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A 28-Day Repeated Dose Toxicity Evaluation of Creatinine Monohydrate and β-Hydroxy β-Methyl Butyrate Combination in Rodent Model

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Abstract

Substance abuse is a global concern, having a significant influence on society, healthcare, and the economy. Athletes are increasingly using substances to improve their performance or to cope with stress. Therefore, having a thorough awareness of the often misused substances, their effects, and potential toxicities is essential. One such chemical is the mixture of Creatinine Monohydrate (CM) and -Hydroxy Methyl Butyrate (HMB), which is widely used by athletes to increase strength and performance. According to studies, the combination can boost athletic performance, build muscle, and offer several health advantages. To determine the chemicals' long-term impacts on human health, more research is required because their safety and toxicity are not well-established. Using TOPKAT(Computer-Aided Toxicity Prediction) and Discovery Studio Version 4.0, this study examined the toxicity and pharmacokinetic profiles of CM and HMB. The interaction of HMB and CM may significantly reduce cell viability compared to either drug alone. There were no negative effects seen in the animal models used for the acute oral toxicity study, but additional research is needed to confirm these results and determine the compounds' long-term safety for human use. The study also performed cytotoxicity studies of individual substances and combinations on VERO and HEP-G2 cell lines and a 28-day repeated dose toxicity study in Wistar albino rats. The study results provide valuable information for healthcare professionals to understand the physiological complications of exposure to such drugs. Overall, the article emphasizes the importance of understanding the potential toxicity of chemical compounds, particularly those used as nutritional supplements.

Keywords: Creatinine Monohydrate, β-Hydroxy β-Methyl Butyrate, Substance Abuse, Toxicity Study

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1. Introduction

Substance abuse and its social impact is a global concern. The impact of physical or psychological dependence on chemical substances causes drastic medical complications. To mitigate such health risks, extensive knowledge of the commonly abused drugs is most needed. In recent times, substance abuse has slowly emerged to be quite common among athletes[1]. Athletes may be susceptible to substance abuse for increased productivity, self-medication for untreated mental illness, dealing with stress, overcoming injury, alleviating physical illness, improved performance, and other professional challenges. Over the decades, substance abuse in sports is omnipresent and poses serious effects, leading to doping arrests, bans, or dire overdoses cases to death[2].

The combination of β-Hydroxy β-Methyl Butyrate (HMB) and creatinine has been established as an effective way for athletes to enhance their strength and improve their athletic performance. When a diet is unbalanced or protein intake is insufficient, HMB may be combined with vitamins, minerals, proteins, or energy supplements to maintain proper dietary balance. Studies have shown that when used with creatinine, HMB can give better results than HMB or creatinine alone. According to published studies, these two most powerful nutritional supplements have additional effects such as strength and muscle building. Creatinine supplements have been shown in many studies which are believed to be associated with weight gain[3,8]. Both HMB and creatinine increase muscle mass through exercise and have been shown to have several health benefits.

In this study, the *in-silico* toxicity and pharmacokinetic profiling of Creatine monohydrate and HMB was done by using *Discovery Studio Version 4.0 and TOPKAT (Computer Aided Toxicity Prediction)* to predict possible toxicity based on structure and correlated with previously available data. The TOPKAT software utilizes a robust and validated QSTR (Quantitative Structure Toxicity Relationship) model to evaluate toxicity and employs a proprietary technique to validate the best prediction range for result interpretation[4]. The software identifies chemicals in its databases that are similar to unknown chemicals, even if they have no known connection in terms of metabolism or carcinogenic mechanisms. DS TOPKAT can quickly and affordably perform various tests such as physical/chemical, genotoxicity, environmental fate, mutagenicity, and sub-chronic reproduction/development. It has a proven track record. Cytotoxicity tests are extensively used in drug discovery and basic research to filter libraries for toxic substances. A literature survey shows the liver and kidney are the most affected cells. Therefore, a cytotoxicity study in VERO cell lines and HEP-G2 cell lines by determining IC50 values was performed. From the kidney of a monkey (*Cercopithecus aethiops*), the VERO cell lines are being used. There are several commercially available VERO cell lines (ie VERO, VERO 76, VERO E6), but all are ultimately obtained from the same source and the protocols in this device can be used with any VERO cell line. In this study, we have used the cells so obtained from NCCS, Pune[5]. HepG2 is the most common human hepatoma used in hepatotoxicity studies and drug metabolism. HepG2 cells are non-carcinogenic cells with great proliferation and epithelial morphology that perform many different liver functions. Cytotoxicity studies of individual substances and combinations were performed on HepG2 and VERO cell lines[6]. A 28-day repeated dose toxicity study of CM and HMB in Wistar albino rats was done[7]. With this toxicological data, understanding the physiological complications that arise in and after exposure to such drugs may be useful for healthcare professionals. The present study was intended to establish a toxicological profile of the CM and HMB combination.

2. Materials and Methods

2.1 Drugs, Chemicals and Cell Lines

Creatine monohydrate was purchased from Himedia Labs Ltd. India and β-hydroxy β-Methyl Butyrate was purchased from TCI Chemicals (India) Pvt. Ltd. Carboxy Methyl Cellulose (CMC) was the used vehicle and was procured from Loba Chemicals Pvt. Ltd., India. All the biochemical diagnostic kits (ErbaMannheim) were procured from Transasia Bio-Medicals Ltd., India.HepG2 and VERO cell lines were procured from *National Centre for Cell Science, Pune, India.*

2.2 *In silico* Pharmacokinetics and Toxicity Screening

Discovery Studio 4.0 (DS) software (Dassault Systemes BIOVIA, France-formerly Accelrys), predicts pharmacokinetic profile and toxicity by an algorithmic model using molecular structures. To understand the pharmacokinetics and toxicity of the selected substances-ADMET and TOPKAT modules were used[8–10]. The MOL files of CM&HMB were obtained from PubChem, NCBI,

USA. The ADMET descriptors module was employed to evaluate the pharmacokinetic profile as per the procedure described by Zeb *et al*[11]. The ADMET_PSA_2D vs. ADMET_AlogP98 plane plot was derived and the results were tabulated. The TOPKAT module was employed to determine the probable toxicities exhibited by the compounds and the procedure described by Selvaraj et al was employed for determining the predictions[12].

