Studies of the Toxicological Potential of Capsinoids IV: Teratology Studies of CH-19 Sweet Extract in Rats and Rabbits

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CH-19 SWEET EXTRACT: TERATOGENICITY

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ABSTRACT

In order to evaluate the safety of CH-19 Sweet extract which contains capsinoids, teratology studies were conducted in pregnant Sprague-Dawley rats (20 rats per group) and pregnant New Zealand White rabbits (17 to 22 animals per group). The test substance was administered to rats by gavage for 11 days on gestation days 7-17 at doses of 0 (vehicle), 1.25, 2.5 and 5.0 mL/kg and to rabbits for 13 days on gestation days 6-18 at doses of 0 (vehicle), 0.25, 0.5 and 1.0 mL/kg. As the concentration of capsinoids in CH-19 Sweet extract was 72.2 - 75.05 mg/mL, the resulting dose of capsinoids administered to rats was 90.25, 180.5 and 361 mg/kg, and to rabbits was 18.76, 37.53 and 75.05 mg/kg in the vehicle, low- mid-, and high-dose groups, respectively

In the rat study, no deaths occurred in any group and there were no test substance-related changes or abnormalities in clinical signs, body weight, food consumption or gross pathological findings. There were no test substance-related changes in the number of *corpora lutea*, number or index of implantations, index of embryo-fetal deaths, number of live fetuses, sex ratio, fetal body weight at the end of the gestation period or abnormalities in the placenta of live fetuses. There were no test substance-related abnormalities or variations in the external, skeletal or visceral examinations of live fetuses. It was concluded that the test article caused neither teratogenic effects nor abnormalities in the progression of ossification.

In the rabbit study, there were no test substance-related effects on clinical signs, body weight, food consumption or necropsy findings. There were neither test substance-related abortions nor test substance-related effects on the number of *corpora lutea*, or number or index of implantations. There were no test substance-related effects on the number of dead embryos/fetuses, the number of live fetuses, sex ratio, body weight of live fetuses, or gross

pathological finding in the placentas. There were no test substance-related external abnormalities or incidences of visceral or skeletal abnormalities or variations, and there were no test substance-related effects on the progress of ossification in any group.

The authors concluded the no-observed-adverse-effect-level (NOAEL) of CH-19 Sweet extract containing capsinoids on pregnant animals and fetal development/growth was >5.0 mL/kg/day (>361 mg/kg/day as capsinoids) in rats and >1.0 mL/kg/day (>75.05 mg/kg/day as capsinoids) in rabbits.

Keywords: Capsinoids, CH-19 Sweet extract, Teratogenicity

INTRODUCTION

Capsaicin (Figure 1) occurs naturally in plants of the *Solanaceae* family and represents the main pungency compound in red pepper fruits. Although commonly used, the strong pungency limits the use of large quantities of capsaicin in food and medicine.

Capsaicin homologues, called capsaicinoids, are groups of acid amides of vanillyl amine and C_8 to C_{13} fatty acids. The major capsaicinoids in pungent red peppers are capsaicin ((*E*)-*N*-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenamide), dihydrocapsaicin, the 6, 7-dihydro analogue of capsaicin and nordihydrocapsaicin, mono-nor homologue of dihydrocapsaicin (Kobata et al. 1999). Recently, novel capsaicinoid-like substances were found in the fruit of CH-19 Sweet, a non-pungent cultivar of *Capsicum annuum* L. (Yazawa et al. 2004). Kobata et al. (1998 and 1999) determined the structures and named these substances capsiate (4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate, Figure 2), dihydrocapsiate (4-hydroxy-3-methoxybenzyl 8-methylnonanoate, Figure 3) and nordihydrocapsiate (4-hydroxy-3-methoxybenzyl 7-methyloctanoate, Figure 4), respectively. These compounds have an ester bond instead of the amide bond between the vanillyl moiety and fatty acid chain normally found in capsaicinoids, and the entire class of these compounds is called capsinoid (Kobata et al. 1999).

CH-19 Sweet extract is an *n*-hexane extracted oil containing capsinoids from CH-19 Sweet. *N*-hexane extraction is frequently employed in the production of chili pepper extracts (*i.e.*, capsicum oleoresin). The concentration of capsinoids of CH-19 Sweet extract is adjusted to approximately 7.5%w/w% by adding medium chain triglyceride (MCT). Capsinoids are non-pungent, making it possible to utilize capsinoids in food applications without the attendant heat effect found in capsaicins (Yazawa et al. 2004). While the acyl residues of capsinoids are identical to those of capsaicinoids, the differences between the sensory properties of capsaicin

and capsiate are due to the way the vanillyl and acyl moieties of the basic structural motif are linked. Specifically, in capsaicin-type compounds these moieties are linked via an amide bond in contrast to the ester bond found in capsiate-type compounds. The lack of this amide bond in capsinoids removes the pungent activity found in capsaicin-containing chili peppers.

As part of an overall safety evaluation, CH-9 Sweet extract has been evaluated for acute toxicity and genotoxicity (Watanabe et al. 2008), subchronic toxicity in rats (Kodama et al. 2008a) and reproductive effects (Kodama et al. 2008b). The findings from these studies are in many ways similar to findings from toxicity studies performed on capsaicinoids (Monsereenusorn 1983; Akagi et al. 1998) with the liver being the apparent target organ. However, since there are no data on the teratogenicity of capsinoids teratology studies of capsinoids were conducted on rats and rabbits using CH-19 Sweet extract. These were conducted at the Bozo Research Center, Inc. (Tokyo, Japan) in compliance with the Law Concerning the Protection and Control of Rats, Law No. 105, October 1, 1973, revised on December 22, 1999, partly revised Law No. 68 on June 22, 2005; Standards Relating to the Care and Management, etc. of Experimental Rats, Notification No. 6, March 27, 1980 of the Prime Minister's Office, Japan, revised on May 28, 2002; and Guidelines for Rat Experimentation, the Japanese Association for Laboratory Rat Science, May 22, 1987. The protocols were reviewed and approved by the Institutional Animal Use and Care Committee.

