

SUPPLEMENTARY MATERIAL

Potential beneficial and/or adverse effects of *Capsicum annum* L. (cv. Fiesta) at two stage of ripening in CD-1 mice

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Potential beneficial and/or adverse effects of *Capsicum annuum* L. (cv. Fiesta) at two stage of ripening in CD-1 mice

Aim of the present study was to evaluate the potential beneficial and/or adverse effects of *Capsicum annuum* L. (cv. Fiesta) extracts at two stage of ripening (immature and mature), and at two dosages (low and high) by evaluation of biochemical profile and oxidative status in CD-1 mice. The extracts were daily administered to mice by oral gavage for 20 days. At the end of the trial, the animals were euthanatized and blood was collected. Evidence of liver damage (increase of AST, ALT, ALP and bilirubin) in the group receiving the higher dosage of immature peppers extract. Even if no adverse effects were seen at the lower doses, also no signs of beneficial effects in term of health status, biochemical profile and oxidative status were detected. These results are in contrast with *in vitro* findings and raise doubts about the possible use of *Capsicum annuum* L. as a nutritional supplement.

Keywords: *Capsicum annuum*; antioxidants; phytotherapy; red pepper; capsaicin

1.Introduction

Capsicum species, belonging to the Solanaceae family, are native to the tropical and humid zones of central and South America. *Capsicum* species are used not only as spices in food industry, but also as food additives to add or change the colour of foodstuffs and for drugs formulation (Sim and Sil 2008; Oyagbemi et al. 2010). Due to its phytochemicals compounds (i.e. capsaicin, carotenoids, and flavonoids) *Capsicum* fruit can be used for its nutritional and biological properties.

Capsaicinoids compounds (i.e. capsaicin and dihydrocapsaicin), responsible for the pungency of pepper fruits, are considered the most representative principles to which physiological and pharmacological properties are attributed (Abdullah et al. 2011).

The use of plant extracts as an alternative to conventional medicine is widespread, but the safety of the extracts should be determined before human consumption. *Capsicum* species have been reported to possess antioxidant (Sim and Sil 2008), anti-inflammatory (Liu and Nair 2010), hypoglycaemic (Tremblay et al. 2016), hypocholesterolemic (Kim et al. 2017), anti-obesity (Tremblay et al. 2016), and chemopreventive (Moore et al. 2003) properties. On the other hand, high doses of capsaicin lead to negative health impacts, e.g. capsaicin was hypothesized to cause liver and gut cancer (Hoch-Ligeti 1951; Toth and Gannett 1992; Erin et al. 2004). In any event, the literature concerning the toxicity of *Capsicum* extracts often reports contradictory results.

Basing on the available data it is not easy to establish a safe level of *Capsicum* in food/feed. The Committee of Experts on Flavouring Substances of the Council of Europe (2006) estimated the safety daily intake of chilli pepper consumers as 0-0.2 mg/kg BW, expressed as total capsacinoids.

Nevertheless, several factors may affect the potential properties of *Capsicum* depending on the light intensity and temperature at which the plant grows the position of the fruit

on the plant and the stage of ripening (Abdullah et al. 2011), as well as the colour of the fruits (Shukla et al. 2016). As reported by Tundis et al. (2013), phytochemical changes occurring during maturation affect the composition of capsicum extracts and, as a consequence, their biological activity. These authors compared extracts from different cultivars of *Capsicum annuum* L. showing significant differences for several *in vitro* effects due to the different stage of maturation. Within them, *C. annuum* L. (cv. Fiesta) showed high scavenging, antioxidant, and hypoglycaemic activity. No data are available about the *in vivo* effects of *C. annuum* L. (cv. Fiesta). Considering all the above facts, the aim of the present study was to evaluate the potential beneficial and/or adverse effects of extracts of *C. annuum* L. (cv. Fiesta) at two stage of ripening (immature and mature), and at two dosages (low and high) by evaluation of biochemical profile and oxidative status in CD-1 mice. As shown *in vitro* (Tundis et al. 2013) it has been hypothesized that the ripening stage of fruits, as well as the dosage of the administered extract, can cause different effects in mice.

