Toxicology Reports 7 (2020) 602–609

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Safety assessment of a fenugreek dietary fiber-based formulation of capsaicinoids-rich red chili (*Capsicum annum*) extract (Capsifen®): Acute and sub-chronic studies

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ARTICLE INFO

Keywords:
Capsifen
Red chili pepper
Capsicum annum
Fenugreek dietary fiber
Sustained-release
Safety

ABSTRACT

Despite the promising health beneficial effects (thermogenic, lipolytic, hypotriglyceridemic, hypocholesterolemic, anti-inflammatory and anticancer) of capsaicinoids-rich red chili pepper, commonly known as cayenne pepper (*Capsicum annum or Capsicum frutescence*), its consumption at physiologically relevant dosage is always hampered by the pungency and stomach discomforts. The present study examined the safety of a pungency-masked and sustained release food-grade formulation of capsaicinoids-rich red chili pepper extract using fenugreek derived galactomannan soluble dietary fiber (Capsifen®). The safety was assessed by oral acute (300, 2000, 5000 mg/kg b. wt. for 14 days) and subchronic (250, 500 and 1000 mg/kg b. wt.) toxicity studies in Wistar rats. None of the group of animals belonging to both acute and subchronic treatments did produce any adverse events in feeding behavior, urine analysis, and in hematology/biochemical parameters when compared to the control. However, a decrease in body weight was observed among 500 and 1000 mg/kg b. wt. treated groups. The terminal autopsy did not reveal any alterations in relative organ weight except for the high dose treated group, where an increase in liver and kidney weight was observed. Histopathology of all the animals was normal. Thus, the Low-observed-adverse-effect level (LOAEL) of Capsifen was determined for 500 mg/kg b. wt. /day.

1. Introduction

Chilies (Capsicum) are known from prehistoric times in Peru and are believed to be originated in the northern Amazon basin around 7500 BCE Chili is said to be the first-ever domesticated crop in America, and one of the first spices used by humans anywhere in the world [1]. Ever since its discovery, Capsicum has been used both as food color and as a flavor [2]. In 1493, Columbus carried a chili seed to Spain and spread cultivation rapidly from Spain to Europe and further to Asia [2]. The major five chili products traded in the world market include paprika (powder form; color and flavor), oleoresin (liquid form; color and pungency), fresh fruits, frozen fruits (whole, diced and pureed) and dried fruits (whole, powdered and smoked) [1]. As per the available information, almost 7 million tons of chilies are grown every year worldwide and India is the largest producer, with approximately 1.2 million tones [2]. Now, more than 400 different varieties of chilies found all over the world. The unique keto carotenoids capxanthin, capsorubin, and cryptoxanthin impart a brilliant red color to ripen chilly pods, and the yellow-orange color comes from β-carotene, zeaxanthin, violaxanthin and β-cryptoxanthin [3]. While the color-rich varieties of chilies with very low pungency levels (Paprika) are primarily grown in China and Spain, the pungent chilies are mainly from India [1]. Pepper varieties from *Capsicum frutescens*, *Capsicum annuum* and *Capsicum chinense* were found to contain 0.22–20 mg capsaicinoids (pungent chili)/g of dry weight [4]. The capsaicinoids (capsaicin, dihydrocapsaicin, and nordihydrocapsaicin along with small amounts of homo-, homodihydro- and nor capsaicin) are responsible for pungency [5].

Several preclinical and clinical studies have demonstrated the potential health benefits of capsaicinoids extracted from red chilies, especially under conditions of obesity. Enhanced energy expenditure, thermogenesis, lipid metabolism, carbohydrate oxidation and reduction in the cumulative *ad libitum* energy and food intake were studied in humans [6]. Besides, capsaicinoids were also shown to be beneficial in conditions of insulin resistance, hyperinsulinemia, type 2 diabetes, hyperlipidaemia, and cancer [7–10]. Capsaicin also stimulates lipid mobilization from adipose tissue and lowers the perirenal adipose tissue weight and serum triglyceride concentration in lard-fed rats [11].

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https://doi.org/10.1016/j.toxrep.2020.04.014
Received 5 September 2019; Received in revised form 21 April 2020; Accepted 30 April 2020
Available online 07 May 2020