MATERIALS AND METHODS

The Test Substance and Dosage Preparation

CH-19 Sweet extract is a yellowish-brown, cloudy fluid. Two lots of test substance (Lot Nos. 050404 and 050530) were supplied by Ajinomoto Co., Inc. (Tokyo, Japan) and employed for evaluating teratology in rabbits and rats, respectively. The concentrations of total and individual

capsinoids in the lots of CH-19 are presented in Table 1. Medium chain triglyceride (MCT, product name: Actor M-2) was purchased from Riken Vitamin Co., Ltd. (Tokyo, Japan) and used as the vehicle. MCT is chemically called Triglyceride C8, which is composed of a glycerol backbone and three of these fatty acids. It was reported that the oral LD_{50} of MCT for male rats was determined to be greater than 36 mL/kg, which is more than 20mL/kg used in this study, and there were no significant findings. (Traul et al 2000). The undiluted test substance was used as the dosing formulation for the high-dose group. For the middle-dose and low-dose groups, the test substance was diluted with the vehicle. Animals in the control group received the vehicle alone.

Teratology Study in Rats

Test Subjects and Mating

Male and female Sprague-Dawley SPF rats (Crl:CD(SD), Atsugi Breeding Center, Charles River Japan, Inc., Kanagawa, Japan were acclimated to laboratory conditions for 5 days. During the acclimation period rats were observed daily. Female rats, determined to be appropriate for mating (i.e., in proestrus) based on their estrous cycle during the acclimation period, were housed on a one-to-one basis with male rats. The presence of vaginal plugs or sperm in the vaginal smear the following morning was taken as confirmation of mating and that day was designated as gestation day 0. At the time of mating, females were 11 or 12 weeks of age and males were 12 or 13 weeks of age. Females that had mated were randomized into 4 groups of 20 females each.

Animal Husbandry

Rats were individually-housed in bracket-type metallic wire-mesh cages.. Animal room environmental conditions included: temperature of 21 to 25°C; relative humidity of 47 to 67%;

ventilation of 10 to 15 air changes per hour with fresh filtered air; and a lighting cycle of 12-hour on (0700-1900):12-hour off lighting cycle. The rats were allowed free access to pelleted diet NMF (radiation-sterilized, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Test Article Administration

The 4 groups were assigned to treatment regimens that included once daily gavage administrations of 0 (vehicle), 1.25, 2.5 or 5.0 mL/kg of CH-19 Sweet extract (the equivalent of 90.25, 180.5 and 361 mg/kg capsinoids, respectively). In order to determine the appropriate dosing volume of MCT, a 4-week repeated dose toxicity study was performed, using a dose volume of 5 mL/kg and 10 mL/kg of MCT, and 10 mL/kg of water (control) in rats. The 10 mL/kg MCT volume was found to be excessive (slight food and weight suppression, lower relative liver weight than controls). Based upon these results, the maximum volume of administration of MCT was determined to be 5 mL/kg (data not shown). Males, non-mated females and mated females remaining after group allocation were excluded from the test system by sacrifice under deep ether anesthesia. The dosing volume was set at 5.0 mL/kg and dosing formulations were administered by gavage using stomach tubes for 11 days, from Day 7 to 17 of gestation. The primary focus of this study was the effect of dihydrocapsiate during the period of organogenesis. Individual dose volume was calculated based on the animals' body weights on day 7 of gestation.

Observation and Examination of Dams

All rats were observed 3 times daily (i.e., before, immediately after, approximately 4 hours post-dosing [during the administration period], and once every morning during the other periods), for general abnormalities (e.g., external appearance, excrement, nutritional condition,

posture and behavior), as well as for gestational abnormalities (e.g., abortion, premature birth). All mated female rates were weighed on gestation days 0, 4 and 7-20. For all rats, the amount of feed supplied and the amount of feed left uneaten was measured for gestational days 0 to 4, 4 to 7, 7 to 10, 10 to 14, 14 to 18, and 18 to 20. Cumulative food consumption and the one-day mean food consumption for these periods were calculated. The amounts of feed supplied and left uneaten were measured. Dams were sacrificed by exsanguination using the abdominal aorta under ether anesthesia in the afternoon of gestation day 20, and major organs and tissues in the thoracic and abdominal cavities were observed macroscopically. At necropsy, the uterus was observed for the presence of *conceptus* to define gestation status. For pregnant rats, the ovaries and uterus were removed. The ovaries were examined for the number of *corpora lutea*. For the uterus, the uterine wall was incised and the uterus was examined for the number of live fetuses and numbers of resorbed/dead fetuses (i.e., implantation sites, resorbed embryos, placental remnants, early macerated fetuses, late macerated fetuses and dead fetuses). The number of implantations was the sum of the number of live fetuses and the number of embryo-fetal deaths. Placentae of live fetuses were observed macroscopically for abnormalities. For 2 dams in the control group and 1 dam in the high-dose group in which implantations were not observed macroscopically, the uterus was made transparent using 2 w/v% NaOH solution for observation of implantation traces. Since there were no implantations, these dams were judged not to be pregnant and all the data on these dams was excluded from the study results.

Observation and Examination of Live Fetuses

All live fetuses were observed for external malformations and sexed using the distance from the anus to the genital papilla. Individual body weights were measured. Fetuses with external abnormalities were weighed, fixed and preserved in phosphate buffered 10% formalin.

For each litter, approximately half of the live fetuses, except those with external abnormalities, were fixed in Bouin's solution. Internal organs were examined for visceral abnormalities/variations by Wilson's free-hand razor method (Wilson 1965) for the cephalic cavity and by Nishimura's microdissection method (Nishimura 1974) for the thoracic and abdominal cavities. All fetuses, except those for visceral examination and those with external abnormalities, were fixed in 70% alcohol and subjected to alizarin red S staining by a modified Dawson's method (Dawson 1926) to prepare clear skeletal specimens. Skeletal specimens were examined with a stereoscopic microscope for skeletal abnormalities/variations and examined for the progress of ossification by counting the numbers of ossified metacarpi, metatarsi, and sacral and caudal vertebrae, and calculating the index of ossified sternebrae.