2.3 *In vitro* Cytotoxicity Studies in Liver and Kidney Cell Lines

Previous data show that the liver and kidney are challenged by the consumption of creatine monohydrate or HMB in overdoses. Therefore, a cytotoxicity study by MTT assay was performed in VERO[12] and HEP-G2[13] cell lines. MTT assay procedure was carried out as per the procedure specified by Senthil Raja *et al*[12]. After twenty-four hours when the cell population is sufficient, cells are centrifuged and pelleted at 100 µl. Various concentrations of the test sample were prepared in the carrier medium in 96 well plates (incubated at 37° C in 5% CO_2). Absorbance was measured at 540 nm wavelength. Per cent, cell viability is calculated using the following formula given in the procedure described by the authors. The assay was performed with CM, HMB and a 1:1 ratio of CM and HMB combination. Percentage cell viability and IC_{50} values were calculated using the following formula given in the procedure described by the authors[14].

2.4 Experimental Animals

An acute oral toxicity study was performed on female Swiss albino mice and a 28-day repeated dose toxicity study was on the Rodent model using both sexes of Wistar albino rats. Wistar Albino Rats of both sexes (180-220 g body weight b.w.) and female Swiss albino mice (18-22 g b.w.) were obtained from Central Animal House facility, JSS College of Pharmacy- Ooty. The animals were lodged separately in the standard polycarbonate rat cages (groups of 3 in a cage). The males and females were housed separately. They were allowed to acclimatize to the day-night cycle with standard rat pellet feed and water ad libitum for 14 days. Animals exhibiting good normal behaviour were chosen for the study. This study was approved by the Institutional Animal Ethical Committee (IAEC) bearing the Ref. no.- JSSCP/OT/IAEC/04/2019-20; by guidelines specified by the *Committee for Control and Supervision of Experiments on Animals.*, India.

2.5 Acute Oral Toxicity Studies

Acute oral toxicity studies of CM, HMB and 1:1 ratio combination of CM-HMB were carried out by The Organization for Economic Cooperation and Development (OECD) Guideline 423[15,16]. A 2000 mg/Kg b.w. dose of CM, HMB and its combination was chosen for the limit test. The administered volume did not normally exceed 1mL/100g of body weight of each animal. Body weight was recorded before and after administration of test chemicals on days 0, 7th, and 15th days after administration. Death and clinical symptoms were observed for fourteen days. Therefore, we have chosen 1/40th, 1/20th and 1/10th of 2000 mg/kg as our treatment doses where 50 mg/kg b.w. is a low dose, 100 mg/kg b.w. as a moderate dose and 200 mg/kg b.w. is a high dose. This study was approved by the Institutional Animal Ethical Committee (IAEC) bearing the Ref. no.- JSSCP/OT/IAEC/04/2019-20; by guidelines specified by the *Committee for Control and Supervision of Experiments on Animals.*, India.

2.6 Repeated Dose Toxicity Study of HMB, Creatine Monohydrate and its Combination

A repeated dose exposure study was conducted under the guidelines of OECD 407[16]. At the end of the study, we observed that no signs of toxicity in the animals. From the results, three different forms of doses were selected for repeated dose exposure. For repeated-dose exposure 50, 100, and 200 mg/kg doses are selected to administer for the animal to observe signs of toxicity. These doses are selected and administered for 28 days and results are observed. Totally 30 male and 30 female rats are used in the study. Each study consisted of 6 animals. Group 1 was control and it received treatment with vehicle, Groups 2, 3 and 4 received HMB at 50, 100 and 200 mg/Kg respectively, Groups 5, 6 and 7 received Creatine Monohydrate at 50, 100 and 200 mg/Kg respectively, while Group 8, 9 and 10 received both HMB and Creatine Monohydrate at 50, 100 and 200 mg/Kg respectively (ratio 1:1)

2.7 Experimental Design

The animals were randomized into 10 groups (n = 6) Group 1: control, Group 2: 50 mg/kg of HMB, Group 3: 100 mg/kg of HMB, Group 4:200 mg/kg of HMB, Group 5: 50 mg/kg of creatinine monohydrate, Group 6: 100 mg/kg of creatinine monohydrate, Group 7: 200 mg/kg

of creatinine monohydrate, Group 8: Receiving 50 mg/Kg of HMB And Creatine Monohydrate Combination (1:1ratio), Group 9: Receiving 100 mg/Kg of HMB and Creatine Monohydrate Combination (1:1ratio), Group 10: Receiving 200 mg/Kg of HMB and Creatine Monohydrate Combination (1:1 ratio)[17]

2.8 Evaluation of Gross Behaviour- Body Weight and Morphological Animal Observations

Body weight was observed and determined every week for both the study groups-acute (days -0, 7 and 14) and repeated doses (0, 7, 14, 21 and 28) accordingly. Digital balance was used to weigh the animals. The procedure was carried out in a manner that ensured the animal was fasted (only water ad libitum) before weighing.

The animal was under observation 2 times daily; before and after administration of the intended drug formulation. At the designated checkpoints, the animal's morphology appearances and gross changes in their hair colour, texture, lesions etc. were observed and photographs were taken as evidence.

2.9 Blood Sampling and Bio-Chemical Analysis

From the retro-orbital plexus, blood samples are taken with isoflurane anaesthesia. The animal was tolerable to recover from the anaesthesia and was replaced in its cage. The blood collected was then either used for the haematological evaluation or clinical biochemical estimations. (OECD n.d) The biochemical evaluation was carried out in serum obtained after centrifugation of all blood without anticoagulation at 5,000 rpm for 10 minutes. Using standard diagnostic kits from Q-Line TM and Ensure Biotech for estimation in the semi-automatic analyser. SGOT, SGPT, cholesterol, creatinine, glucose, total proteins, albumins and urea were estimated[18].

2.10 Histopathological Studies

After the duration of the study or at the death of animals; the organs- Liver and Kidney from the study group were subjected to Histopathological examinations. After fixing the tissues in 10% v/v formaldehyde the tissues were dehydrated and paraffin blocks were made. Then sectioning was done at about 5-7 µ. Routine histopathology was performed by using the Haematoxylin stain. The tissues were then observed for inflammation, cell necrosis and cell deformities along with characteristic observations.