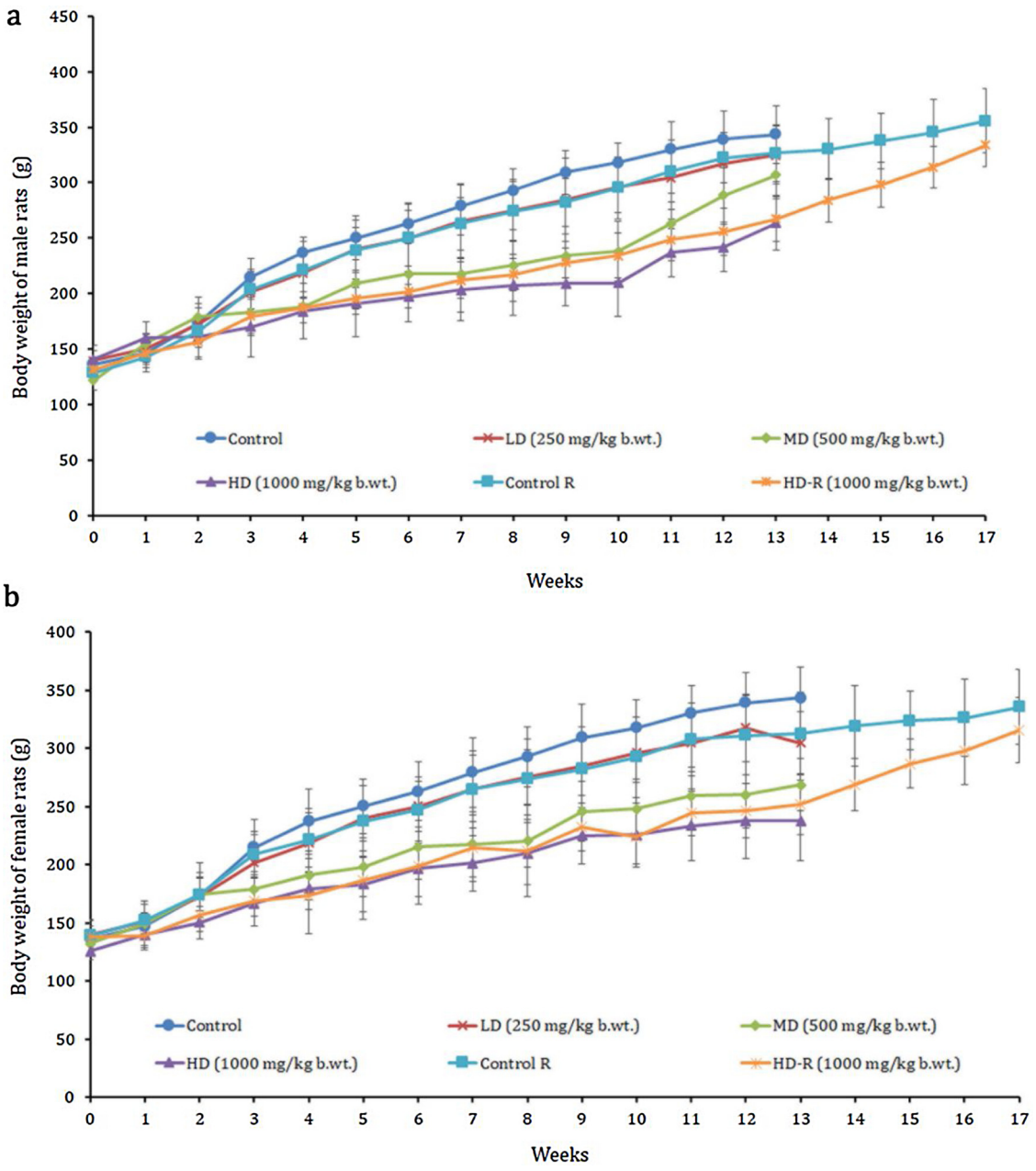

Fig. 1. Effect of oral administration of Capsifen on the body weight of (a) male and (b) female rats during 90-days repeated dose toxicity study. Data are expressed as mean ± SD and analyzed by two-way ANOVA, as compared to a respective parameter value of the control.

However, prolonged usage of capsicum may lead to different kinds of toxicities in human beings. It may cause irritation, diarrhea, vomiting or even ulcer in the mucous membranes throughout the gastrointestinal tract, from mouth to anus [12]. Handling difficulties due to skin irritations such as burning and pain, upper respiratory discomforts and allergy have been reported with pungent chilies. It has been found that the severity of capsaicinoids depends on its concentration and the length and frequency of exposure by a person [13]. Thus, there exists a great demand and need for safe oral delivery forms of capsaicinoids or pungent red chili extracts to translate its therapeutic and nutritional properties to the clinic. Capsifen is a pungency-masked and sustained-release oral delivery form of standardized capsicum extract or capsicum oleoresin obtained by solvent extraction of pungent red chilies. Debitterised fenugreek dietary fiber rich in galactomannans was used as a matrix for the encapsulation of capsicum extract employing the patented FenuMAT™ technology. Fenugreek (*Trigonella foenum graecum*), a popular kitchen spice generally recognized as safe (GRAS) by US Food and Drug Administration (FDA), is a rich source of soluble dietary fibre composed of galactose and mannose units (galactomannan) and has been successfully used for the oral delivery of curcuminoids with enhanced bioavailability [14]. The present study hypothesized that the formulation 'Capsifen' would be highly tolerant and safe for oral consumption due to its natural beadlet structure and controlled intestinal release of capsaicinoids. The study design involves acute (14 days), and sub-chronic (90-days) toxicity studies in Wistar rats.

2. Materials and methods

2.1. General

Capsifen beadlets were obtained from Akay Flavors & Aromatics Pvt. Ltd., Cochin, India. In the present study, Capsifen having 3.06 % of capsaicinoids [sum of capsaicin, dihydrocapsaicin (DC) and nordihydrocapsaicin (NDC)] was used. It was formulated with 25 % (w/w) of

Table 1
Effect of 90-days of repeated dose Capsifen administration on body weight of Wistar rats.

