbal remedies has increased globally [24–27]. Around 80% of people in Bangladesh depend on herbal medicine for their primary healthcare [28, 29], since it has fewer adverse effects and is less expensive [30] as 85% of the country's population lives in rural areas [31].

Long term, safe and symptomatic medication without side effects is one of the main approaches of alternative system of medicine comprising herbal products [32]. The plant-based formulation is enriched with various essential phytochemicals and enormous antioxidants, serrates to prevent diseases [33,34]. We developed a

novel, low-cost herbal formulation composed of medicinal plants and spices. Our previous study upon animals showed that this formulation does not produce any toxic effects and safe for therapeutic medication [35,36]. Here we tried to apply this traditional medicine to maintain the essential protein level altered experimentally by CCl_4 treatment.

2. MATERIAS AND METHODS

2.1 Chemicals

Carbon tetrachloride (CCl_4) and TRIS buffer were obtained from Merck, India. PBS pH 7.4 was procured from Sigma-Aldrich. Biochemical determination kits i.e., total protein, albumin and globulin were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

2.2 Preparation of Extract –

Fresh parts of the medicinal plants were first air-dried after cleaning with double distilled water and kept in an oven at 80ºC for 10 min and 60ºC for 30 min and grounded by a blade mill to a fine powder. After that the polar fraction was extracted by modified method of Adhikari *et al.* (2018) [37].

2.3 Examination of colour, odour and taste

Color: Five grams AKSS16-LIV01 of were placed in watch glasses and placed in a white tube light against a white background. Their color was visible to the naked eye.

Scent: Two grams of Trasina scent.

Taste: A pinch of Trasina was taken and its taste was explored with the tongue taste.

2.4 Calculating the loss after drying

2 g of soil material were weighed into a dry petri dish (a tar evaporating dish) to quantify loss on drying. The soil was dried at 105-110 oC until two subsequent weights did not deviate by more than 5 mg. After drying, the weight was measured, and the drying loss was computed. The proportion was given in weight percent for the air-dried sample [38].

2.5 Measurement of the overall ash

In a pre-weighed crucible, 1 g of ground, air-dried material was utilized, and its ash content was determined by gradually heating it to between 500 and 600^{o}C until it was carbon-free. After cooling off, it was dried and weighed. The total ash was calculated as a mass percent of the mass of air-dry material [38].

2.6 Calculating the extractive value of water

5 g of AKSS16-LIV01that had been accurately weighed were immersed in an Erlenmeyer flask with a glass lid. 100 cc of chloroform water was added, and the mixture was soaked and agitated continuously for 6 hours. It was quickly filtered after standing for 18 hours, and 20 ml of the filtrate was added to a plate with a flat bottom and tar on top. The dish was dried out on a boiling water bath after 24 hours. The dish was dried at 105°C for 6 hours, and then it was cooled and weighed. When compared to the air-dried sample, the weight percentage of the water-soluble extract in the residue was calculated [38].

2.7 Bacterial load

The standard recommended outlined technique was utilized to determine the microbial burden with a little modification. We adhere to the accepted Indian Pharmacopoeia procedure [39].

2.8 Calculation of pH

A 100 ml volumetric flask was filled with distilled water and 1 g of LIV01-SSA-23 powder. For around ten minutes, sonicate the solution. We used a digital pH meter to measure pH.

2.9 Animals

Twenty-four young, healthy Swiss albino mice weighing 25g ± 5g have been randomly included for the study. The animals have been housed in healthy atmospheric conditions (12 h light and dark cycles, at 25±2 ºC and 50-60% humidity), normal feeding, drinking, and medical care based on the CPCSEA guidelines. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any infection. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. 261/JU/s/IAEC/Pharma/2018).

2.10 Experimental procedure

The mice were randomly assigned to four major groups of six mice each according to their body weights such that each group was made up of mice within the close range of body weight. The groups are as follows: Group-I serve as control, Group-II received Multi herbal formulation (AKSS16-LIV01) 400 mg/kg/day, Group-III received carbon tetrachloride (CCl_4) 1 ml/kg-bw and Group-IV received CCl_4 along with AKSS16-LIV01 (400 mg/kg).

2.11 Body weight, food consumption and water intake

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by body weight gain. Water intake was determined by subtracting the remaining of water found in the drinking bottle from the initial water given to the animals.

2.12 Blood Collection and serum preparation

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow colour serum was separated and used for further analyses.