2.11 Statistical Analysis

P values < 0.05 were considered statistically significant. The values are expressed as Mean ± SEM. Data were analysed by one-way ANOVA followed by Tukey's post-test. The software used here is Graph Pad Prism. The results obtained are tabulated and presented graphically on the following pages[19].

3. Results

3.1 Evaluation of ADMET Descriptors and Toxicity Prediction (TOPKAT) - *In Silico Studies*

The ADMET predictor was the in-silico method used to predict the potential for toxicity and pharmacokinetic parameters of compounds to be identified for molecules. The evaluation of ADMET descriptors and Toxicity Prediction (TOPKAT) of calcium HMB and Creatine monohydrate were carried out in Discovery Studio software version 4.1.

The results of ADMET descriptors and Toxicity Prediction (TOPKAT) of these compounds are shown in Table 1 and Table 2 respectively.

Table 1. ADMET descriptors of calcium HMB and creatine monohydrate

Compound	HMB	Creatine Monohydrate
Solubility	1.246	0.912
Solubility Level	5	5
BBB Level	4	3
Ext Hepatotoxicity	-4.01443	-7.58534
Ext Hepatotoxicity Prediction	TRUE	FALSE
Ext Hepatotoxicity Applicability	10.5704	7.67782
Ext Hepatotoxicity Applicability Mdpvalue	0.0221826	0.950501
AlogP98	-1.52	-1.095
ADMET PSA 2D	55.417	91.096
Unknown AlogP98	0	0
Absorption level	1	0

Table 2. Toxicity Prediction (TOPKAT) of calcium HMB and creatinine monohydrate

Compound	HMB	Creatine Monohydrate
Rat Female NTP Prediction	Non-Carcinogen	Non-Carcinogen
Probability	0.451269	0.451336
Enrichment	0.991425	0.991571
Score	-2.39791	-2.39583
Rat male NTP Prediction	Non-Carcinogen	Non-Carcinogen
Probability	0.53303	0.551095
Enrichment	1.04747	1.08297
Score	-2.00347	-1.60627
Rat Female FDA	Multi-Carcinogen	Multi-Carcinogen
Rat male FDA	Non-Carcinogen	Non-Carcinogen
WOE Prediction	Non-Carcinogen	Non-Carcinogen
Probability	0.496156	0.409074
Enrichment	0.963609	0.794483
Score	-1.11037	-3.48078
TD 50 rat	101.692	12.3454
Unit	mg/kg_ bodyweight/day	mg/kg_ bodyweight/day
Ames	Non-Mutagen	Non-Mutagen
Rat Oral LD50	0.911121	0.253684
Units	g/kg body weight	g/kg body weight

3.2 Evaluation of *in vitro* Cytotoxicity Studies

3.2.1 Determination of IC50 by MTT Assay

The in vitro MTT assay has been extensively applied to predict drug sensitivity for in vivo studies. So, the substance Calcium HMB, creatine monohydrate and its combinations are tested in liver and kidney-based cell lines. The VERO and HEP-G2 are identified as cell lines for the kidney and liver respectively. The concentration (250-15.625 µg/ml) of these substances is tested in these cell lines. The IC 50 values of VERO and HEP-G2 are shown in Table 3. The % cell viability of VERO and HEP-G2 are calculated and recorded shown in Figures 1 to 6.

Table 3. HMB and creatine monohydrate-IC50 values in VERO and HEP-G2 cell lines

Sl No.	Sample Description	VERO IC50 µg/ml	HEP-G2 IC50 µg/ml
1.	HMB	108.20	30.10
2.	Creatine Monohydrate	126.13	53.28
3.	HMB and Creatine Monohydrate Combination	112.36	25.86

3.2.2 VERO Cell Lines

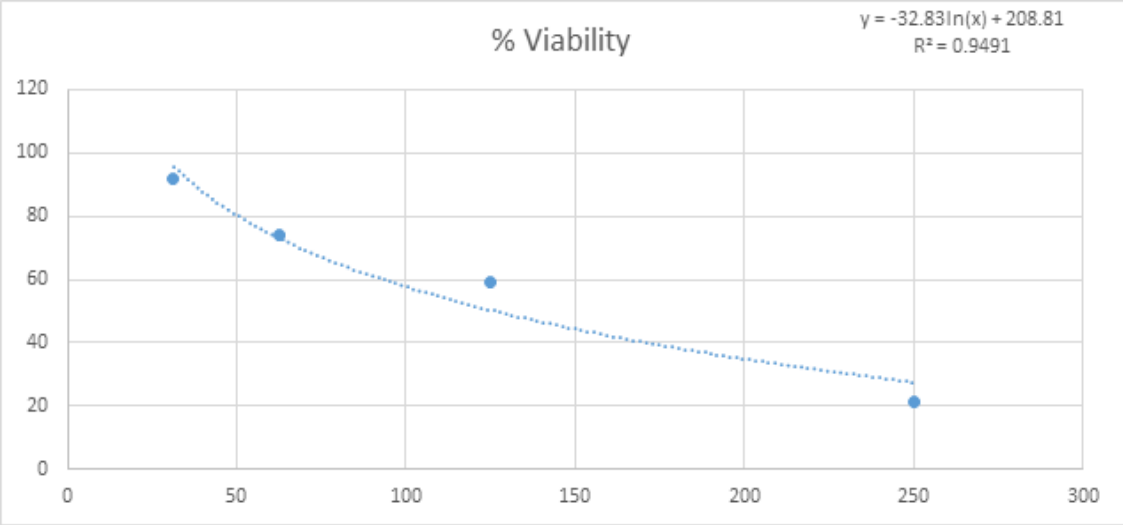


Figure 1. Percentage % cell viability of Creatine monohydrate in VERO cell line.