2.Results and discussion

In Table S1 the results of blood chemistry parameters and oxidative status analyses of the five groups were reported.

The effects of the group on body weight and on average daily gain are reported in Figure S1A and S1B and mean feed intake and mean water consumption gain are reported in Figure S2A and S2B.

In a previous report (Morittu et al. 2016), testing in mice *C. annuum* L. (cv. Fiesta) at the doses corresponding to the mean consumption of chili in Europe the extract was well tolerated but did not show beneficial effects. In this study, the administration of

higher doses of both mature and immature *C. annuum* L. (cv. Fiesta) still showed no clear beneficial effects whereas some adverse findings were evidenced.

Regarding blood chemistry parameters, the increase of AST and ALT detected in the group receiving the highest dosage of immature peppers extract (HI) is suggestive of liver damage. In addition, independently from the ripening stage, both groups receiving high dosage of extract (HI and HM) showed the highest values of bilirubin. These results suggest that the metabolic capacity of the liver has been exceeded or hepatocellular damage occurred.

A decrease of cholesterol was observed only in HI and LM groups. An hypocholesterolemic effect has been described both for capsaicinoids and flavonoids. The analysis of the extracts content of these substances suggests that the decrease of CHOL observed in those groups should not be ascribed to the flavonoid content, but, also considering the capsaicinoids content of the two extracts, such decrease is hard to explain and probably needs further studies.

It has been reported that dietary flavonoids intake is inversely associated with lower cholesterol serum levels and, consequently, lower mortality from coronary heart disease (Hertog et al. 1993). A study conducted by Arai et al. (2001) on 115 Japanese women volunteers measured the flavonoids intake in the diets. Results showed that diets rich in flavonoids were inversely correlated with the plasma total cholesterol concentration. Flavonoids, by modulating lipid absorption and lipogenesis, may improve dyslipidemias (Galleano et al. 2012). In our study, the two groups (HI and LM) that showed such effect contained the highest and the lowest dose of flavonoids, respectively. Therefore, the absence of a cholesterol decrease in the other two groups suggests that the decrease of cholesterol should not be ascribed to these substances.

On the other hand, several authors reported that capsaicinoids are able to lower blood cholesterol levels (Sirivasan 2015). A high-cholesterol diet containing 0%, 0.010%, 0.015%, 0.020% and 0.030% capsaicinoids was administered to hamsters for 6 weeks and capsaicinoids reduced plasma total cholesterol, regardless the tested doses (Liang et al. 2012). The effect of *Capsicum* on dietary hypercholesterolaemia was also observed in male gerbils at a dose of 75 mg/kg body wt/day. In gerbils a reduction of serum cholesterol and triglycerides by 70% and 66% was observed with the experimental diet characterized by the inclusion of *Capsicum annum* L. at the dosage of 75 mg/kg body wt/day (Gupta et al. 2002). Srinivasan et al. (1980) observed a decrease of cholesterol by administering 0.5, 1.0, 2.0 and 5.0% of red pepper to rats in a synthetic diet. There are many hypothesis about how capsaicinoids may affect cholesterol in serum.

Capsaicinoids were hypothesized to reduce high cholesterol levels by breaking blood cholesterol down, preventing its accumulation and promoting its fecal excretion (Huang et al., 2014). Instead, Srinivasan and Sambaiah, (1991) observed that dietary capsaicinoids stimulate hepatic conversion of cholesterol to bile acids possibly mediated by up-regulation of cholesterol 7 α -hydroxylase and down-regulation of liver X receptor alpha, an important pathway for elimination of cholesterol from the body. Despite these considerations, in this study the hypocholesterolemic effect was not detected for the HM group that resulted the richest in capsaicinoids. Therefore, considering the total capsaicinoids content, a decrease of cholesterol was found at the doses of 0.95 (LM) and 3 (HI) mg/kg BW but not at the dose of 3.8 (HM)/kg BW. The hypothesis of a possible interaction between capsaicinoids and flavonoids cannot be excluded but needs further studies.