Teratology Study in Rabbits

Animals and Animal Husbandry

Nulliparous female New Zealand White SPF rabbits (Kbl:NZW, Kitayama Labes Co., Ltd., Nagano, Japan) were purchased at 14 weeks of age and acclimated to laboratory conditions for 19 to 28 days. They were observed daily during the acclimation period. Females in estrus showing swollen and dark-purple vulva were housed together with male rabbits for mating on a 1:1 basis in circles for mating (650 mm in diameter × H 500 mm). Thirty-one male New Zealand White SPF rabbits (Kbl:NZW Kitayama Labes Co., Ltd.) which had been housed at the test facility were used for mating. The females, for which copulation was confirmed twice by the presence of sperm in the vagina and around the orifice, were regarded as animals that had copulated and the day was designated as gestational day 0. Females that had copulated were randomized into 4 groups, each comprised of 24 copulated females. The groups were assigned to

treatment regimens that included once daily oral administrations of 0 (vehicle), 0.25, 0.5 or 1.0 mL/kg of CH-19 Sweet extract (the equivalent of 18.76, 37.53 and 75.05 mg/kg capsinoids, respectively). The maximum dose volume was based on a preliminary repeated-dose study in non-pregnant rabbits. In that study, general toxicological changes at a dose volume of 2.5 mL/kg, but not at 1.0 mL/kg was observed. As a result, a dose volume of 1.0 mL/kg was selected as the maximum volume. Males, unmated females and mated females remaining after group allocation were excluded from the test system and kept in the testing facility as possession animals. The dosing volume was set at 1 mL/kg and dosing formulations were administered orally using Nelaton catheters from Day 6 to 18 (period of organogenesis) of gestation. Organogenesis effects from the test article were the primary focus of this study. Individual dose volume was calculated based on the most recently recorded body weights of animals during the administration period.

Rabbits were individually-housed in aluminum animal cages.. Animal room environmental conditions included temperature: 21 to 23°C; relative humidity: 51 to 74%; ventilation: 10 to 15 air changes per hour with fresh filtered air; and a 12-hour on: 12-hour off lighting cycle. The animals were allowed free access to pelleted diet RC4 (Oriental Yeast Co., Ltd.) and tap water.

Observation and Examination of Dams

All female rabbits were observed daily (before, immediately after, and 4 hours after dosing during the administration period) and once every day (morning) during the other periods for abnormalities in general condition and external appearance, excrement, nutritional state, posture and behavior. All female rabbits were weighed on days 0, 3, 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 26 and 28 of gestation. For all rabbits, food consumption was measured on days 1, 3, 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 26 and 28 of gestation. One-day food consumption was calculated

as the difference between the amount of food left uneaten on the day of measurement and the amount of food supplied on the day before. On day 28 of gestation, surviving females were sacrificed by exsanguination via the abdominal artery/vein under anesthesia by intravenous administration of pentobarbital sodium solution (Pentobarbital sodium, Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan and physiological saline, Otsuka Pharmaceutical Factory Inc., Osaka, Japan), and major organs/tissues in the thoracic and abdominal cavities were macroscopically observed. Since there were no macroscopic abnormalities, organs/tissues were not removed or preserved. The female that aborted (0.25 mL/kg group) was subjected to necropsy in the same manner as for the other animals after abortion was found. At necropsy, the uterus was examined for implantations to determine gestation status. For pregnant females, the ovaries and uterus were removed. For the ovaries, the number of corpora lutea was counted. For the uterus, the uterine wall was cut and the following noted: numbers of live fetuses, dead embryos, fetuses with its stage (resorbed embryo, placental remnant, early macerated fetuses, late macerated fetuses, and dead fetuses); the total number of live fetuses and dead embryos/fetuses was calculated as a percentage of implantations. The placentae of live fetuses were observed for macroscopic abnormalities and weighed individually. For the female that aborted (0.25 mL/kg group), the ovaries and uterus were observed in the same manner as for the other animals. The uterus of animals in which no implantations were observed macroscopically was stained in 10 vol% ammonium sulfide solution to observe for implantation sites. Since no implantation sites were observed in any of the animals, they were judged to be non-pregnant and the uterus was discarded after the end of observation.

Observation and Examination of Live Fetuses

All live fetuses were observed for external abnormalities, including those in the oral cavity, and weighed individually. For all fetuses (except 2 fetuses that showed external abnormalities at cesarean section and were fixed in phosphate buffered 10% formalin), internal organs in the thoracic cavity (except internal observation of the heart) and those in the abdominal cavity were observed macroscopically for abnormalities using fresh specimens. Sex determination was done by examining internal organs. The brain and the heart were removed, fixed in phosphate buffered 10% formalin, and observed for dilatation of the ventricle by Wilson's macro-dissection method (Wilson 1965) for the brain and for visceral abnormalities/variations by Nishimura's micro-dissection method (Nishimura 1974) for the heart. After the end of observation, visceral organs/tissues were preserved in phosphate buffered 10% formalin.

Following the observation of the viscera of fresh specimens, the skin and fatty tissues were removed, and the fetuses were fixed in 95% alcohol and subjected to alizarin red S staining by Dawson's method (Dawson 1926) to prepare clear skeletal specimens. The skeletal specimens were observed for skeletal abnormalities/variations and examined for the progress of ossification by counting the numbers of ossified sternebrae, metacarpi, metatarsi, phalanges (proximal, medial and distal), and sacral and caudal vertebrae. Specimens for skeletal observation were preserved in 50% glycerin solution (containing 0.5 mg/mL thymol) after the end of observation.