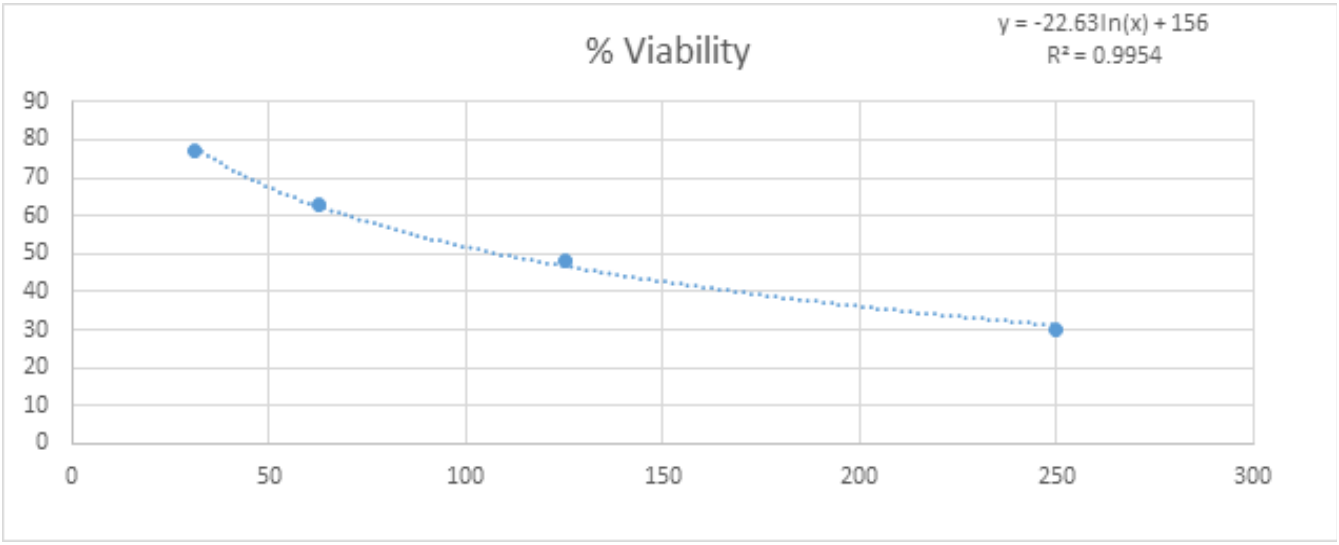


Figure 2. Percentage % cell viability of HMB in VERO cell line.

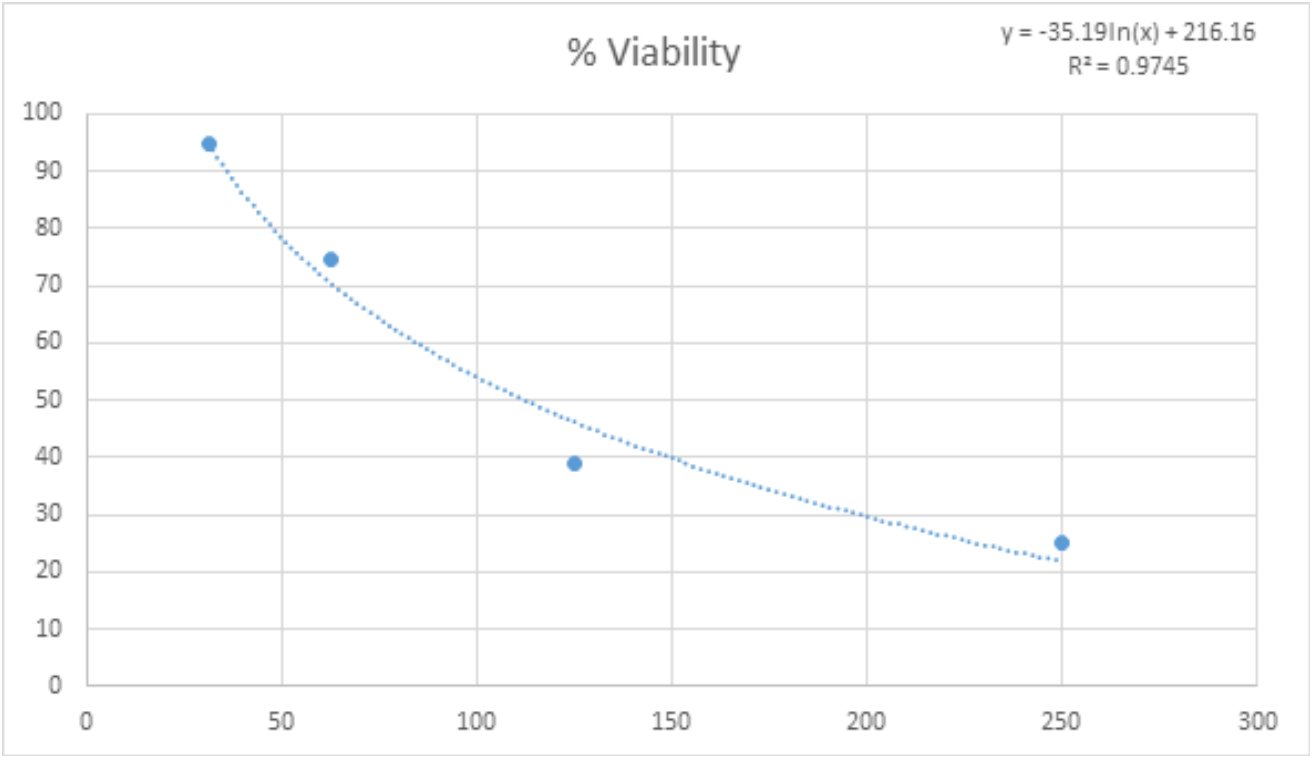


Figure 3. Percentage % cell viability of HMB and creatine monohydrate combination in VERO cell line.