No differences were detected between control and treated groups regarding the in life evaluations (body weight, on average daily gain, feed and water consumption). The

differences registered for time effect for all the experimental groups could be attributed to the age of the mice, young subjects in the growth phase.

Our data were in contrast with some authors that testing *Capsicum* L. did not find any adverse effects. Zimmer et al. (2011) showed that the administration of 200mg/kg BW/day of ethanol extract of *Capsicum baccatum* L. in mice by oral gavage for 60 days did not affect specific aspects of CF1 mice physiology. There was no significant difference among groups in: ALT, ALP, TP and albumin, LDH, triglycerides, cholesterol, HDL and LDL, urea, and creatinine at the end of the experiment. No statistical differences among groups were observed in final mean body weight and food intake during the administration period. Jang et al. (1992) tested *Capsicum annuum* L. at levels of 0.5, 1.0, 2.5, 5.0, 7.5 or 10% body weight (w/w) in mice added in the diet for the 4-week feeding period. General health, body weight and feed intake were apparently not adversely affected at any level of pepper consumption. The authors concluded that red chilli was relatively non-toxic at the doses tested in male B6C3F1 mice.

In a study performed by Akagi et al. (1998) a mixture of 64.5% capsaicin and 32.6% dihydrocapsaicin at dose levels of 0, 0.0625, 0.125, 0.25, 0.5, and 1% (in the diet) was administered to B6C3F1 mice. At the end of the experiment (13 weeks) significant decrease of feed intake and body weight gain in all groups were registered. Hsu et al. (2016) administered capsaicin once a day for 28 consecutive days: 205 mg/kg; 410 mg/kg and 1025 mg/kg by oral gavage to female mice. In that study, it was demonstrated that capsaicin supplementation could be beneficial to the liver function; the kidney-related indexes including BUN, creatinine, and UA were all improved by capsaicin supplementation.

On the other hand, our data are in accordance with some studies in which larger quantities of *Capsicum* L. or capsacinoids have been tested. Monsereenusorn (1983) administered in rats 50 mg/kg body weight/day of capsaicin by gavage or 50 mg/kg /day of an extract of capsicum fruit for 60 days. The results showed that starting from day 30, up to the end of the trial plasma total cholesterol levels were significantly reduced. Besides there were significant reductions of the growth rate in both treated groups compared to the control, feed intake increased steadily throughout the experimental periods with a tendency for Capsicum treated animals to show more adverse effects. Kodama et al. (2008) conducted in rats a 26-week oral toxicity study of capsacinoids. A reasonably clear adverse finding in this study was an effect in the livers of high-dose male rats. This conclusion was supported by increases in some clinical chemistry parameters (AST, ALT, LDH) observed at the highest dosage 5.0 mL/kg/day (capsacinoids 365.75 mg/kg). The adverse effects we have encountered, similar to those of the studies mentioned above, might be due to the higher levels of capsaicin and dihydrocapsaicinin *C. annuum* L. (cv. Fiesta) compared to other varieties (Tundis et al. 2013).

Concerning the antioxidant properties of *Capsicum annuum* L. no difference was seen for both d-ROMs and BAP, thus suggesting no antioxidant effect. Our results are in contrast with *in vitro* studies (Khan et al. 2014). Sim and Sil (2008) investigated *in vitro* the antioxidant activities of *Capsicum annuum* L., pericarp and red pepper extracts; both extracts resulted highly effective for the antioxidant properties assayed (i.e. DPPH radical-scavenging activity, superoxide anion radical scavenging, hydroxyl radical scavenging). Farhoudi et al. (2017) studying five different Jalapeno peppers found a significant correlation between capsacinoids and the antioxidant activity.