Weeks	Control	LD (250 mg)	MD (500 mg)	HD (1000 mg)	Control R	HD-R (1000 mg)
a) Male animals						
0	136.4 ± 12.38	140 ± 13.2	122.1 ± 9.26	141.06 ± 12.52	128.6 ± 5.12	131.6 ± 10.06
1	147.2 ± 11.33	151 ± 12.5	155.21 ± 9.09	160.11 ± 14.25	143.2 ± 13.36	146.6 ± 13.39
2	174 ± 13.72	173 ± 17.82	179.46 ± 17.88	160.96 ± 18.28	166.2 ± 14.3	156.8 ± 15.41
3	215 ± 16.37	201.6 ± 18.63	183.25 ± 21.18	170.4 ± 26.98*	203.8 ± 17.83	179.8 ± 15.41*
4	237.2 ± 13.48	218.8 ± 21.81	188.37 ± 28.99	184.1 ± 24.88*	221.2 ± 25.46	187.6 ± 13.79*
5	250.2 ± 19.51	239.8 ± 26.84	209.9 ± 27.95*	191.18 ± 29.8*	239.2 ± 20.39	195.8 ± 9.52*
6	263 ± 17.94	250.2 ± 31.19	217.83 ± 30.83*	197.38 ± 22.61*	250 ± 25.12	201.8 ± 7.04*
7	279.2 ± 19.37	265.2 ± 32.79	218.02 ± 34.88*	203.45 ± 27.71*	263 ± 23.63	212.2 ± 16.08*
8	292.8 ± 19.39	275.4 ± 27.03	225.36 ± 32.28*	207.99 ± 27.87*	274 ± 27.06	217.2 ± 14.82*
9	309.2 ± 19.69	284.6 ± 37.25	234.01 ± 20.11*	209.27 ± 20.11*	282.2 ± 21.89	228 ± 13.37*
10	317.8 ± 17.88	296 ± 22.57	238.25 ± 26.87*	209.7 ± 29.76*	295.2 ± 27.55	234.4 ± 20.28*
11	330.2 ± 24.38	304.4 ± 28.48	263.14 ± 27.05*	237.08 ± 21.42*	310.4 ± 27.7	248.6 ± 18.83*
12	339 ± 25.4	317.6 ± 27.93	288.64 ± 26.68*	242.09 ± 22.1*	322.6 ± 22.95	255.8 ± 21.41*
13	343.4 ± 26.28	324.8 ± 25.89	307.09 ± 21.17*	263.57 ± 24.4	326.8 ± 25.45	267.31 ± 19.96*
14	–	–	–	–	330.2 ± 27.25	284.2 ± 19.94*
15	–	–	–	–	337.6 ± 24.92	298.2 ± 20.36*
16	–	–	–	–	345.4 ± 29.88	314.2 ± 18.62
17	–	–	–	–	355.6 ± 29.06	333.8 ± 19.58
b) Female animals						
0	132.4 ± 7.44	137.85 ± 7.75	129.39 ± 4.62	127.86 ± 7.80	139 ± 14.27	135.2 ± 13.48
1	144.2 ± 18.79	153.93 ± 4.30	149.64 ± 8.88	139.58 ± 9.30	152.2 ± 16.57	138.8 ± 11.88
2	168.6 ± 17.13	171.1 ± 16.17	174.46 ± 13.87	150.43 ± 13.80*	174.2 ± 19.11	149.4 ± 14.10*
3	210.5 ± 23.55	197.6 ± 24.19	178.97 ± 15.26*	166.78 ± 11.29*	208.6 ± 20.23	168.6 ± 21.03*
4	231.2 ± 28.08	221.8 ± 25.83	190.92 ± 20.90*	179.47 ± 18.51*	221.2 ± 27.40	173.2 ± 32.38*
5	253.2 ± 23.16	242.8 ± 27.73	197.85 ± 25.09*	182.91 ± 23.29*	237.4 ± 30.14	186.6 ± 33.72*
6	267.8 ± 25.27	247.2 ± 21.34	215.45 ± 27.89*	196.5 ± 23.85*	247 ± 28.50	199 ± 33.38*
7	283.2 ± 29.79	271.2 ± 28.44	217.8 ± 27.92*	201.14 ± 23.95*	265 ± 33.07	214.4 ± 28.71*
8	286.8 ± 25.42	278.4 ± 23.40	220.56 ± 22.42*	209.66 ± 26.34*	273.6 ± 34.49	211.6 ± 39.25*
9	302.7 ± 28.48	289.6 ± 25.40	245.56 ± 25.62*	224.98 ± 24.47*	282 ± 36.47	232.4 ± 20.64*
10	307.8 ± 23.99	291.6 ± 25.35	247.99 ± 25.21*	225.69 ± 27.51*	292.4 ± 34.66	223.6 ± 23.36*
11	326.2 ± 23.46	295.4 ± 24.15	259.42 ± 24.81*	233.56 ± 30.05*	307.8 ± 31.52	245 ± 20.24*
12	332.6 ± 26.43	311.6 ± 29.10	260.4 ± 28.50*	237.65 ± 31.91*	311.2 ± 33.90	246.4 ± 23.81*
13	335.4 ± 26.14	304.8 ± 26.99	268.4 ± 22.40*	237.71 ± 34.74*	312.6 ± 35.26	251.6 ± 25.79*
14	–	–	–	–	319.2 ± 34.44	269.2 ± 22.39*
15	–	–	–	–	323.8 ± 25.46	286.8 ± 20.84*
16	–	–	–	–	325.2 ± 33.02	306.2 ± 29.72*
17	–	–	–	–	337.4 ± 32.21	319.8 ± 28.17

Data are expressed as mean ± SD and analyzed by two-way ANOVA, as compared to a respective parameter value of the control.

chili extract, 60 % (w/w) fenugreek dietary fiber, and 15 % (w/w) cellulose gums. All solvents used for analysis were of HPLC grade and that for extraction were reagent grade, from Merck, India. Milli Q Plus (Millipore) purified water was used for all experiments. Analytical standard of capsaicin (CAS No. 404-86-4) was purchased from Sigma-Aldrich, Bangalore, India. Capsaicin contents measured by a validated high-performance liquid chromatography (HPLC) method carried out on a Shimadzu LC 20 AT system, with the M20A Photodiode array (PDA) detector (Shimadzu Analytical Pvt. Ltd., Mumbai, India), using reverse- phase C18 Phenomenex column (250 × 4.6 mm, 3 µm).

2.2. Animals

The animals (Wistar rats, 150 − 200 g body weight) were procured from the Veterinary College, Mannuthy, Kerala, India, and were acclimatized for a period of 14 days in ventilated cages and housed in an air-conditioned room at 21 to 24°C temperature under standard laboratory conditions with adequate fresh air supply using IVC system (air changes 15 per hour) and relative humidity 57–65 %, with 12 h light/dark cycle, at the animal house facility of M/s CARe Keralam Ltd, Kerala, India. All animals experiments were approved by the Institutional Animal Ethics Committee (IAEC) recognized by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (IAEC No: 1620/PO/RcBi/S/12/ CPCSEA). Animals were provided with a pellet diet (Laboratory animal feed manufactured by VRK nutritional solutions, Maharashtra, India) and water *ad libitum*. Deep bore-well water passed through an activated charcoal filter and exposed to ultraviolet rays in aqua guard water filter cum purifier (manufactured by Eureka Forbes Ltd., Mumbai, India) was provided in plastic water bottles with stainless steel sipper tubes.

2.3. Toxicity studies

2.3.1. Acute toxicity

Acute toxicity study was performed according to the Organization for Economic Cooperation and Development (OCED) 423 guidelines [15]. Forty Wistar rats (175 ± 20 g) were divided into four groups, each group comprising five animals of both sexes. After fasting overnight, an aqueous solution of Capsifen was administered orally to each group at single doses (300, 2000 and 5000 mg/kg b. wt.) and same volume of distilled water was given to the Control group. After administration, all animals were observed individually for mortality and changes in general behavior during the first 30 min, then at 2, 4, 6, 10, and 24 h after administration. The LD_{50} value was determined according to the method described by the OECD-423 [15]. In the next phase of the study, the animals were administered with Capsifen for 14 days and were observed for the changes in the skin, fur, the occurrence of secretion, excretion and autonomic activity along with a change in body weight and rate of food consumption. On 14th day (end of study), necropsy of all animals was performed.