3.2.3 Hep-g2 Cell Lines

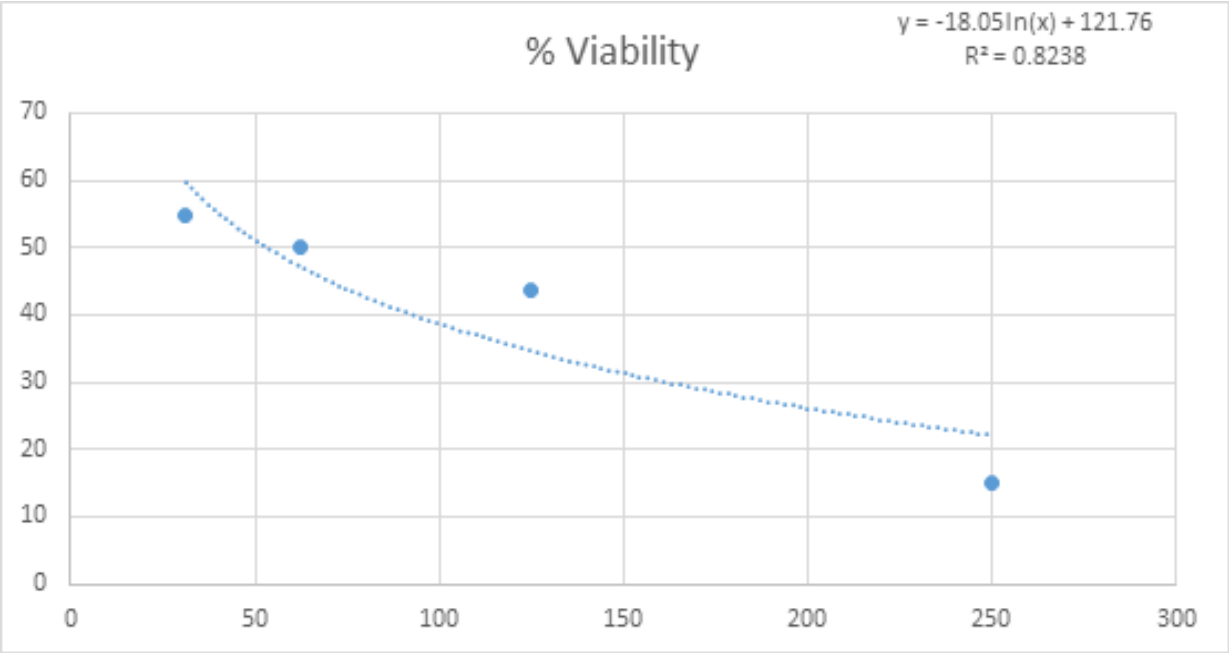


Figure 4. Percentage% cell viability of creatine monohydrate in HEP-G2 cell line.

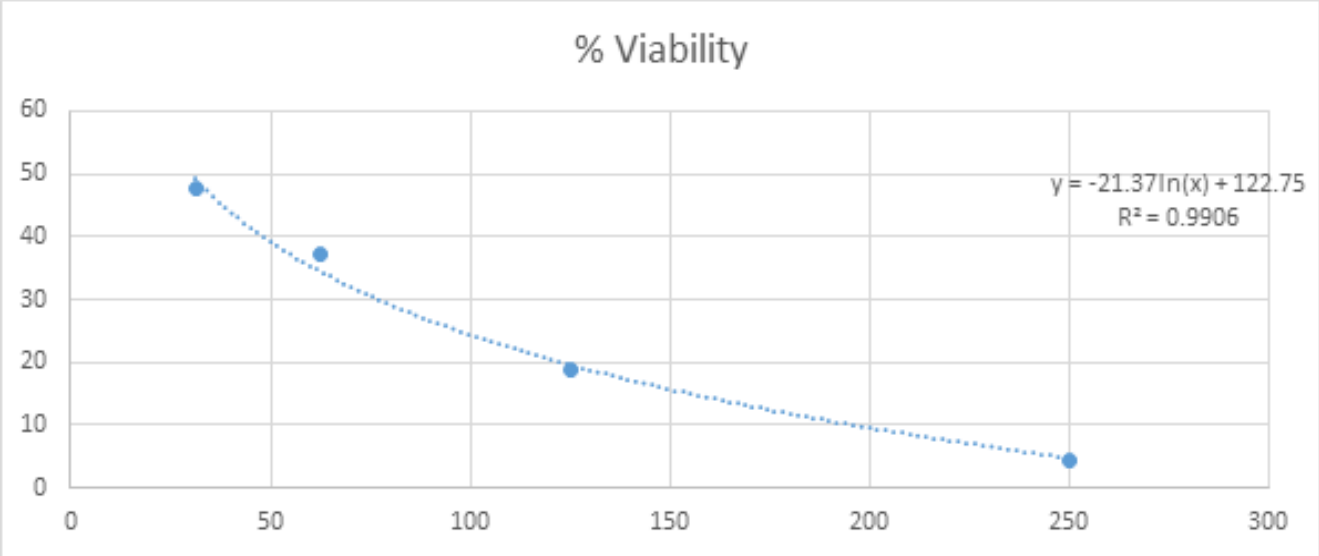


Figure 5. Percentage % cell viability of HMB in HEP-G2 cell line.

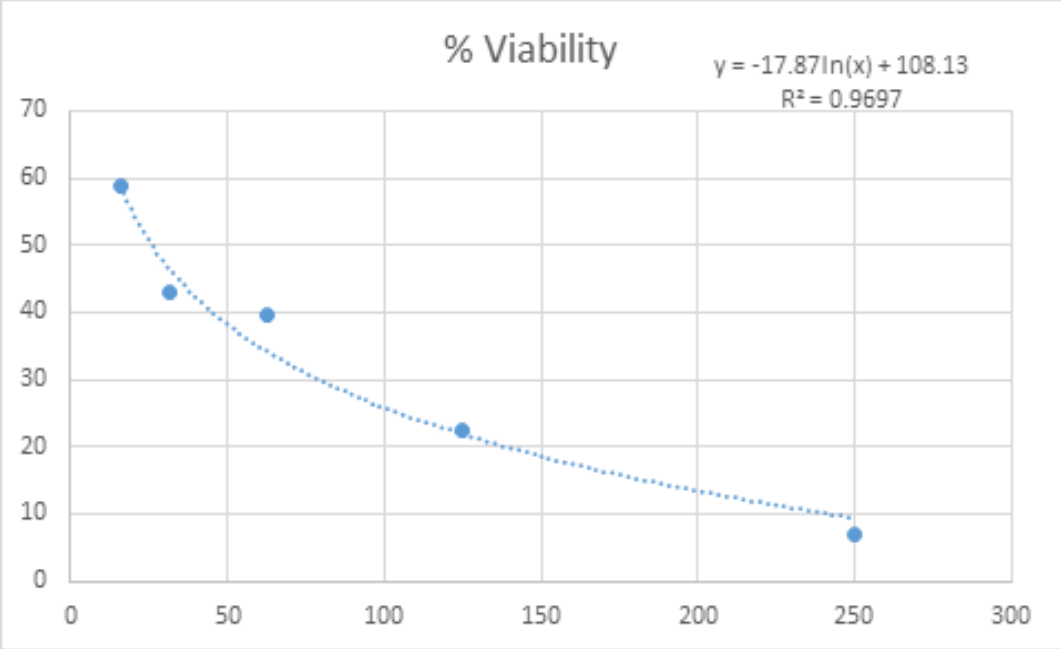


Figure 6. Percentage % cell viability of HMB and creatine monohydrate combination in HEP-G2 cell line.