Tundis et al. (2013) comparing extracts from different cultivars of *Capsicum annuum* L. at different stage of ripening founded higher scavenging effects in immature samples, probably due to the higher phenols and flavonoids content (Pulido et al. 2000) compared to the mature ones. Indeed, also mature extracts possesses antioxidant property as demonstrated by the β -carotene bleaching test.

3.Experimental

3.1. Extracts preparation

Fruits at two different ripening stages, namely mature (M) and immature (I), of *C. annuum* L. (cv. Fiesta) were used in this study. The peppers were purchased from the farm Miceli s.r.l. (Calabria, Italy). The fruits, deprived of stalks and seeds, were cut into small pieces and soluble components were extracted with a maceration in ethanol as previously reported by Tundis et al. (2013). As reported in this study, immature fruits were characterized by a total phenols and flavonoids content of 781.4 mg of chlorogenic acid equivalents/100 g of fresh weight (FW) and 115.7 mg of quercetin equivalents/100 g FW, respectively. Mature fruits showed a lower content of phenols and flavonoids in comparison to immature fruits, but demonstrated a high content of capsaicin and dihydrocapsaicin (1280.2 and 422.5 μ g/g FW, respectively).

3.2. Animals

A total of 30 male mice (Hsd: ICRCD-1®, Envigo, The Netherlands) were used in the study. Animals were housed 3 per cage into polycarbonate cages on wood chips bedding (Lignocel BK 8-15, J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg) placed in an air-conditioned room environmentally controlled (temperature 21.0 ± 1.0 °C; humidity: 40.0 ± 5.0 %) with a 12:12-h light:dark cycle (lights on, 0700 to 1900).

Excursions from these ranges during the experimental period were minimal and considered to have had no impact on the study. The animals had free access to water and were fed *ad libitum* with standard rodent chow (2018 Global rodent diet, Teklad) for one week before assignment to one of five experimental groups. All animal studies were approved by the Italian Health Ministry (1150/2015-PR) and by the local ethics committee.

3.3. *Experimental design*

At 5 weeks of age (day “-7”), the mice were weighed, marked with ear punch for recognition, and randomly assigned to five experimental groups. From day “0” to day “19”, each animal received once daily by oral gavage one of the following substances: Group High Immature (HI, n=6): 200 mg/kg body weight of immature peppers extract dissolved into 0.2 ml of water.

Group Low Immature (LI, n=6): 50 mg/kg body weight of immature peppers extract dissolved into 0.2 ml of water.

Group High Mature (HM, n=6): 200 mg/kg body weight of mature peppers extract dissolved into 0.2 ml of water.

Group Low Capsicum Mature (LM, n=6): 50 mg/kg body weight of mature peppers extract dissolved into 0.2 ml of water.

Group Control (C n=6): mice received 0.2 ml of water to minimize false positive results.

3.4. *In-life evaluations*

During the experimental period, mice were observed daily, before and after dosing, for abnormalities in clinical signs. Body weights were registered individually at day 0-5-10-

15-20. Diet and water consumption were recorded for each cage every 5 days during the experimental period.

3.5. Laboratory analysis

At the end of the trial, the animals were euthanized and blood samples were collected. Blood chemistry analyses were performed by an automatic biochemical analyser (AMS Autolab, Diamond Diagnostics - USA) using reagents from Spinreact (Girona, Spain) to determine: total proteins (TP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CREA), glucose (GLU), aspartate amino transferase (AST), alanineaminotransferase (ALT), gamma-glutamyltransferase (GGT), bilirubin (BIL), lactic dehydrogenase (LDH), creatine kinase (CK), alkaline phosphatase (ALP), cholesterol (CHO) and triglycerides (TRI). Reagents from Diacron International s.r.l. (Grosseto, Italy) were used to assess reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests.