2.3.2. Sub-chronic toxicity

Sub-chronic toxicity study was conducted as per OECD guideline No. 408 [16]. Sixty Wistar rats (30 males and 30 females) of average weight between 150–200 g were divided into six groups, each consisting of five males and five females as follows:

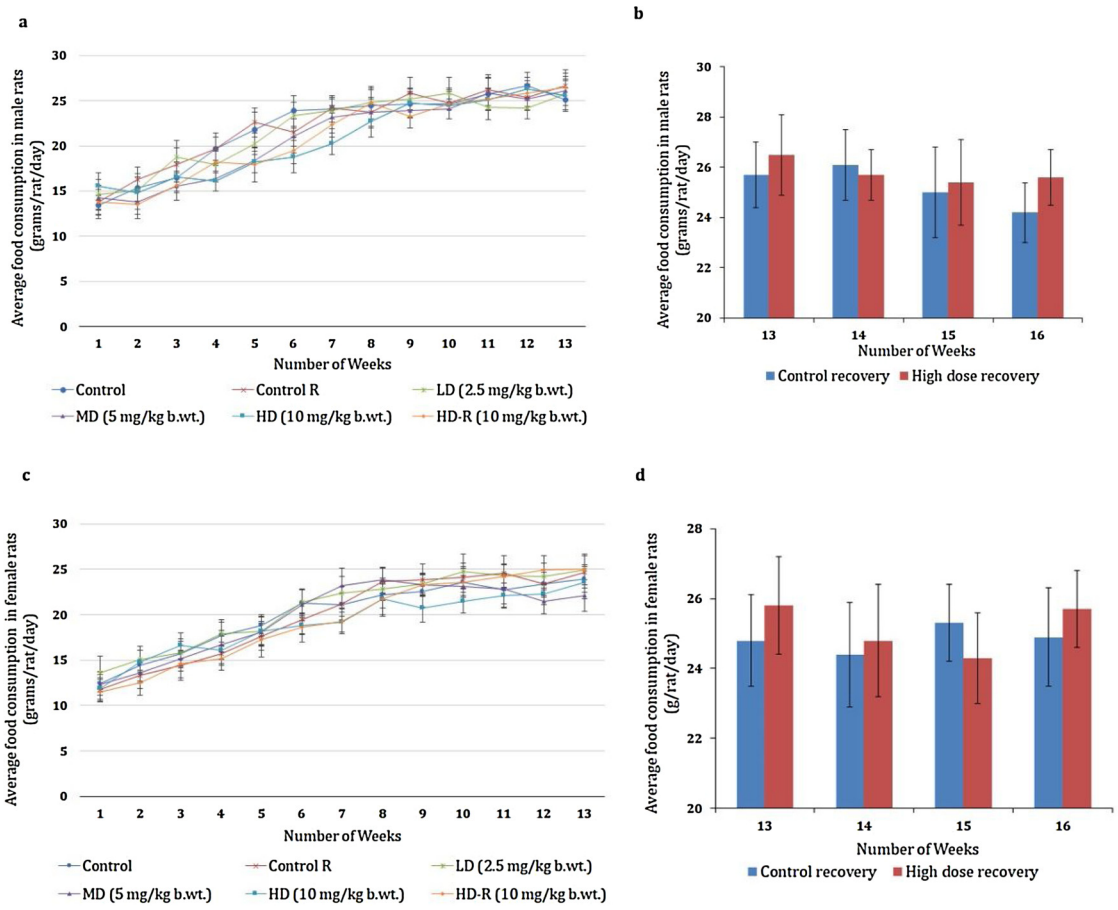

Fig. 2. Food intake pattern of (a) male (c) female rats during the administration of Capsifen for 90-days whereas (b) and (d) represents the food consumption during 28-days of the recovery period for both male and female rats respectively.

Table 2
Effect of Capsifen on administration on relative organ weight of Wistar rats.

Groups	Liver (%)	Kidney (%)	Heart (%)	Spleen (%)
Male group				
Control	2.60 ± 0.22	0.57 ± 0.08	0.29 ± 0.03	0.36 ± 0.05
Control R	1.99 ± 0.26	0.41 ± 0.04[a]	0.24 ± 0.01	0.27 ± 0.06[a]
LD (250 mg)	2.80 ± 0.41	0.57 ± 0.09	0.27 ± 0.04	0.36 ± 0.06
MD (500 mg)	1.54 ± 1.06[a]	0.57 ± 0.09	0.28 ± 0.08	0.29 ± 0.07[b]
HD (1000 mg)	3.39 ± 0.94[b]	0.81 ± 0.20[b]	0.40 ± 0.07[a]	0.39 ± 0.07
HD-R (1000 mg)	1.94 ± 0.23	0.37 ± 0.03	0.23 ± 0.03	0.23 ± 0.03[c]
Female group				
Control	2.64 ± 0.23	0.57 ± 0.07	0.29 ± 0.03	0.36 ± 0.07
Control R	2.00 ± 0.33[a]	0.44 ± 0.09[a]	0.25 ± 0.04	0.27 ± 0.04[a]
LD (250 mg)	2.76 ± 0.31	0.60 ± 0.05	0.30 ± 0.04	0.35 ± 0.07
MD (500 mg)	3.05 ± 0.51	0.61 ± 0.16	0.30 ± 0.06	0.29 ± 0.05
HD (1000 mg)	3.41 ± 0.38[b]	0.86 ± 0.10[b]	0.40 ± 0.03[a]	0.35 ± 0.06
HD-R (1000 mg)	2.02 ± 0.35[c]	0.42 ± 0.07[c]	0.26 ± 0.03	0.25 ± 0.03[b]

Values are mean ± standard deviation, expressed as the percentage of organ weight/100 g of body weight. 5 animals/sex/group, unless otherwise specified. The values having a superscript significantly differ at $p < 0.05$ with respective controls.

Group I – Control – 5% Tween 80 with distilled water (v/v)

Group II – Control Recovery (Control R) – 5 % Tween 80 with distilled water (v/v)

Group III – Low dose (LD) – Capsifen 250 mg/kg b. wt.