2.13 Preparation of tissue homogenate

A small portion of the liver and kidney tissues was homogenized in ice-cold 0.9% w/v saline using a homogenizer to obtain 20% homogenate. Aliquots of the liver homogenate were stored at 4^0 C prior to biochemical analysis.

2.14 Determination of serum, liver and kidney protein

Serum and tissue homogenate were used for the determination of total protein, albumin and globulin. Total protein, albumin and globulin were determined according to the standard biochemical protocol with slight modification using colorimetric kit obtained from Thermo Scientific, USA.

2.15 Statistical analysis

Data are presented as mean ±SE. Statistical analysis of the data was carried out using two-way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis. Statistical significance was acceptable to a level of $p< 0.05$.

3. RESULTS

3.1 Effect of multi herbal formulation (AKSS16-LIV01) on Physical observation

Our developed a novel multi herbal formulation (AKSS16-LIV01) which is composed of six Indian medicinal plants and three medicinal spices those are used in Indian traditional system of medicine. The formulation consists of *Tinospora cordifolia*-20 mg, *Terminalia chebula*-20 mg, *Azadirachta indica*–50mg, *Andrographis paniculata*-50 mg, *Aloe barbadensis Miller* - 50 mg, Curcuma *longa*-20 mg, *Trigonella foenum-graecum*-10 mg, *Piper nigrum*-10 mg and *Elettaria cardamomum* -10mg (Table 1).

Table 1: Details ingredient(s) present in the newly developed multi herbal formulation (AKSS16-LIV01)

Sl. No.	Botanical Name	Common Name	Family	Quantity used in extract
1.	*Tinospora cordifolia*	Guduchi	Menispermaceae	20 mg
2.	*Terminalia chebula*	Haritaki	Combretaceae	20 mg
3.	*Azadirachta indica*	Neem	Meliaceae	50 mg
4.	*Andrographis paniculata*	Kalmegh	Acanthaceae	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	Liliaceae	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Zingiberales	20 mg
7.	*Trigonella foenum-graecum*	Methi	Fabaceae	10 mg
8.	*Piper nigrum*	Black pepper	Piperaceae	10 mg
9.	*Elettaria cardamomum*	Cardamom	Zingiberaceae	10 mg

* Amount depicted in the table are required for preparation of 5 ml extract.

3.2 Effect of multi herbal formulation (AKSS16-LIV01) on analytical parameters

Organoleptic Parameters of AKSS16-LIV01maintain Indian Pharmacopeia standard (Table 2). The range of lime was seen for all chemical parameters, including loss on drying, total ash, acid-soluble ash, and pH (Table 3). The preparations of developed formulation (AKSS16-LIV01) disintegration times fell within acceptable pharmacopoeia bounds as well (Table 3).

Table no. 2: Organoleptic Parameters of AKSS16-LIV01 (multi-herbal formulation)

Organoleptic characters	Observation
Odour	Typical Herbal dust smell
Colour	Light pale brown l
Test	Characteristic
Texture	Soft Powder

Table 3: Different analytical parameters of AKSS16-LIV01 (multi-herbal formulation)

Parameters	Specification	Results
Disintegration	NMT 30 min.	11.55
Loss on drying at 105°C	NMT 10% w/w	1.97% w/w
Total Ash at 450°C	NMT 10% w/w	12.48% w/w
Acid soluble ash	NMT 10% w/w	1.86% w/w
pH	6.0 – 8.0	6.05
Total Bacterial Count	NMT $1x10^5$ cfu/gm	9.75

According to the analytical examination, the product satisfies each of the pharmacopoeia's test requirements.

3.3 Effect of multi herbal formulation (AKSS16-LIV01) on Body weight, Food Consumption and Water Intake

Gross body weights and relative changes, food consumption and water intake were presented in table 4. Administration of carbon tetrachloride (CCl_4) (1 ml/kg-bw) significantly reduced ($p<0.001$) the body weight, food intake and water intake capacity as compared with control animals. Treatment with multi herbal formulation (AKSS16-LIV01) 400mg/kg/day normalized the body weight, daily food intake and water intake capacity and reduced the liver weight as compared with control animals. Administration of AKSS16-LIV01 did not show any abnormal changes as compared with control animals.