3.3 Evaluation of Gross Behaviour- Body Weight and Morphological Animal Observations in Acute Oral Toxicity Study

In the Acute Oral Toxicity study of drugs, all animals survived. They were found to be active and healthy throughout the study. This is correlative to the gross body weight observations on the 0, 7th and 14th days. In the acute oral toxicity study, the 2000 mg/Kg b.w. was chosen to study the therapeutic effect in experimental animal models. The results are depicted as follows:

Table 4. Morphological changes in acute oral toxicity study of HMB, creatine monohydrate and its combination

Parameter	Control	*HMB* (2g/kg)	*CREATINE MONOHYDRATE* (2g/kg)	*COMBINATION (1:1 RATIO)* (2g/kg b.w.)
Skin changes	-	-	-	-
Fur changes	-	-	-	-
Lacrimation	-	-	-	-
Ptosis	-	-	-	-
Tremor	-	-	-	-
↓Stool consistency	-	-	-	-
Lethargy	-	-	-	-
Sleep	-	-	-	-
Coma	-	-	-	-

Note: (-) represents the absence of Parameters, (+) represents the Presence of Parameters.

3.4 Repeated Dose Toxicity

3.4.1 Evaluation of Gross Behaviour- Body Weight and Morphological Animal Observations

Table 5. Effect of HMB, creatine monohydrate and its combination on body weight (in grams) at a designated checkpoint (n = 6)

Treatment		DAY 0	DAY 7	DAY 14	DAY 21	DAY 28
Control	M	105 ± 1.13	106 ± 1.66	110 ± 1.33	120 ± 2.05	127 ± 2.02
	F	85.50 ± 1.47	90.00 ± 1.04	92.00 ± 1.28	96.00 ± 1.28	103.0 ± 1.63
HMB-1	M	107 ± 2.23	109.0 ± 2.70	116.0 ± 1.13**********	123.0±1.13	133.0±4.08*
	F	85 ± 1.24	94.50 ± 1.83	97.00 ± 1.12	106.00 ± 2.14	114.0 ± 2.00
HMB-2	M	109.0 ± 1.14	111.0 ± 2.39	115.0 ± 2.00	125.0 ± 2.16	135.0 ± 2.57
	F	86.0 ± 1.38	94.50 ± 1.67	99.0 ± 1.52	108.00 ± 1.63	116.0 ± 2.00
HMB-3	M	112.0 ± 1.66	116.0 ± 3.00	119.0 ± 2.23	126.0 ± 2.03	136.02 ± 1.63
	F	87.0 ± 2.3	96.0 ± 1.23	105 ± 1.2	109.0 ± 1.3	120.0 ± 1.7
CM-1	M	128.5 ± 3.05	135.0 ± 2.33	150.0 ± 3.2	160.0 ± 2.65	200.0 ± 1.2
	F	112.0 ± 1.2	125.0 ± 1.13	145.0 ± 2.3	155.0 ± 3.1	170.0 ± 1.2
CM-2	M	132.0 ± 2.13	142.0 ± 4.58	160.0 ± 5.3	165.0 ± 1.2	222.0 ± 2.1
	F	120.0 ± 3.1	132.0 ± 2.3	151.0 ± 3.6	160.0 ± 4.1	180.0 ± 2.5
CM-3	M	145.0 ± 1.3	150.0 ± 4.23	168.0 ± 2.5	178.0 ± 1.29	234.0 ± 1.57
	F	130.0 ± 1.59	145.0 ± 2.2	162.0 ± 4.23	175.0 ± 2.70	195.0 ± 2.76
CH-1	M	180 ± 5.17	116.7 ± 3.86***	109.3 ± 7.17	124 ± 4.53	129.3 ± 5.50
	F	228.7 ±16.59	193.7 ± 2.96****	201.3 ± 3.18	226 ± 2.30***	231.3 ± 2.40***
CH-2	M	221.7 ± 15.90	176.7 ± 10.93	168.3 ± 10.67	172.7 ± 14.34	184 ± 5.04
	F	194.3 ± 2.1	193.3 ± 3.84****	196.7 ± 2.18	193.3 ± 4.25****	218.3 ± 2.33**
CH-3	M	210.3 ± 1.76	225.7 ± 2.52	231.7 ± 2.00	242 ± 1.08	251 ± 2.81
	F	200.7 ± 2.33	221 ± 3.21**	230.7 ± 4.17	243 ± 1.15*	254.3 ± 2.90****

The parameters used for assessing the effect on gross behaviour are- body weight and gross animal observations. The values are expressed as Mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's posttest.

Table 6. Effect of HMB, creatine monohydrate and its combination on clinical biochemistry after 28 days of treatment (n = 6)