Group IV – Mid dose (MD) – Capsifen 500 mg/kg b. wt.

Group V – High Dose (HD) – Capsifen 1000 mg/kg b. wt.

Group VI – High Dose Recovery (HD-R) – Capsifen 1000 mg/kg b. wt.

All treated rats were observed for mortality and clinical signs during the 90-days study period, whereas recovery groups (Control R & HD-R) were observed for up to 119 days (reversal period of 28 days). The food consumption was determined every week up to 90 days by measuring the left-over feed after 24 h and was noted for a single cage of five animals. The animals were sacrificed at the end of the study period and organs were collected, washed with saline, weighed, and examined for structural abnormalities. The control recovery and high-dose recovery group were kept for post-observation for 28 days.

2.3.3. *Hematological and biochemical analyses*

The blood from all experimental animals was collected in EDTA coated vials and analyzed for red blood cells (RBCs) count, white blood cells (WBCs) count, platelet count and hemoglobin (Hb) content using the hematological analyzer (Model-Diatron, Wein, Austria). For the measurement of biochemical parameters, serum was separated by centrifuging at 5000 rpm for 10 min at −4°C and was stored at −20°C. Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated using kinetic method kits supplied by M/s Raichem, India, using a Micro lab 300 auto-analyzer (Merck, Mumbai, India) and the total protein concentration was determined by the Biuret method [17]. Creatinine; the kidney function marker, was estimated by Jaffe's kinetic method [18]. The total cholesterol was estimated as per the method of Deeg et al. [19], triglycerides by the GPO–PAP (glycerol-3-phosphate oxidase-phenol + amino phenazone) method [20], HDL cholesterol by precipitation with phosphotungstic acid and LDL cholesterol by the equation LDL = total cholesterol − (HDL + VLDL) [21]. Electrolyte such as sodium and potassium were estimated using Flame photometer [22]

Table 3
Effect of Capsifen administration on hematological parameters of Wistar rats.

	Control	Control R	LD (250 mg)	MD (500 mg)	HD (1000 mg)	HD-R (1000 mg)
Male groups						
RBC (10^6 cells/μL)	10.9 ± 4.6	7.2 ± 1.8	7.2 ± 1.8	11.8 ± 4.3	9.2 ± 4.0	12.3 ± 4.4
WBC (10^3 cells/μL)	15.3 ± 0.4	11.8 ± 2.6	12.1 ± 2.6	11.2 ± 4.0	14.5 ± 3.1	13.76 ± 2.6
Hb (g/dL)	12.6 ± 0.9	12.5 ± 1.0	12.7 ± 1.0	13.8 ± 0.9	12.8 ± 0.5	11.96 ± 0.5
Platelet (10^3 cells/μL)	603 ± 126	579 ± 169	644 ± 169	539 ± 152	555 ± 134	660 ± 137
Female groups						
RBC (10^6 cells/μL)	9.3 ± 4.3	14.1 ± 4.2	9.8 ± 4	11.4 ± 5.2	10.2 ± 4.6	7.6 ± 0.3
WBC (10^3 cells/μL)	14.8 ± 2.9	15 ± 1.6	14.8 ± 3.3	14.5 ± 2.0	13.4 ± 2.1	10.3 ± 2.6
Hb (g/dL)	13.2 ± 1	12.2 ± 0.8	12.7 ± 0.7	12.6 ± 0.8	12.1 ± 0.3	13.3 ± 1.3
Platelet (10^3 cells/μL)	662 ± 164	671 ± 69	569 ± 126	627 ± 84	741 ± 122	685 ± 131

Abbreviations: RBC - Red Blood Cells; WBC - White Blood Cells; Hb - Hemoglobin. The values are expressed as mean ± standard deviation. 5 animals/sex/group unless otherwise specified.

Table 4
Effect of Capsifen administration on biochemical parameters of male Wistar rats.

	Control	Control R	LD (250 mg)	MD (500 mg)	HD (1000 mg)	HD-R (1000 mg)
TC (mg/dL)	60.4 ± 06.4	56.3 ± 9.3	51 ± 11.3	63.6 ± 10.5	61.7 ± 7.2	51 ± 16.4
TG (mg/dL)	48.7 ± 12.1	43.6 ± 20.9	44.3 ± 10.9	48.6 ± 9.3	49.9 ± 14.7	48.3 ± 18.8
HDL (mg/dL)	26.4 ± 4.2	28.2 ± 5	28 ± 2.3	29.4 ± 5.8	27.2 ± 3.8	27.1 ± 5.7
LDL (mg/dL)	12.7 ± 2.9	20.2 ± 9	23.3 ± 15.1	13 ± 7.8	15.9 ± 74.5	19.6 ± 11.9
ALT (U/L)	58.9 ± 20.9	61.5 ± 14	58.3 ± 12.6	76.8 ± 20.3	69.2 ± 18.7	70.3 ± 21.3
AST (U/L)	146.6 ± 21.4	127.6 ± 22.3	137.6 ± 11.5	134 ± 27.7	138.4 ± 20.9	140.6 ± 25.8
ALP (U/L)	94.8 ± 66.2	94.8 ± 36.5	134.3 ± 56.4	141.9 ± 54.1	89.6 ± 24.3	99.4 ± 77.7
TP (mg/dL)	7.7 ± 0.2	7.7 ± 0.9	7.7 ± 0.4	7.6 ± 0.6	7.7 ± 0.7	7.6 ± 0.5
Cr (mg/dL)	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Na (mmol/L)	133.4 ± 4.7	134.6 ± 2.7	133.4 ± 4.3	131.8 ± 4.3	134.6 ± 6	136.6 ± 1.7
K (mmol/L)	3.8 ± 0.3	4.1 ± 0.1	3.5 ± 0.7	3.5 ± 0.3	4.1 ± 0.5	3.5 ± 0.4

Abbreviations: TC, Total Cholesterol; TG, Triglycerides; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; ALP, Alkaline Phosphatase; TP, Total Protein; Cr, Creatinine; Na, Sodium; K, potassium. The values are expressed as mean ± standard deviation. 5 animals/sex/group, unless otherwise specified.