Statistical Analysis of Data

The data of pregnant rats and rabbits and were analyzed between the control group and each dose group for statistical significance. The levels of significance for each test were 5 and 1%, two-tailed. All the data of non-pregnant animals, including dead animals, was excluded from statistical analyses. Body weights, body weight gain (rat: days 7 to 18 of gestation, days 18 to 20

of gestation; rabbits: days 6 to 19, and 19 to 28 of gestation), food consumption, number of *corpora lutea*, number of implantations, number of live fetuses, body weights of live fetuses, and the numbers of ossified metacarpi, metatarsi, phalanges (rats only) and sacral and caudal vertebrae were analyzed for homogeneity of variance by Bartlett's test (Sokal and Rohlf 1969). Homogeneous data were analyzed by Dunnett's method (Dunnett 1964) and heterogeneous data were analyzed by a Dunnett-type method (Hollander and Wolfe 1973). Body weights of live fetuses (for each sex) and the number of ossified bones were analyzed after obtaining the mean value for each litter. Implantation index, index of embryo-fetal deaths, index of placental abnormalities, index of external abnormalities, sex ratio (rabbits only), index of visceral abnormalities, index of visceral variations, index of skeletal abnormalities, index of skeletal variations, and index of ossified sternebrae were analyzed by a Dunnett-type method after obtaining the index for each litter using following formulas. In the rat, sex ratio was analyzed by chi-square test with Yates' continuity correction (the levels of significance: 5% and 1%, two-tailed) (Yates 1934) after calculating the sum of live male fetuses and live female fetuses for each group.

RESULTS

Teratology Study in Rats

Effects on Dams

No deaths occurred in any group. Salivation was observed in 2 females in the 5.0 mL/kg group on day 17 of gestation. Otherwise, there were no clinical observations during the gestation period in any group. Results of body weight are shown Table 2. There were no significant differences between the control group and any test substance administration group in body weight on any day of measurement during the gestation period or body weight gain during the

administration period (days 7 to 18 of gestation). In the 2.5 mL/kg group, body weight gain after the end of the administration period (days 18 to 20 of gestation) was significantly higher than in the control group. However, body weight gain in the 1.25 and 5.0 mL/kg groups was not significantly different from that of the control group. Food consumption from days 14 to 18 of gestation was significantly higher in the 1.25 and 2.5 mL/kg groups than in the control group, and tended to be higher in the 5.0 mL/kg group than in the control group. However, during the other periods, food consumption was comparable between the control group and each test substance administration group and there were no significant differences (Table 3). There were no gross pathological findings in the main organs/tissues in the thoracic or abdominal cavity in any group (Table 4).

Effects on Embryo/Fetal Development

Results of cesarean section are shown in Table 5. There were no significant differences between the control group and any test substance administration group in the number of corpora lutea, number of implantations, implantation index, index of dead embryos/fetuses, or number of live fetuses. There were no significant differences between the control group and any test substance administration group in the sex ratio or body weight of live fetuses. For external abnormalities, short trunk with vestigial tail was observed only in 1 fetus in the 5.0 mL/kg group. There were no macroscopic abnormalities in the placentae of live fetuses and placental weight was comparable between the control group and each test substance administration group. Results of visceral examination of live fetuses are shown in Table 6. The number of live fetuses with visceral abnormalities was 5 in 4 litters (3.8 ± 8.0 %) in the control group, 1 in 1 litter (0.6 ± 2.8 %) in the 1.25 mL/kg group, 7 in 7 litters (5.4 ± 7.7 %) in the 2.5 mL/kg group and 2 in 2 litters (1.5 ± 4.5 %) in the 5.0 mL/kg group. The abnormalities observed were dilatation of

lateral ventricle, abnormal origin of left pulmonary artery, ventricular septal defect, and abnormal lobulation in the liver; however, their incidences were comparable between the historical control data in the testing facility (3.1 ± 7.1 %) and the control data in this study, and also between the control group and each test substance administration group. The number of live fetuses with visceral variations was 7 in 6 litters (5.7 ± 9.7 %) in the control group, 12 in 8 litters (8.5 ± 12.6 %) in the 1.25 mL/kg group, 7 in 6 litters (4.8 ± 8.2 %) in the 2.5 mL/kg group and 8 in 8 litters (6.0 ± 7.7 %) in the 5.0 mL/kg group. The variations observed included thymic remnant in neck, dilatation of renal pelvis, and dilatation and convolution of the ureter. However, incidences of these variations were comparable between the historical control data in the testing facility (7.1 ± 11.2 %) and the control data in this study, and also between the control group and each test substance administration group. Results of skeletal examination of live fetuses are shown in Table 7. As a skeletal abnormality, only wavy rib was observed in 1 fetus in 1 dam (0.6 ± 2.8 %) in the 1.25 mL/kg group. This incidences was comparable with the historical control data in the testing facility (0.2 ± 2.4 %). As for ossification, there were no significant differences between the control group and any test substance administration group in the index of ossified sternebrae, or the number of ossified metacarpi, metatarsi or sacrococcygeal vertebrae.

Teratology Study in Rabbits

Effects on Dams

The number of non-pregnant females was 2 to 7 in each group. No deaths occurred in any group. Abortion was observed in 1 animal in the 0.25 mL/kg group. A decrease in the amount of feces was observed in each group, and the incidence was 3 in the control group on days 14-19, 20 or 28 of gestation, 3 in the 0.25 mL/kg group on days 1, 15-17 or 26-28 of gestation, 1 in the 0.5 mL/kg group on day 14 of gestation, and 4 in the 1 mL/kg group on days 9, 10 or 15-19 of

gestation; however, there were no differences between the control group and any test substance administration group. The female that aborted only showed a decrease in the amount of feces on day 1 of gestation. Results of body weight are shown in Table 8. There were no significant differences between the control group and any test substance administration group in body weight on any day of measurement or body weight gain for any period. For food consumption, there were no significant differences between the control group and any test substance administration group (Table 9). In necropsy, there were no gross pathological abnormalities in any animal in any group (Table 10). Results of cesarean section are shown in Table 11. There were no significant differences between the control group and any test substance administration group in the number of corpora lutea, number or index of implantations, or number or index of embryo-fetal deaths.