Groups	Sex	SGPT	SGOT	CHOL	GLU	TP	CR	UREA
CONT	M	33.42 ± 1.845	62.12 ± 3.671	62.89 ± 4.02	79.17± 0.76	6.10 ± 0.06	37.35 ± 1.12	56.25 ± 4.96
	F	30.05 ± 1.54	76.97 ± 3.587	57.00 ± 3.85	116.3 ± 0.53	6.44 ± 0.16	25.83 ± 0.29	64.09 ± 2.57
HMB-1	M	41.19 ± 1.72	95.87 ± 1.528*	50.45 ± 0.37	130.4 ± 2.37	5.88 ± 0.03	32.78 ± 0.63	50.65 ± 7.24
	F	34.77 ± 1.88	89.81 ± 2.084*	56.65 ± 6.27	152.4 ± 2.97	6.80 ± 0.42	22.37 ± 1.80	86.15 ± 3.14
HMB-2	M	42.87 ± 2.19	89.46 ± 2.426*	52.82 ± 2.89	156.3±5.37*	6.58 ± 0.13	32.85 ± 0.88	71.13 ± 8.76
	F	39.16 ± 2.37	92.50 ± 2.371*	57.87 ± 4.30	88.08±8.39	5.49 ± 0.50	22.05 ± 1.26	50.63 ± 7.65
HMB-3	M	44.23 ± 3.39	93.17 ± 2.026**	61.75 ± 6.03	101.5 ± 1.25	6.47 ± 0.20	21.94 ± 1.50	85.65 ± 2.52
	F	38.48 ± 1.68	95.88 ± 2.147**	53.92 ± 8.16	123.4 ± 4.96	5.68 ± 0.05	19.07 ± 1.17	44.80 ± 1.90
CM-1	M	34.50 ± 3.50	106.7 ± 5.92**	80.17 ± 7.51	120.5 ± 1.18	6.41 ± 0.16	25.30 ± 0.10*	93.19 ± 2.21
	F	32.69 ± 4.14	107.9 ± 4.53**	59.73 ± 8.22	87.07 ± 1.43	5.74 ± 0.21	20.96 ± 2.93	38.26 ± 6.03
CM-2	M	69.94 ± 5.84***	167.6 ± 1.71**	65.15 ± 4.64	108.2 ± 9.38	7.46 ± 0.69	29.41 ± 2.16	94.95 ± 4.61
	F	39.12 ± 3.77	161.1 ± 7.14**	55.22 ± 3.75	81.44 ± 4.73	5.49 ± 0.25	24.96 ± 1.48	82.48 ± 1.94
CM-3	M	77.79 ± 7.24***	174.4 ± 2.81**	55.01 ± 4.39	113.8 ± 9.74	5.80 ± 0.23	28.95 ± 1.54	84.30 ± 2.48
	F	37.87 ± 3.01	151.6 ± 16.00**	53.13 ± 5.15	100.3 ± 2.99	6.91 ± 0.55	23.40 ± 0.44	71.57 ± 1.52
CH-1	M	77.56 ± 6.18***	178.9 ±14.9**	72.72 ± 1.25	93.52 ± 1.88	6.29 ± 0.15	23.38 ± 0.63*	74.26 ± 2.93
	F	52.96 ± 5.27	157.0 ± 13.53**	54.26 ± 5.60	110.5 ± 1.54	5.91 ± 0.77	24.43 ± 2.49	56.15 ± 9.93
CH-2	M	183 ± 8.0***	201 ± 5.50***	67.89 ± 3.19	119.4 ± 5.90	6.78 ± 0.21	24.12 ± 1.62	84.34 ± 2.91
	F	61 ± 7.2	162 ± 9.8**	50.6 ± 4.12	96.24 ± 21.0	5.22 ± 0.30	24.72 ± 0.61	64.18 ± 2.56
CH-3	M	234 ± 6.8***	204 ± 4.8***	66.74 ± 4.68	133.7 ± 28.09	8.17 ± 0.45**	22.73 ± 4.20	108.5 ± 1.92
	F	72 ± 20.9	171 ± 30.4**	56.20 ± 2.17	128.1 ± 12.87	6.86 ± 1.33	25.23 ± 1.43	63.02 ± 1.16

The values are expressed as Mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's post-test. ($P < 0.05$ was considered significant)

3.5 Morphological Animal Observations

The toxic effect of HMB, Creatine monohydrate and its combination with morphological changes were assessed for treatment groups by comparing with the control. The creatine monohydrate low dose group (50 mg/kg) and HMB and creatine monohydrate combination groups produced adverse events in animals.

3.6 Evaluation of Clinical Biochemistry

Various biochemical parameters were evaluated after the 28-day treatment with all three dose regimens. On the 28th day, Biochemical evaluation is carried out in serum. Determination of the following biochemical parameters was done using standard diagnostic kits: Serum Glutamate Oxaloacetate (SGOT), Serum Glutamate Pyruminate Transaminase (SGPT), cholesterol, creatinine, glucose, total proteins and urea. The level of clinical biochemical estimated was significantly elevated ($p < 0.05$) in the treatment group compared to the Control group. The measurements of biochemical parameters are recorded in Table 6.

4. Discussion

For any country, substance abuse is a major health concern. In addition to that, the issues related to substance abuse problems among athletes go unnoticed. Often, athlete consumes pharmaceuticals, nutraceuticals and protein supplements for increasing their chances of success in competitions[29]. But without the knowledge of ingredients listed in these products, it could become a health hazard in the long run. Currently, athletes suffer from toxic effects produced due to their dependence on substances such as alcohol, steroids and stimulants. Now the problem of dietary supplements being consumed on a chronic basis is showing up in our literature search. The lack of toxicological knowledge on these compounds when consumed without proper dosing or nutrition specialist expertise, the situation is still an unsolved question[20].

Protein supplement formulations are easily available in bulk quantities to the common man through e-commerce platforms. Flash signs and pamphlets attract consumers without any inhibitions. We found very less restrictions and regulations imposed on the sale of the products. These products contain stand-alone or a mixture of protein-related supplements in them. Concurrently, most of them offer quick results in terms of body conditioning. A 2015 survey conducted abroad by US FDA has revealed that in a decade, around 1,000 people aged 25 years and younger have reported a health issue associated with the consumption of dietary supplements. Around forty per cent of those cases were categorized as severe health issues that are resultant in hospitalizations and deaths. In developed countries like the USA, it was observed that athletes seek a dietician or a nutrition specialist before consuming these products. However, athletes in developing countries like India had lesser access to factual information and professional expertise before their consumption[21].

Unfortunately, the intended purpose of these supplements is not met. However, the US Food and Drug Administration tracks reports of illness, injury, or reactions that arise from the consumption of dietary supplements. And we found such reports on different products that are being sold in their market. No such data is available on combinations of these products. Unlike drugs and other related chemical substances, dietary supplements like protein supplements may not be mandatorily tested for their toxicity on long-term usage. In this study, we chose to study two such supplements- Creatine Monohydrate (CM) and Beta-Hydroxy beta Methyl Butyrate (HMB). However, we hypothesise that when the products are used under prescribed conditions, they may improve health and help reduce the risk of diseases. This is only achievable if the toxicity profile of Creatine Monohydrate (CM) and Beta-Hydroxy beta Methyl Butyrate (HMB) and its combination is studied thoroughly. Hence, we studied the three facets of toxicity testing- in silico using molecular structural profiling by software, in vitro testing on probably affected tissues namely liver, and kidney cell lines and lastly by in vivo repeated dose toxicity study on a rodent model[22].