Table 5
Effect of Capsifen administration on biochemical parameters of female Wistar rats.

	Control	Control R	LD (250 mg)	MD (500 mg)	HD (1000 mg)	HD-R (1000 mg)
TC (mg/dL)	58.5 ± 15.9	60.9 ± 23.3	56.4 ± 10.4	45.3 ± 3	54.7 ± 18.5	45.6 ± 9
TG (mg/dL)	51.6 ± 36.3	43.6 ± 19.7	58.8 ± 19	38.7 ± 14.5	39.2 ± 8.1	37.9 ± 6.6
HDL (mg/dL)	26.7 ± 5.2	31.5 ± 6.3	32.3 ± 7.2	26.4 ± 1.8	24.9 ± 4.3	25.1 ± 7.2
LDL (mg/dL)	14.7 ± 8.3	18.1 ± 10.4	10.1 ± 3.7	24 ± 9.9	18.7 ± 7.3	11.5 ± 9.4
ALT (U/L)	49.9 ± 14.4	47.2 ± 13.9	53.7 ± 17.5	60.4 ± 8.6	51.7 ± 14.3	56.3 ± 18.6
AST (U/L)	132.4 ± 19	129.6 ± 24.3	126.8 ± 30.4	138 ± 31.9	131.4 ± 6.2	132.4 ± 24.1
ALP (U/L)	126.3 ± 28.9	127.6 ± 57.8	108.5 ± 38.4	117 ± 30.4	119 ± 25.8	100.6 ± 52.7
TP (mg/dL)	7.3 ± 0.2	7.6 ± 0.6	7.8 ± 0.5	8.1 ± 0.5	7.4 ± 0.2	7.7 ± 0.6
Cr (mg/dL)	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
Na (mmol/L)	132.6 ± 2.2	131 ± 5.1	133 ± 1.9	136 ± 0.9	129.4 ± 4	134.2 ± 2.6
K (mmol/L)	3.7 ± 0.2	3.7 ± 0.3	3.7 ± 0.6	4 ± 0.4	3.5 ± 0.5	3.5 ± 0.6

Abbreviations: TC, Total Cholesterol; TG, Triglycerides; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; ALP, Alkaline Phosphatase; TP, Total Protein; Cr, Creatinine; Na, Sodium; K, potassium. The values are expressed as mean ± standard deviation. 5 animals/sex/group, unless otherwise specified.

2.3.4. Histopathological studies

All animals were subjected to gross necropsy, which includes the examination of the thoracic organs, an external surface, and all the internal organs for any type of abnormalities. Organ weights were recorded (liver, kidney, heart, and spleen). The tissue samples were fixed in 10 % formalin, subjected to dehydration process, embedded in paraffin and were sectioned into slices of 2 μm followed by hematoxylin-eosin (E&H) staining for histopathological examinations. The pathological observations of all tissues were performed on gross and microscopic basis using an optical microscope of 100× magnifications (Olympus-Magnustrinocular microscope, Tokyo, Japan).

2.3.5. Urine analysis

Urine test was conducted in all rat groups according to Liju et al. [23]. Briefly, five male and five female rats were orally administered with single dose of Capsifen at various concentrations (250, 500, 1000 mg/kg b. wt.) b. wt. and urine samples were collected during 24 h of post-administration. The animals were deprived of food during this 24 h time period, but provided with water *ad libitum*. The volume and pH of the urine samples were measured. Glucose and albumin content of the urine was analyzed using Magistik-GP urinalysis strip and microscopic evaluations of the sediments were carried out to detect calcium or phosphate crystals.

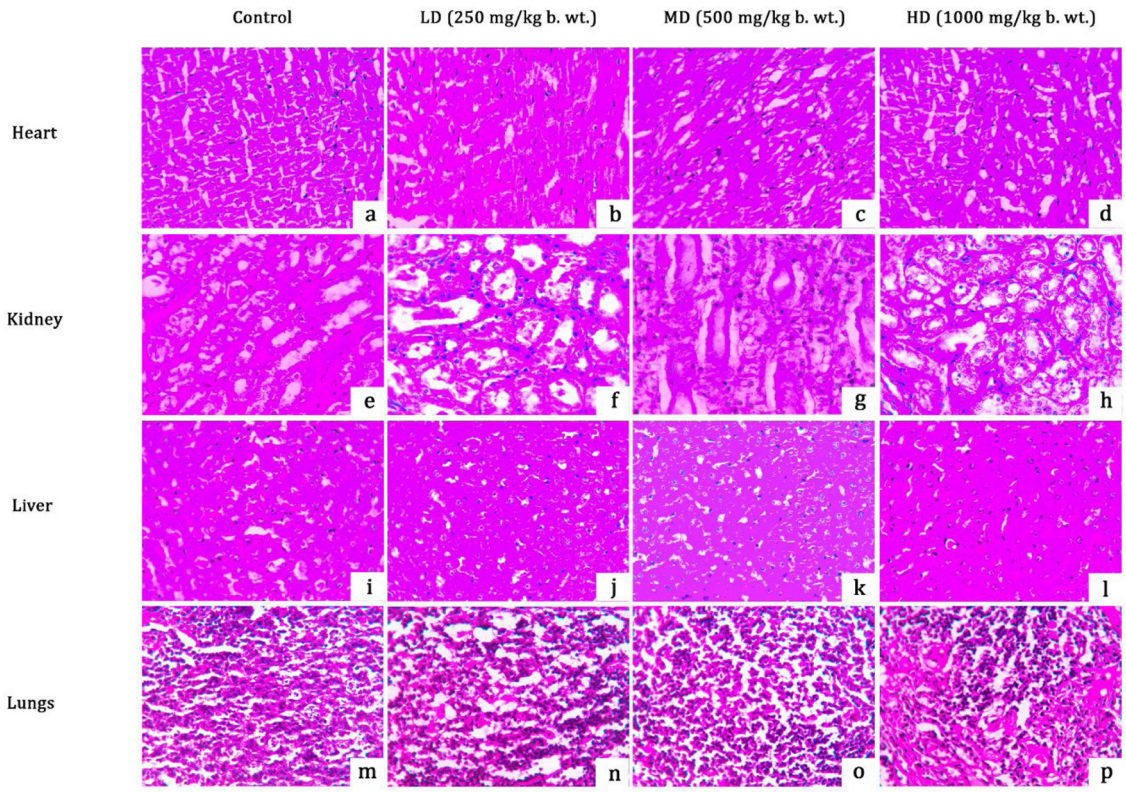

Fig. 3. Effect of Capsifen on the histological examination heart (a–d), liver (e–h), kidney (i–l), and lung (m–p) tissues of rats during 90-days toxicity study. Photomicrographs a, e, i, m represents the control group; b, f, j, n represents the LD (250 mg/kg b. wt.); c, g, k, o represents the MD (500 mg/kg b. wt.); d, h, l, p represents the HD (1000 mg/kg b. wt.).