Effects on Embryo/Fetal Development

Results of cesarean section are shown in Table 11. There were no significant differences between the control group and any test substance administration group in the number of male or female live fetuses, sex ratio, or body weights of male or female live fetuses. There were neither gross pathological abnormalities in the placentae in any group nor significant differences in placental weight between the control group and any test substance administration group. External abnormalities of the fetuses which were observed included meningocele or sirenomelia in the forelimb each in 1 fetus in the control group, cleft of abdominal wall in 1 fetus in the 0.5 mL/kg group and club foot in 1 fetus in the 1 mL/kg group. However, there was no significant difference in the incidence of any change between the control group and any test substance administration group. Results of visceral examination of live fetuses are shown in Table 12. The number of live fetuses with visceral abnormalities was 3 in 2 litters (2.0 ± 6.3 %) in the control

group, 6 in 6 litters (3.9 ± 7.0 %) in the 0.25 mL/kg group, 6 in 3 litters (3.2 ± 8.9 %) in the 0.5 mL/kg group and 2 in 2 litters (1.3 ± 3.8 %) in the 1 mL/kg group. There were no significant difference in the incidence of these changes between the historical control data in the testing facility (2.4 ± 2.2 %) and the control data in this study, and also between the control group and any test substance administration group. The number of live fetuses with visceral variations was 46 in 15 litters in the control group, 77 in 19 litters in the 0.25 mL/kg group, 63 in 16 litters in the 0.5 mL/kg group and 60 in 16 litters in the 1 mL/kg group. There were no significant difference in the incidence of these changes between the control group and any test substance administration group. For each variation observed, the incidence of thymic remnant in neck was significantly higher in the 1 mL/kg group than in the control group. Results of skeletal examination of live fetuses are shown in Table 13. The number of live fetuses with skeletal abnormalities was 5 in 4 litters (3.6 ± 7.8 %) in the control group, 1 in 1 litter (0.5 ± 2.4 %) in the 0.25 mL/kg group, 1 in 1 litter (0.7 ± 3.1 %) in the 0.5 mL/kg group and 4 in 4 litters (3.0 ± 5.6 %) in the 1 mL/kg group. There were no significant difference in the incidence of these changes between the historical control data in the testing facility (2.7 ± 1.7 %) and the control data in this study, and also the control group and any test substance administration group. Regarding the progress of skeletal ossification, there were no significant differences between the control group and any test substance administration group.

DISCUSSION

Teratology studies of capsinoids-containing CH-19 Sweet extract were conducted in pregnant Sprague-Dawley rats and pregnant New Zealand White rabbits. The concentration of capsinoids and dihydrocapsiate in the lots of CH-19 Sweet extract used in rats and rabbits was 72.2-75.05 mg/mL, respectively, which was equivalent with 16.2 mg/mL and 15.2 mg/mL of

dihydrocapsiate , respectively. The test substance was administered orally for 11 days from days 7 to 17 of gestation in rats at dose levels of 0 (vehicle), 1.25, 2.5 or 5.0 mL/kg (the equivalent of 20.l9, 40.38, 80.75 mg/kg dihydrocapsinate, respectively) and for 13 days from days 6 to 18 of gestation in rabbits at dose levels of 0 (vehicle), 0.25, 0.5 or 1.0 mL/kg (the equivalent of 3.8, 7.6, and 15.2 mg/kg dihydrocapsinate, respectively).

There were neither deaths nor dams with premature delivery/abortion in any group. Salivation was observed in the 5.0 mL/kg group on day 17 of gestation; however, since it was observed transiently only in 2 animals, it was judged to be of no toxicological significance. Body weight gain after the end of the administration period was significantly higher in the 2.5 mL/kg group than in the control group, and food consumption from days 14 to 18 of gestation was greater (sometimes achieving statistical significance) in each test substance administration group than in the control group. However, since the degree of changes in body weight gain or in food consumption was not dose-related, and the increase in food consumption was transient, these changes were judged to be incidental. Necropsy revealed no abnormalities in any group. In the fetal development/growth, there were no test substance-related effects on the number of corpora lutea, number of implantations, implantation index, index of embryo/fetal deaths, number of live fetuses, body weight or sex ratio. There were no test substance-related gross or macroscopic findings or variations in the external differentiation, visceral organs, skeleton or placenta of live fetuses, and there were no test substance-related effects on the number of ossified metacarpi, metatarsi or sacrococcygeal vertebrae, or percentage of ossified sternebrae which are the indices for ossification in live fetuses.

In rabbits, there were no test substance-related effects on clinical signs, body weight, food consumption or necropsy findings in any group for dams. For effects on the reproductive

functions of dams, abortion was observed in 1 dam in the 0.25 mL/kg, but inhibition of implantation was not observed and there were no test substance-related effects on the maintenance of pregnancy or embryo-fetal viability. For embryo-fetal development, there were no differences in the number of live fetuses, body weights of live fetuses or sex ratio between groups, and, thus, there were no test substance-related embryo-fetal deaths or growth inhibition. In the morphological observation of fetuses, there were no findings in the external, visceral or skeletal abnormalities which suggested teratogenicity of the test substance. However, the incidence of thymic remnant in neck, a visceral variation, and the incidence of unossified talus, a skeletal variation, were significantly higher in the 1 mL/kg group than in the control group (thymic remnant in neck: 24.1%; unossified talus: 3.5%), and they were slightly higher than the range of background data of the test facility (In the years from 2001 to 2004; No. of studies: 15; No. of litters: 271; lower limit - upper limit / Mean ± S.D., thymic remnant in neck: 4.8-19.8%/13.4±4.3; unossified talus: 0-2.3%/0.42±0.67). Of these variations, thymic remnant in neck is thought to be a change suggesting growth inhibition rather than teratogenicity, and unossified talus is related to the progress of ossification. It is said that the indices for evaluation of growth of fetuses are related to morphological development of external differentiation and viscera and progress of ossification. However, in this study, since there were no changes suggestive of growth inhibition except the aforementioned two changes, the increases in the numbers of visceral and skeletal variations were thought to be incidental and not test substance-related.