3.6. Statistical analysis

Haematochemical parameters were analysed with the General Linear Model Univariate procedure including only the group as fixed effect. Body weight, average daily gain, feed intake and water consumption data were analysed with the General Linear Model for Repeated measures, including the effects of the “group” as between factor, of the “day” as within factor and the interaction term “group x day”.

The Bonferroni post-hoc test was used for comparison of the mean values calculated for different levels of the same independent variable. All statistical procedures were performed using SPSS (version 20.0). The significance level was set at $P < 0.05$.

4.Conclusions

Our results are in contrast with *in vitro* findings and raise doubts about the real efficacy of *C. annuum* L. (cv. Fiesta) as a nutritional supplement for treating and preventing diseases such as, obesity, diabetes and others as well as those pathological conditions that increase ROS production.

Acknowledgments

The authors would like to thank Dr. Raffaele Maglione (LABFORVET, Naples, Italy) and Dr. Maria Ferrara for their technical support.

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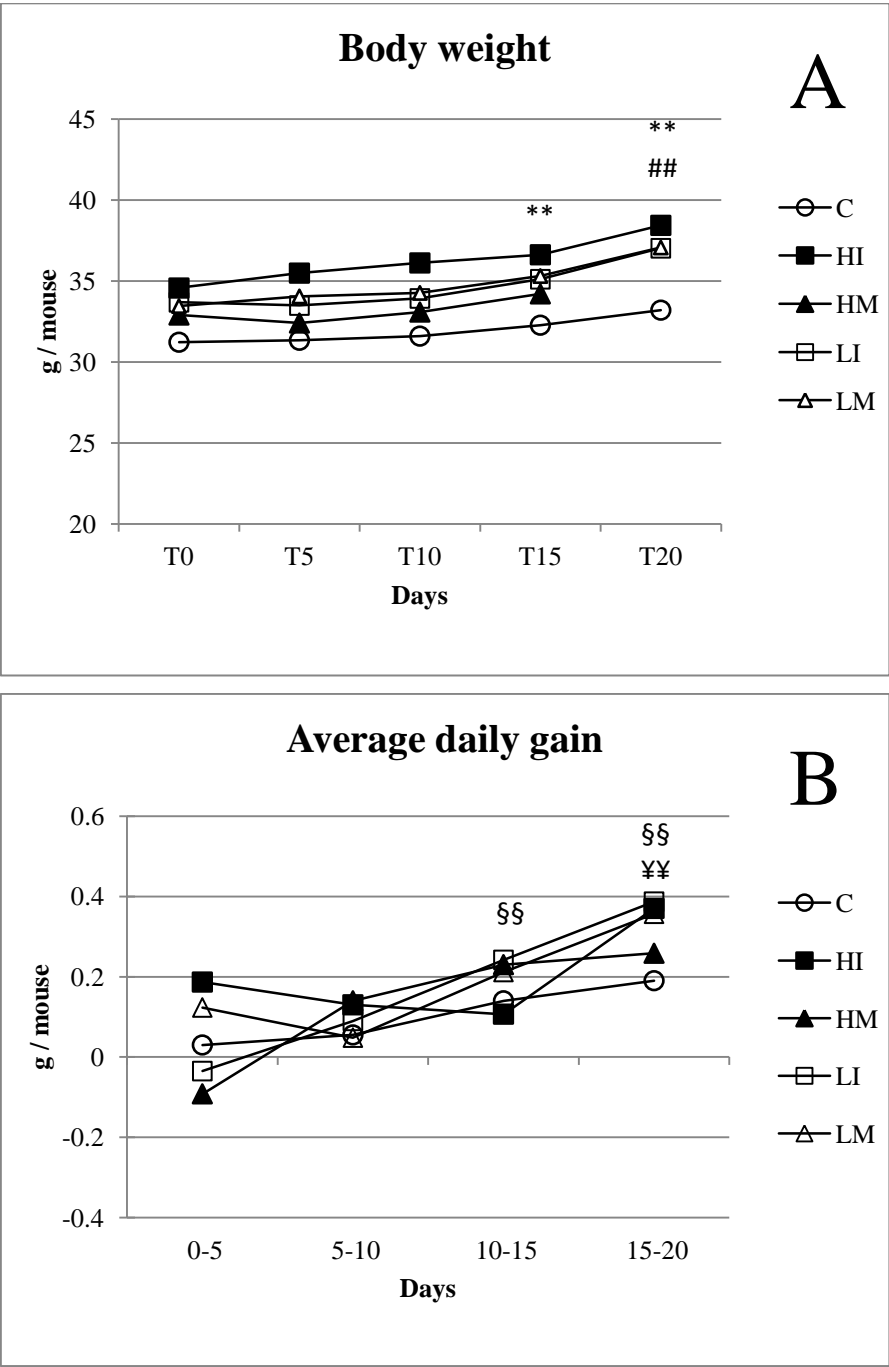
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Table S1. Blood chemistry parameters and oxidative status of the mice after 20 days of daily consumption of Capsicum extracts by oral gavage.