Table-4: Effect of multi herbal formulation (AKSS16-LIV01) on body weight, food consumption and water intake

Parameters	Mice			
	Group-I	Group-II	Group-III	Group-IV
Body weight (g) Initial	26.35±1.91	26.51±2.35	26.71±4.2	26.68±5.1
Body weight (g) Final	37.84±2.03	36.94±1.69	21.81±2.41$^{\#}$	36.97±1.67*
Body weight (g) gain or loss	11.49±0.06	10.43±0.04	4.90±0.006	10.29±0.03
Food consumption (g)	4.52±0.05	4.37±0.07	2.94±0.06$^{\#}$	5.11±0.04*
Water intake (ml)	4.01±0.04	4.25±0.04	3.01±0.02$^{\#}$	4.31±0.06*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. $^{\#}$Significantly different from the control group at $p<0.001$ and *Significantly different from (CCl_4) group values at $p<0.001$

3.4 Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney total protein

Figure 1 shows the mean serum, liver and kidney total protein (TP) levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney total protein compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline total protein levels when compared with CCl_4

treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney protein levels when compared with control group.

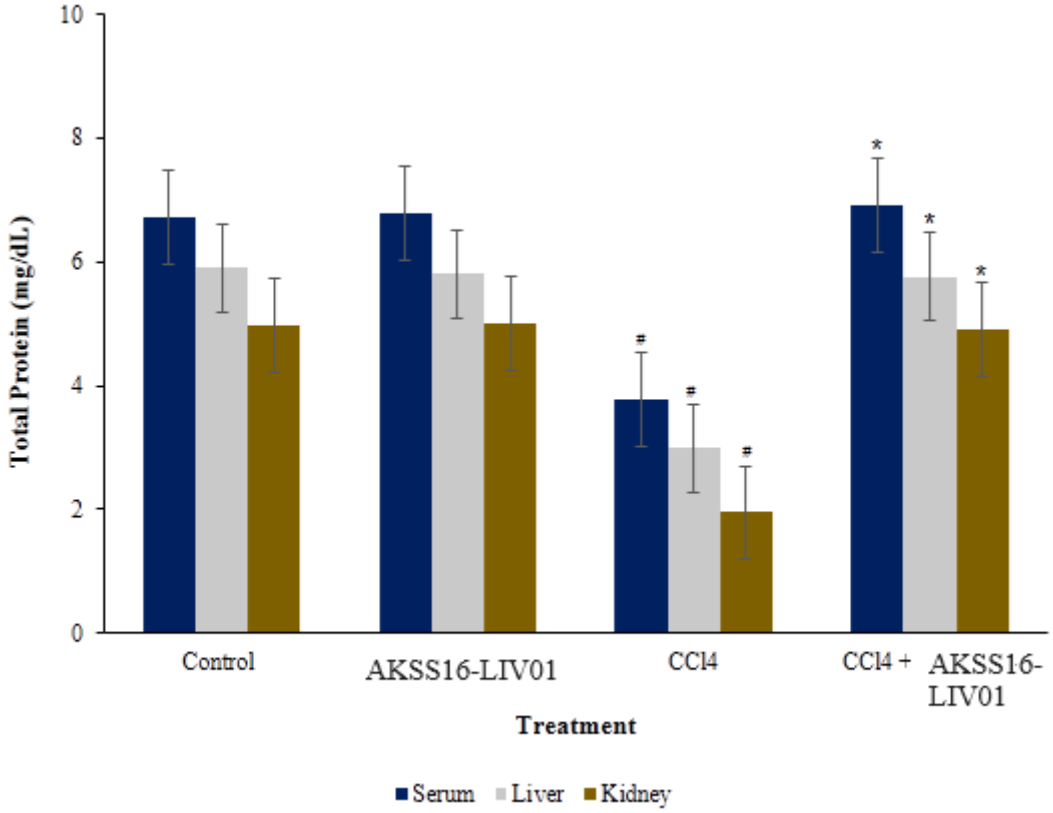

Figure 1: Effect of multi herbal formulation (AKSS16-LIV01) on Total protein levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *significantly different from (CCl_4) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

3.5 Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney albumin

Figure 2 shows the mean serum, liver and kidney albumin levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney albumin compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline albumin levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney albumin levels when compared with control group.