Failure in pharmacokinetics and the occurrence of toxicity are often the reasons for the forfeiture of lead molecules in drug discovery. Given this, it makes sense to use the in-silico method to predict the potential for toxicity and pharmacokinetic parameters of compounds to be identified for molecules[23].

Bajic *et al.*, states that *in silico* testing of chemicals complement existing toxicity procedure to forecast and categorize these different chemical molecules based on their toxicity. Which, enables us to minimize failures in drug discovery. Algorithm-based toxicity prediction with a particular emphasis on toxicity can help us review a large number of chemical substances towards new research directions. The same strategy can be employed to study already used compounds such as HMB or CM[24].

Yang *et al.*, have reviewed different in silico drug design software that can predict toxicity before synthesis. A software suite such as Discovery Studio was listed as one of the better software for analyzing the same. It is said to incorporate predictive models to screen chemical substances with toxicity risk[25]. In the further literature survey, we chose Discovery Studio 4.0 to be the choice of our in-silico studies. And HMB and creatine monohydrate were assessed by the ADMET module in Discovery Studio. Zhang *et al.*, have extensively used this software to determine the ADME and Toxicity parameters[26]. They have screened for HMB, Creatinine monohydrate and its combination for its impurities based on the structure alerts. The Polar Surface Area (PSA) play is quite a game changer for absorption across membranes. The data showed that PSA of HMB has moderate absorption and high permeability but creatine monohydrate has good absorption and low permeability. Due to higher PSA, creatine monohydrate has a greater propensity towards comparable intestinal absorption. HMB results are undefined due to poor aqueous solubility. Our preliminary solubility test shows that HMB and Creatine Monohydrate are poorly soluble in water. The HMB is identified as hepatotoxic and creatine monohydrate is not hepatotoxic. These data are contradicting the safety issues when consumed. However, the drawback of this study was that we were not able to assess the ADMET prediction for a combination of the two molecules as to how it is consumed by athletes. Yet, this sight was helpful to study the *in vitro* cytotoxicity studies conducted on the liver and kidney[27].

By in vitro studies, these compounds- HMB, CM and its combination induced cell cytotoxicity in a concentration-dependent manner, as illustrated in the results. This may be indicative of the synergistic toxicity of a combination of drugs. To understand this clearly, *in vivo* repeated dose toxicity study in a rodent model was conducted[28].

Upon literature survey, it was found that a toxicological profile of this combination was not yet established. Therefore, the present study was designed to assess the

toxicological profile of this combination as per OECD guidelines Test number 407. OECD prescribes specific guidelines to establish toxicity profiles of such substances or combinations. We have carried out acute oral toxicity studies to obtain three different dose levels for conducting a 28-day repeated dose toxicity testing procedure in rats. Body weight, weekly food Consumption, gross animal observations, and clinical biochemistry were carried out. It is observed that after the administration of the study drug combination, all the animals (both males and females) were highly active. By the end of the study, we observed that body weight changes were not conclusive in the animals. Further studies warrant understanding the possible mechanism due to which the weight gain is claimed[29].

Various biochemical parameters were evaluated after the 28-day treatment with all three dose regimens. On the 28th day, Biochemical evaluation is carried out in serum obtained for Serum Glutamate Oxaloacetate (SGOT), Serum Glutamate Pyruminate Transaminase (SGPT), cholesterol, creatinine, glucose, total proteins and urea. These values are compared with the control animals. There is no significant difference in urea, protein, glucose, cholesterol, SGPT and SGOT levels. Clinical parameters prepared by Giknis and Clifford clearly state the normal range of serum albumin in males and females was 3.8 to 4.8 g/dL and 3.7 to 5.5 g/dL concentrations respectively[30]. Low albumin levels are associated with inflammation, shock, and malnutrition. However, a case study by Bernardi *et al*., has clearly stated that hypoalbuminemia with less than 3.0 g/dL is associated with liver cirrhosis[31]. Our results were indicative of decreased levels of albumin that may be indicative of liver damage to a degree. Biochemical estimations exhibit there is a decrease in albumin content in serums collected. Low albumin levels are often seen in cases with inflammation or diseases such as Crohn's disease. The liver is the organ which produces albumin. Low albumin levels are often seen in cases of liver failure, liver cirrhosis or hepatitis[32]. Hence, we can state that the drugs have produced liver damage to some extent. The histopathological examination may help us to understand the extent of damage[33].

Overall, the article presents valuable information on the use of in-silico methods for predicting the potential for toxicity and pharmacokinetic parameters of chemical compounds. The study's results indicate that the ADMET descriptors of Calcium HMB and Creatine Monohydrate are similar, but their toxicity prediction differs[34]. The in vitro cytotoxicity studies suggest that the combination of the two compounds has a greater inhibitory effect on cell viability than either compound alone[35]. Additionally, the acute oral toxicity study of the two compounds and their combination showed no adverse effects in the animal models[36].

5. Conclusion

Several drugs and combinations have been enlisted by FDA, NCAA, FSSAI, WDAA and NIDA as abusive combinations among athletes. Attempts have been made to document the toxicity profiles of such substances and their combinations. The information about the toxicity of such substances or combinations is highly useful in understanding the pathogenesis of toxicity. It will help identify the possible intervention sites in pathogenesis to develop a remedial strategy. It will help to create awareness in athletes regarding the hazardous influence of such combinations. It may pave the way for developing the de-addiction and rehabilitation strategies. In this study, we chose one such unexplored supplement combination for determining the toxicity profile when given together. By *in silico*, the algorithm predicts pharmacokinetics and toxicities in HMB and creatine monohydrate, the results show that HMB displays hepatotoxicity. Both compounds are non-mutagens but HMB is hepatotoxic. I*n vitro,* studies portrayed that HMB and creatine monohydrate combination has possibilities for synergistic hepatotoxicity. Further studies may be needed to understand the actual mechanism of action of these medicines to show the effect of body-building protein supplements. In our study, the toxicity study was just a limit test to identify the possible toxicity of protein supplement abuse. To further substantiate our results, an in-depth molecular-level examination for exploring the mechanism of HMB and Creatine monohydrate may be taken up further. In conclusion, the study highlights the importance of evaluating the toxicity of chemical compounds through in-silico methods and in vitro and in vivo studies. These approaches can provide valuable information for the development of safe and effective drugs.

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