2.4. Statistical analysis

Data on body weight, food intake, organ weights, hematology, and clinical chemistry were analyzed using SPSS software version 25 and expressed as mean ± SEM. The statistical significance was compared between control and experimental groups by one-way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnet multiple comparison tests). Data of Capsifen treated animals were compared with that of control animals and the differences between the groups were considered to be significant when $p < 0.05$.

3. Result

3.1. Studies on acute toxicity

Administration of Capsifen at doses 300, 2000, 5000 mg/kg b. wt. did not produce any mortality, visible clinical or behavior changes up to the dose of 5000 mg/kg b. wt. The body weight significantly decreased at dosage 2000 and 5000 mg/kg b. wt. but food/water consumption remained unchanged. Necropsy at the end of study did not reveal any gross pathological abnormalities suggesting the safety of Capsifen at the tested dose of 5 g/kg/day for rats (data not shown)

3.2. Study on subchronic toxicity

Treated and control groups of both the sexes were examined daily for 90 days, for general appearance, behavior, signs of toxicity, morbidity, and mortality. No mortality was observed during the study period. All the animals of both the treated and control groups remained active and healthy during the experiment period.

3.2.1. Effect on body weight

The mean body weight of male and female rats during the experimental period showed a significant decrease ($p < 0.05$) when compared with the control groups [Fig. 1a & b and Table 1]. The percentage difference were as 5.83, 16.61 and 23.55 % ($p = 0.022$) respectively for 250, 500 and 1000 mg/kg b. wt. dosages in male rats and were 1.9, 18.01 and 24.87 % ($p = 0.006$) respectively for the corresponding dosages in female rats, when compared with control groups [Fig. 1a & b and Table 1a & b]. Post 28-days observation of both the sexes showed no abnormal clinical signs (general behavior) of the animals as compared to control. A significant difference ($p = 0.033$) was observed in female high dose recovery groups when compared to control recovery and no significant change ($p = 0.078$) was observed in male high dose recovery groups [Fig. 1a & b and Table 1].

3.2.2. Effect on food/water consumption

The food consumption behavior showed no significant difference ($p > 0.05$) upon Capsifen administration when compared to the control irrespective of the dosage [Fig. 2a & c]. Post 28-days observation of both the sexes (male and female) showed no substantial difference in food consumption [Fig. 2b & d]. Water consumption of the Capsifen treated animals also remained unchanged when compared with control animals (data not shown).

3.2.3. Urine analysis

There were no substantial changes in either the pH (6.5–7.5) or the volume of urine collected from the treated animals at various dosages (250, 500, 1000 mg/kg b. wt.) when compared with control animals. Microscopy of the urinary sediment did not reveal any calcium or phosphate crystals. Urinary glucose, albumin and keto acids were also absent in both treated and control groups.

3.2.4. Necropsy and organ weights

The relative organ weights (liver, kidney, heart, and spleen) of Capsifen treated animals showed significant ($p > 0.05$) increase at

high dose (1000 mg/kg) when compared with the control group of both the sexes (Table 2). Other doses showed no significant change except Kidney and Spleen (significant reduction) compared to control. Necropsy of various organs and tissues of treated animals was normal.

3.2.5. Hematological parameters

As shown in Table 3, Capsifen treated groups in both the sexes showed no significant ($p > 0.05$) changes in hematological parameters. The parameters such as hemoglobin, WBC, RBC, and platelet counts in treated groups were in the normal range when compared to the control group. In the same way, high dose recovery group animals also showed no significant alteration in hematology parameters.

3.2.6. Serum biochemical parameters

The biochemical parameters (lipid profile, hepatic and electrolyte) of treated animals with Capsifen did not produce any significant changes when compared with the control group. After 90 days of supplementation, the renal function (serum creatinine) and electrolytes (Sodium & potassium) in both male and female rats showed similar effects (Tables 4 and 5). Hepatic marker (total protein) were not altered in Capsifen treated rats of both sexes (Tables 4 and 5). Similarly, no significant variation was seen in the lipid profile (total cholesterol, HDL & LDL cholesterol, triglycerides, VLDL) of treated rats in both sexes when compared to control group (Tables 3 and 4). Nonetheless, the values remained within laboratory ranges.

3.2.7. Histopathological analysis

The histopathological examination of various organs of treated rats of Capsifen showed normal cellular architecture when compared to the control group (Fig. 3). Normal portal triads and the central venous system was observed in all treated animals; normal hepatocytes and Kupffer cells with normal sinusoidal spaces which are identical with that of untreated animals in the liver section. The tissue sections of kidney in the Capsifen treated groups showed normal glomeruli with Bowman's capsule adrenal tubules. The interstitial tissues of the lung section appeared with no apparent abnormalities when compared with the tissues of the control group. The section of heart in both the treated and control group showed a normal myocardial fibre in a longitudinal section featuring central nuclei and syncytial arrangement of the fibres.

4. Discussion

The present study was designed to demonstrate the safety of a pungency-masked and sustained release food-grade formulation of capsaicinoids-rich red chili pepper extract, employing fenugreek derived galactomannan soluble dietary fiber as a matrix (Capsifen). The results demonstrated that the consumption of Capsifen at its highest tested dosage of 1000 mg/kg b. wt. was tolerable to animals, as it did not produce any changes in behavior, food/water intake or clinical parameters except the body weight reduction. However, body weight, feeding behavior, necropsy, organ weights and histopathology supported the safety of Capsifen at 500 mg/kg b. wt. indicating it as the LOAEL.