ITEM		C (n=6)	HI (n=3)	HM (n=6)	LI (n=5)	LM (n=6)	P	SEM
AST	<i>U/L</i>	75.2 bc	122.3 a	91.2 b	71.4 c	70.0 c	<0.001	1.912
ALT	<i>U/L</i>	52.3 bc	148.3 a	69.5 b	58.6 b	55.2 b	<0.001	3.677
ALP	<i>U/L</i>	79.8 b	130.0 ab	85.2 b	140.4 a	95.8 ab	0.007	5.665
BIL	<i>mg/dL</i>	0.38 c	1.13 a	0.82 ab	0.54 bc	0.43 c	<0.001	0.040
GLU	<i>mg/dL</i>	117.0	121.3	114.0	117.0	110.8	0.863	3.008
TP	<i>g/dL</i>	6.53	6.20	6.50	6.34	6.47	0.841	0.094
ALB	<i>mg/dL</i>	3.27	3.07	3.23	3.24	3.25	0.771	0.046
UREA	<i>mg/dL</i>	40.8 a	33.3 ab	35.0 ab	31.6 b	34.5 ab	0.031	0.918
CREA	<i>mg/dL</i>	0.33	0.33	0.30	0.34	0.32	0.986	0.027
LDH	<i>U/L</i>	2516.3	2025.3	2514.7	1647.6	1919.2	0.543	205.305
CPK	<i>U/L</i>	50.7	104.3	97.0	70.4	69.3	0.330	8.871
CHOL	<i>mg/dL</i>	219.8 a	179.3 b	202.5 ab	215.2 a	181.0 b	0.001	3.222
TRI	<i>mg/dL</i>	219.4	249.7	237.3	238.2	246.8	0.864	9.392
d-ROMS	<i>U</i>							
	<i>CARR</i>	102.0	111.0	70.3	93.4	99.2	0.611	8.589
BAP	<i>μmoli/L</i>	2622.3	2515.7	2454.8	2401.4	2456.8	0.986	141.300

C, control received only water; groups treated with high dose of immature (HI) or mature (HM) peppers extract; groups treated with low dose of immature (LI) or mature (LM) peppers extract.

Figure S1. Mean BW (A) and mean ADG (B) in five mice groups



C, control received daily only water; groups treated daily with high dose of immature (HI) or mature (HM) peppers extract; groups treated daily with low dose of immature (LI) or mature (LM) peppers extract] during 20 days of treatment.