Chanda et al. (2006) reported that trans-capsaicin administered to pregnant rats and rabbits via skin induced no gross external, soft tissue, or skeletal fetal alterations (malformations or variations) except for delay in skeletal ossification in rat fetuses. On the other hand, this study

is the first reported teratology study for capsinoids.. However, in general, the results reported herein suggest the capsinoids, in the form of CH-19 Sweet extract, are relatively non-toxic when it comes to teratogenicity (or general toxicity). This is consistent with the findings of Watanabe et al. (2008) who reported low single dose acute toxicity in rats limited to transient salivation or decreased spontaneous movement immediately following gavage administration $\geq$10 mL/kg BW and an LD_{01} >20 mL/kg as CH-19 Sweet extract (>1425 mg/kg or >285 mg/kg as capsinoids or dihydrocapsiate, respectively).

A two-generation reproduction study performed in rats using CH-19 Sweet extract and a high dose of 5 mL/kg (equivalent to approximately 354 mg/kg/day capsinoids or 70 mg/kg/day dihydrocapsinate) reported no reproductive or general toxicity effects (Kodama et al. 2008b).

Traurig (1984) reported the effects of neonatal capsaicin treatment on growth and subsequent reproductive function in the rat. Capsaicin-treated rats had normal estrous cycles, but mated significantly less frequently than age-matched controls. Confirmed matings in capsaicin-treated female rats resulted in significantly fewer pregnancies compared to controls. Male rats treated with capsaicin as neonates produced significantly fewer pregnancies when mated with untreated females compared to controls. Ovulation, sperm transport, and fertilization occur normally in capsaicin-treated rats. Traurig (1988) suggested that adult female rats, treated with capsaicin as neonates, exhibit decreased fertility following mating and diminished sensitivity to the induction of pseudopregnancy following copulomimetic electrical stimulation of the cervix. However, reproductive toxicity of capsinoids was not detected. What relevance if any, the Traurig findings have for food or medicinal use, given route and timing of test article administration in these studies, remains to be seen.

General toxicity was not observed until Kodama et al. (2008a) administered 5.0 mL/kg/day of CH-19 Sweet extract (equivalent to a dose of approximately 360 mg/kg/day capsinoids or 73 mg/kg/day dihydrocapsinate to rats. Even here, the toxicity was not severe and was limited to the liver.

CH-19 Sweet extract has been reported to be non-genotoxic having exhibited negative results in both *in vitro* and *in vivo* tests (Watanabe et al. 2008). The testing included a bacterial reverse mutation test performed employing *S. typhimurium* and *E. coli,* in the presence (+S9) or absence (-S9) of metabolic activation. Similarly, negative findings were observed in an *in vitro* chromosome aberration test conducted using Chinese hamster lung cultured cells wherein treatment with CH-19 Sweet extract failed to induce chromosome aberrations in either short-term or continuous treatment scenarios, with or without metabolic activation (-S9 +S9). Finally, in an *in vivo* micronucleus test using BDF_1 male mice, CH-19 Sweet extract failed to increase the incidence of micronucleated polychromatic erythrocytes (MNPCE) or decrease the ratio of polychromatic erythrocytes (PCE) in any of the treatment groups. The general findings that the capsinoids have low potential toxicity and the liver is its target organ are consistent with what is known regarding the toxicity of the capsaicinoids. Monsereenusorn (1983) reported that rats given 50 mg/kg bw of capsaicin or 500 mg/kg bw of a crude aqueous extract of *Capsicum annuum* L. by gavage for 60 days were observed to have signs of general toxicity and hepatotoxicity in particular. Akagi et al. (1998) conducted a feeding study using capsaicinoids in mice for 13 weeks (at 0, 93.75, 187.5, 375, 750 and 1500 mg/kg bw). General toxicity was observed and in particular, liver toxicity with a tendency for males to be more affected than females.

CONCLUSION

In order to evaluate the safety of CH-19 Sweet extract which contains capsinoids, teratology studies were conducted in pregnant Sprague-Dawley rats (20 rats per group) and pregnant New Zealand White rabbits (17 to 22 animals per group). The test substance was administered to rats by gavage for 11 days on gestation days 7-17 at doses of 0 (vehicle), 1.25, 2.5 and 5.0 mL/kg and to rabbits for 13 days on gestation days 6-18 at doses of 0 (vehicle), 0.25, 0.5 and 1.0 mL/kg.

In the study, as described above, the NOAEL for CH-19 Sweet extract containing capsinoids on pregnant rats and fetal development/growth was 5.0 mL/kg/day (361 mg/kg/day and 80.75 mg/kg/day as capsinoids and dihydrocapsiate, respectively), the highest dose tested.

In the study, as described above, the NOAEL for CH-19 Sweet extract containing capsinoids on pregnant rabbits and fetal development/growth was 1.0 mL/kg/day (75.05 mg/kg/day and 15.2 mg/kg/day as capsinoids and dihydrocapsiate, respectively), the highest dose tested.

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FIGURE 1

Chemical structure of capsaicin

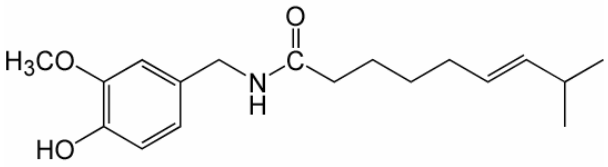

FIGURE 2

Chemical structure of capsiate

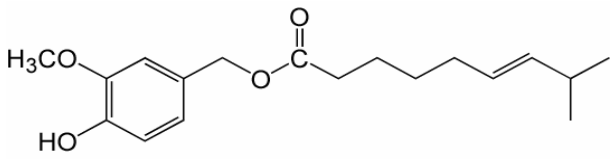

FIGURE 3

Chemical structure of dihydrocapsiate

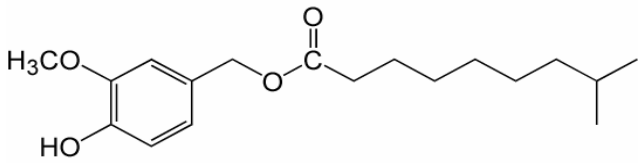

FIGURE 4

Chemical structure of nordihydrocapsiate

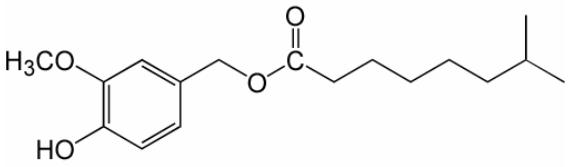

TABLE 1

Total and individual capsinoids concentrations in CH-19 Sweet extract

		Lot No. 050404	Lot No. 050530
Content (mg/mL)	Capsiate	53.2	50.35
	Dihydrocapsiate	15.2	16.15
	Nordihydrocapsiate	6.65	5.7
	Total of capsinoids	75.05	72.2