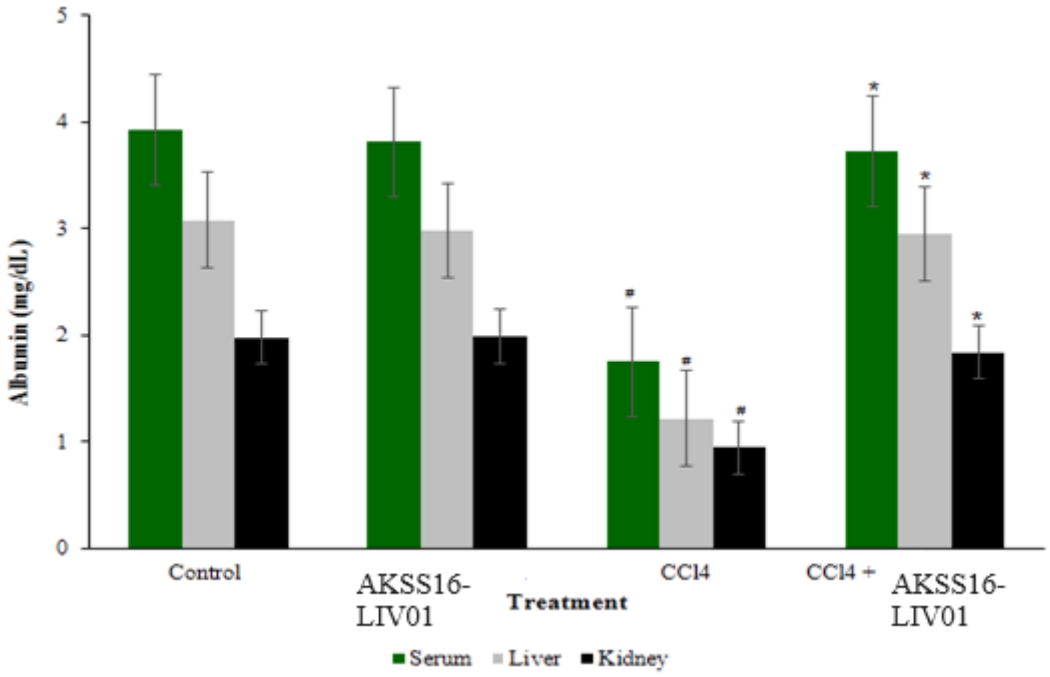

Figure 2: Effect of multi herbal formulation (AKSS16-LIV01) on albumin levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *significantly different from (CCl_4) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

3.6 Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney globulin

Figure 3 shows the mean serum, liver and kidney globulin levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney globulin compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline globulin levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney globulin levels when compared with control group.

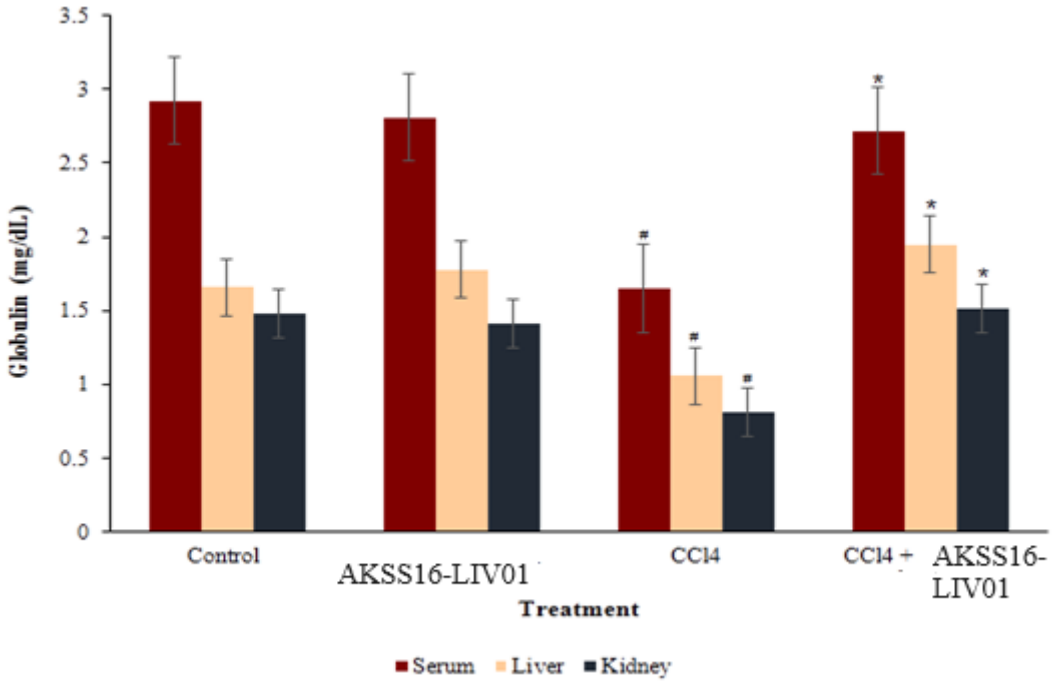

Figure 3: Effect of multi herbal formulation (AKSS16-LIV01) on globulin levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

3.7 Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney albumin/globulin ratio

Table 2 shows the mean serum, liver and kidney albumin/globulin ratio in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney albumin/globulin ratio compared with the control (p<0.001). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline albumin/globulin ratio levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney albumin/globulin ratio levels when compared with control group.