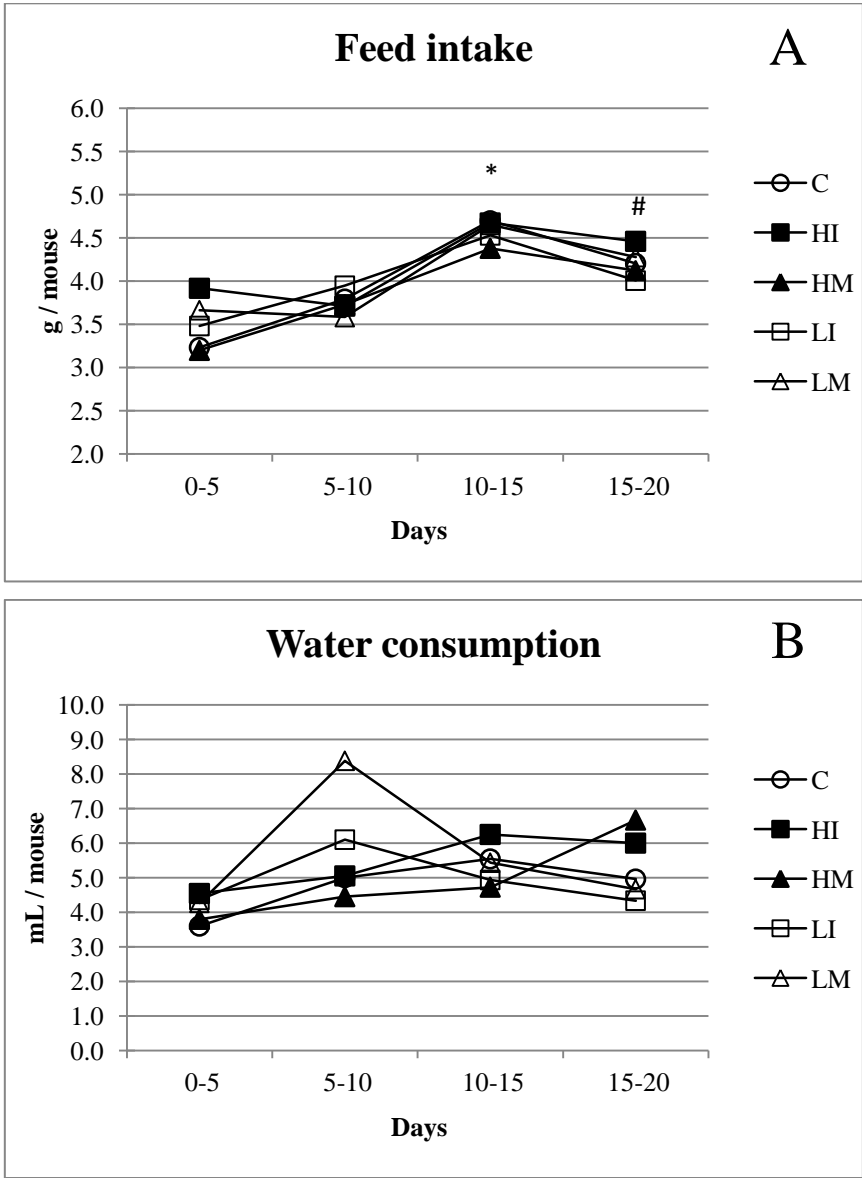
**Statistically significant if compared with Day 0, 5, and 10 ($P<0.001$).

Statistically significant if compared with Day 15 ($P<0.001$).

§§ Statistically significant if compared with period 0-5 ($P<0.01$)

¥¥ Statistically significant if compared with period 5-10 and 10-15 ($P<0.01$)

Figure S2. Mean Feed intake (A) and mean Water consumption (B) in five mice groups



C, control received daily only water; groups treated daily with high dose of immature (HI) or mature (HM) peppers extract; groups treated daily with low dose of immature (LI) or mature (LM) peppers extract during 20 days of treatment.

*Statistically significant if compared with Day 0-5 and Day 5-10 periods ($P < 0.05$).

Statistically significant if compared with Day 0-5 ($P < 0.05$).