TABLE 2

Body weights of pregnant rats dosed with CH-19 Sweet extract during gestation[a]

Dose (mL/kg/day)	Statistic	Gestation days																Gain		
		0	4	7	8	9	10	11	12	13	14	15	16	17	18	19	20	7-18	18-20	
0	Mean	255.8	284.8	301.7	302.3	309.6	316.7	321.3	327.8	332.4	339.4	345.2	354.4	367.2	384.1	402.3	419.0	82.4	34.9	
	S.D.	10.1	11.9	12.2	13.5	13.0	14.6	14.8	15.2	15.5	15.6	19.0	18.5	19.6	20.8	22.1	21.8	12.0	5.7	
1.25	Mean	257.5	288.0	304.9	306.1	313.8	319.8	327.0	332.9	338.9	345.7	352.6	364.2	377.8	393.6	411.8	430.6	88.7	37.0	
	S.D.	9.9	12.2	15.3	17.2	16.9	17.6	18.8	19.3	19.6	20.0	20.6	22.5	23.3	23.6	26.3	29.3	14.7	7.4	
2.5	Mean	259.3	286.0	303.3	306.0	311.9	317.4	324.4	329.7	334.2	342.1	349.1	361.5	375.8	391.5	411.8	432.8	88.2	41.3	**
	S.D.	15.0	15.9	18.0	18.0	19.2	19.5	19.1	19.3	20.3	21.9	22.6	22.2	23.9	25.0	28.2	30.7	11.1	6.7	
5.0	Mean	259.3	287.9	303.8	305.0	312.3	319.4	324.7	331.6	336.5	343.0	350.7	361.9	375.3	391.1	410.6	482.2	87.4	37.1	
	S.D.	13.3	13.7	16.5	17.3	17.3	18.3	18.6	19.1	20.1	20.2	21.9	23.0	23.9	24.3	25.5	27.6	12.2	6.0	

[a]Data expressed as mean (g) and standard deviation (*S.D.*): N=20 rats/group.
**p<0.01 (significantly different from control group; Dunnett's test).

TABLE 3

Food consumption by pregnant rats dosed with CH-19 Sweet extract during gestation[a]

Dose (mL/kg/day)	Statistic	Gestation days 0-4	4-7	7-10	10-14	14-18		18-20
0	Mean	25.1	27.1	23.7	24.6	22.9		29.7
	S.D.	1.7	2.2	2.3	2.3	6.4		2.0
1.25	Mean	25.3	27.2	24.2	25.4	27.0	*	30.9
	S.D.	2.0	2.3	3.2	3.3	2.6		3.0
2.5	Mean	24.8	27.8	24.1	25.1	26.9	*	31.3
	S.D.	4.9	2.2	2.3	1.8	2.4		2.8
5.0	Mean	25.2	27.2	24.1	24.6	26.4		30.9
	S.D.	2.1	2.4	3.1	2.8	3.3		3.0

[a]Data expressed as mean (g) and standard deviation (*S.D.*): N=20 rats/group.
*$p<0.05$ (significantly different from control group; Dunnett-type rank test).

TABLE 4

Macroscopic observations in pregnant rats dosed with CH-19 Sweet extract[a]

	Dose (mL/kg/day)			
Finding	0	1.25	2.5	5.0
No. of dams examined	20	20	20	20
No. of dams with abnormal findings	0	0	0	0

[a]Data are expressed as frequency of observation.

TABLE 5

Intrauterine parameters examined at cesarean section on gestation day 20 of pregnant rats dosed with CH-19 Sweet extract

	0 mL/kg/day	1.25 mL/kg/day	2.5 mL/kg/day	5.0 mL/kg/day
No. of pregnant animals examined				
Total	20	20	20	20
No. of corpora lutea				
Total	305	323	320	307
Mean±*SD*	15.3±1.9	16.2±2.1	16.0±1.8	15.4±1.8
No. of implantations				
Total	293	306	309	295
Mean±*SD*	14.7±1.7	15.3±1.8	15.5±1.7	14.8±2.4
Implantation index				
%, Mean±*SD*	96.3±5.7	95.0±4.9	96.8±5.3	95.5±7.3
No. of dead embryo or fetuses				
Total	19	16	13	15
%, Mean±*SD*	6.5±7.3	5.8±9.1	4.2±4.8	5.2±5.4
Dead fetuses				
Total	0	0	0	0
Macerated fetuses				
Total	1	0	1	0
Resorbed fetus				
Total	18	16	12	15
No. of live fetuses				
Total	274	290	296	280
Mean±*SD*	13.7±1.9	14.5±2.4	14.8±1.8	14.0±2.3

[a]Number of males / number of liveborn pups.
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 5 (continued)

	0 mL/kg/day	1.25 mL/kg/day	2.5 mL/kg/day	5.0 mL/kg/day
Sex ratio				
Male:Female	134:140	158:132	150:146	147:133
Male ratio [a]	0.49	0.54	0.51	0.53
Fetal body weight (g)				
Male (Mean±*SD*)	3.95±0.18	3.91±0.24	3.98±0.25	3.98±0.22
Female (Mean±*SD*)	3.75±0.22	3.75±0.20	3.78±0.25	3.76±0.18
No. of fetuses with external abnormalities				
Total	0	0	0	1
%, Mean±*SD*	0.0±0.0	0.0±0.0	0.0±0.0	0.3±1.4
Placental weight (g)				
Male (Mean±*SD*)	0.48±0.05	0.48±0.06	0.48±0.06	0.51±0.05
Female (Mean±*SD*)	0.46±0.06	0.47±0.07	0.46±0.06	0.48±0.04
Gross evaluation of placenta				
No. of dams with abnormal findings	0	0	0	0