Single dose acute toxicity studies at 2000 and 5000 mg/kg b. wt. caused a significant body weight reduction without any significant changes in food/water intake indicated the tolerability and bioactivity of Capsifen. The normal necropsy at the end of the study period (14 days) also supported the absence of any pathological abnormalities even at 5000 mg/kg b. wt. The subchronic toxicity study also showed similar effects at doses of 500 mg/kg and 1000 mg/kg b. wt., indicating the systemic absorption of capsaicinoids from Capsifen in both male and female rats. wt., indicating the systemic absorption of capsaicinoids from Capsifen in both male and female rats. However, no significant change was observed in organ weights at dose of 500 mg/kg b. wt., as observed with an earlier study by Das et al. [24]. Further observation that animals did not show any behavioral changes and food/water intake during subchronic studies supported the tolerability of Capsifen. Necropsy and histopathology analysis also indicated the safety of Capsifen, since no morphological abnormalities of vital organs was observed.

The daily consumption of Capsifen for 90 days did not induce any significant changes in ALT, AST, and ALP levels, indicating the lack any hepatotoxicity. In fact, capsaicin has been reported to possess significant hepatoprotective effect in an LPS-induced hepatotoxic model of rats [25]. The lipid profile of the Capsifen treated animals was also similar to the control group indicating normal lipid metabolism. Thus, the observed body weight reduction among the high dose Capsifen treated animals without changing the lipid profile may be due to a thermogenesis mechanism of action, as observed by Fattori et al. [26]. Normal fluid balance and kidney function among Capsifen treated animals was also clear from the normal levels of sodium, potassium and creatinine. The high dose recovery groups in both sexes also showed similar results. Thus, the hematological and biochemical analysis demonstrated the safety of Capsifen at the investigated dosage of 500 mg/kg b. wt.

High dose recovery group of animals (1000 mg/kg b. wt.) followed a normal food intake indicating the normal metabolism. The normal food/water intake and the absence of inflammation, mucosal membrane damage or hemorrhage in the gastrointestinal tract indicated a healthy digestive tract for the recovery group. The body weight reduction was also found to be restored among recovery group of animals. So, high dosage Capsifen induced body weight change is not due to any permanent organ damage or metabolic disorder, but is reversible. Moreover, clinical parameters also remained in the normal range indicating the absence of toxicity.

Saito and Yamamoto reported the oral LD_{50} of capsaicin as 118.8 mg/kg for male and 97.4 mg/kg b. wt. for female mice. In the case of rats, the LD_{50} was reported to be 161.2 mg/kg and 148.1 mg/kg b. wt. respectively for male and female animals [27]. Hypotension and respiratory paralysis were the major reasons for mortality. Johnson et al. also reported an oral LD_{50} of 118 mg/kg b. wt. for capsaicin in rats and were found dead and showed hemorrhage in the gastric fundus [28]. Glinsukon stated that acute toxicity of capsaicin showed a large variation depending upon the mode of administration; *i.e.* in male mice, the LD_{50} varies from 0.56 mg/kg b. wt. (Intravenous) to 60 − 75 mg/kg b. wt. (in ethanol) and 190 (122–294) mg/kg b. wt. (in dimethyl sulfoxide), following intragastric intubation [29]. Nopanitaya and Nye reported that intraduodenal and intragastric administration of 10 % capsicum (0.014 % capsaicin + 0.85 % saline) to rats showed morphological damages in the duodenal mucosa [30]. The highest single dose used in the present study was 5000 mg/kg b.wt of Capsifen which is equivalent to 150 mg/kg b.wt of Capsaicinoids (Capsifen contained 3.06 % of total capsaicinoids). It was observed that the animals were safe at the dosage of 5000 mg/kg body weight without any mortality and any adverse behaviour or heamatological/biochemical changes, except a body weight reduction. Though the LD_{50} of capsaicin (pure capsaicin) was reported as 100 mg/kg (Glinsukon et al., 1979), the safety of Capsifen may be due to the sustained intestinal delivery of capsaicinoids from Capsifen. The capsaicinoids in Capsifen were tightly bound and encapsulated in the fenugreek soluble dietary fiber (galactomannan) matrix. Upon ingestion, Capsifen beadlets swells in the gastrointestinal fluid and submicronsed colloidal capsaicinoids leaches out for absorption. In the case of pure capsaicin, it is administered in a soluble form and resulted in faster and quick absorption. The direct contact of capsaicin with the gastrointestinal tract may also cause mucosal membrane damage, ulcers, and inflammation. The fenugreek dietary fiber has been separately investigated for its toxicity and efficacy [31,32]. A number of clinical trials at 5–20 g/day have been reported with significant benefits on blood-sugar, lipid profile and digestive tract [33].

5. Conclusion

The present study reported the safety assessment of a novel natural formulation of capsaicinoids-rich red chili pepper (*Capsicum annum*) extract using fenugreek dietary fiber as shown by LD_{50}, acute (14 days) and subchronic (90 days) oral gavage at 1000 mg/kg b. wt. The Capsifen administration did not produce any significant change in food and water consumption, hematological or biochemical parameters, except the bodyweight which indicated a decreasing trend at doses 500 mg/kg and 1000 mg/kg b. wt. Histopathological examination indicated the absence of any morphological abnormalities of vital organs. Considering the safety of Capsifen in the present study, it may be considered for safe human consumption and further clinical trials.

CRediT authorship contribution statement

Ashil Joseph: Formal analysis. **Johannah NM:** Writing - original draft. **Suresh Kumar:** Investigation. **Syam Das S:** Writing - review & editing. **Balu Maliakel:** Resources. **Krishnakumar IM:** Project administration, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors Ashil Joseph, Johannah NM, Syam Das S, Krishnakumar IM & Balu Maliakel are employees at Akay who developed and manufactured Capsifen.

Acknowledgment

Authors SK is thankful for M/s Akay Flavours & Aromatics, Pvt. Ltd, Cochin, India for the financial assistance under the program of development of Spiceuticals® – functional ingredients from spices' and also for the sample of Capsifen® used in the present study.

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