Table 5: Effect of AKSS16-LIV01 on serum, liver and kidney albumin/globulin in CCl_4 induced toxicity

Groups	**Albumin/Globulin ratio**		
	Serum	**Liver**	**Kidney**
Control	1.32±0.12	1.45±0.14	1.48±0.11
AKSS16-LIV01	1.31±0.11	1.37±0.16	1.41±0.12
CCl_4	1.76±0.16	1.58±0.14	1.57±0.15
CCl_4 + AKSS16-LIV01	1.26±0.13	1.43±0.19	1.50±0.18

4 DISCUSSIONS

Ayurveda's core beliefs are prolonging life in health and avoiding needless pain. Ayurveda uses natural methods, such as diet, herbs, spices, minerals, exercise, meditation, yoga, mental hygiene, sounds, smells, and mechano -procedures, to eliminate the disease's root cause by restoring balance and, at the same time, create a healthy lifestyle to prevent the recurrence of imbalance [40,41]. This is in contrast to allopathic medicine, which primarily uses synthetic chemicals designed for specific target receptors and only provides symptomatic relief. In order to avoid sickness and promote wellbeing, longevity and happiness [42], ayurveda is said to be holistic, since it strives to integrate and balance the body, mind, and spirit [43].

Various secondary metabolites of the medicinal plants are mainly responsible for therapeutic effects [44]. Poly herbal drug are very useful for treatment of various diseases due to synergistic effects of different compounds present in the plants [45]. Phenolic compounds and flavonoids present in the aromatic plants are mainly responsible for pharmacological functions and prevent oxidative stress [46].

According to the analytical examination, the product satisfies each of the pharmacopoeia's test requirements. Our study showed that administration of carbon tetra chloride (CCl_4) inhibits normal body growth, food consumption and water intake. Co administration of our developed formulation retained the body weight, food consumption and water intake.

Protein is responsible for normal body growth and development. Abnormal protein level inhibits the body growth which may be occur when subject exposed with environmental toxin [47,48]. Total serum protein is an indicator in liver and kidney damage [49]. In the present study we observed that carbon tetra chloride (CCl_4) significantly decreased the serum, liver and kidney protein levels. Co-administration with AKSS16-LIV01 maintained the normal serum, liver and kidney protein levels.

Albumin play a crucial role to maintain physiological activities of human body [50,51]. It is one of the liver biomarkers as it generates from the liver cells. Low level of albumin is responsible for poor nutrition [52-56]. In this study we observed that chronic administration of CCl_4 declines normal albumin levels in serum, liver and kidney, which was recovered when animals pre-treated with novel multi herbal formulation (AKSS16-LIV01). The result clearly indicates that AKSS16-LIV01 capable to maintain the normal albumin level against the environmental toxicant like CCl_4. On the other hand, scientific study revealed that serum globulin is involved in chronic inflammation. Recent study showed that carbon tetra chloride (CCl_4) alters the serum, liver and kidney globulin and disrupt normal homeostasis. Our study also confirms that application of CCl_4 decreased normal globulin levels in serum, liver and kidney. Treatment with the developed formulation (AKSS16-LIV01) normalized the globulin level in experimental animals.

CONCLUSION

The findings of this investigation demonstrated that the developed formulation includes considerable levels of crucial phytochemicals. The dosage form complies with all of the pharmacopoeia's test requirements, according to the analytical investigation. Chronic administration of carbon tetrachloride (CCl_4) suppressed the normal body growth and reduced normal food and water intake capacity in mice. This environmental toxin reduced the total protein, albumin and globulin levels both in serum and tissues. Our developed novel multi herbal formulation helps in maintaining the normal essential protein levels and prevent the CCl_4 induced deleterious effects in mice. Thus, we believe that the developed formulation composed of medicinal herbs and medicinal spices can be used as a therapeutic medicine in future in serum and tissue protein alterations.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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