[a]Number of males / number of liveborn pups.
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 6

Observations made during visceral examinations of fetuses in rats delivered by cesarean section from dams dosed with CH-19 Sweet extract[a]

	0 mL/kg/day	1.25 mL/kg/day	2.5 mL/kg/day	5.0 mL/kg/day
No. of dams	20	20	20	20
No. of fetuses				
Total	132	141	140	134
No. of dams with abnormality				
Total	4	1	7	2
No. of fetuses with abnormality				
Total	5	1	7	2
(%, Mean±*SD*)	(3.8±8.0)	(0.6± 2.8)	(5.4±7.7)	(1.5±4.5)
Dilatation of lateral ventricle				
Total	1	0	0	0
(%, Mean±*SD*)	(1.0±4.5)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Abnormal origin of left pulmonary artery				
Total	1	0	1	0
(%, Mean±*SD*)	(0.8±3.7)	(0.0±0.0)	(0.6±2.8)	(0.0±0.0)
Ventricular septal defect				
Total	0	0	1	1
(%, Mean±*SD*)	(0.0±0.0)	(0.0±0.0)	(0.8±3.7)	(0.6±2.8)
Abnormal lobulation of liver				
Total	3	1	5	1
(%, Mean±*SD*)	(2.0±6.3)	(0.6±2.8)	(4.0±7.1)	(0.8±3.7)
No. of fetuses with variation				
Total	7	12	7	8
(%, Mean±*SD*)	(5.7±9.7)	(8.5±12.6)	(4.8±8.2)	(6.0±7.7)
Thymic remnant in neck				
Total	5	6	5	5
(%, Mean±*SD*)	(4.1±9.2)	(4.2±8.1)	(3.4±7.7)	(3.8±6.9)
Dilatation of renal pelvis				
Total	0	4	2	1
(%, Mean±*SD*)	(0.0±0.0)	(2.9±5.9)	(1.3±4.1)	(0.7±3.2)
Dilatation of ureter				
Total	0	0	0	1
(%, Mean±*SD*)	(0.0 ± 0.0)	(0.0±0.0)	(0.0±0.0)	(0.6±2.8)
Convoluted ureter				
Total	2	4	1	1
(%, Mean±*SD*)	(1.6±4.8)	(2.9±7.5)	(0.7±3.2)	(0.8±3.7)

[a]Data expressed as mean±*SD* (percentage).
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 7

Observations made during skeletal examinations of fetuses in rats delivered by cesarean section from dams dosed with CH-19 Sweet extract[a]

	0 mL/kg/day	1.25 mL/kg/day	2.5 mL/kg/day	5.0 mL/kg/day
No. of dams	20	20	20	20
No. of fetuses				
Total	142	149	156	145
No. of fetuses with abnormality				
Total	0	1	0	0
(%, Mean±*SD*)	(0.0±0.0)	(0.6±2.8)	(0.0±0.0)	(0.0±0.0)
Wavy ribs				
Total	0	1	0	0
(%, Mean±*SD*)	(0.0±0.0)	(0.6±2.8)	(0.0±0.0)	(0.0±0.0)
Variations				
Cervical rib				
Total	3	0	1	2
(%, Mean±*SD*)	(2.1±5.0)	(0.0±0.0)	(0.6±2.8)	(1.3±4.1)
14th rib				
Total	26	16	21	27
(%, Mean±*SD*)	(17.8±18.3)	(10.6±18.5)	(14.1±19.5)	(19.8±19.8)
Splitting of thoracic vertebral body				
Total	0	4	2	2
(%, Mean±*SD*)	(0.0±0.0)	(3.8±8.6)	(1.2±3.6)	(1.3±3.8)
Splitting of sternebra				
Total	1	0	0	0
(%, Mean±*SD*)	(0.7±3.2)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Lumbarization of sacral vertebra				
Total	1	2	1	0
(%, Mean±*SD*)	(0.7±3.2)	(1.4±4.4)	(0.7±3.2)	(0.0±0.0)

[a]Data expressed as mean±*SD* (percentage).
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 7 (continued)

	0 mL/kg/day	1.25 mL/kg/day	2.5 mL/kg/day	5.0 mL/kg/day
Progress of ossification				
No. of ossified sternebrae				
1st [Total (%, Mean±*SD*)]	142 (100.0±0.0)	149 (100.0±0.0)	156 (100.0±0.0)	145 (100.0±0.0)
2nd [Total (%, Mean±*SD*)]	142 (100.0±0.0)	149 (100.0±0.0)	156 (100.0±0.0)	145 (100.0±0.0)
3rd [Total (%, Mean±*SD*)]	142 (100.0±0.0)	149 (100.0±0.0)	156 (100.0±0.0)	145 (100.0±0.0)
4th [Total (%, Mean±*SD*)]	142 (100.0±0.0)	149 (100.0±0.0)	156 (100.0±0.0)	145 (100.0±0.0)
5th [Total (%, Mean±*SD*)]	126 (88.4±16.2)	135 (90.1±10.6)	140 (90.0±13.2)	135 (93.6±6.6)
6th [Total (%, Mean±*SD*)]	140 (98.3±7.4)	149 (100.0±0.0)	156 (100.0±0.0)	143 (98.7±4.1)
No. of ossified metacarpi				
Right (Mean±*SD*)	4.00±0.00	4.00± 0.00	4.00±0.00	4.00±0.02
Left (Mean±*SD*)	4.00±0.00	4.00± 0.00	4.00±0.00	3.99±0.03
No. of ossified metatarsi				
Right (Mean±*SD*)	4.83±0.20	4.71± 0.31	4.85±0.20	4.78±0.29
Left (Mean±*SD*)	4.84±0.17	4.71± 0.31	4.85±0.25	4.79±0.27
No. of ossified sacrococcsgeal vertebrae				
Mean±*SD*	8.69±0.36	8.71± 0.31	8.83±0.42	8.76±0.43

[a]Data expressed as mean±*SD* (percentage).
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 8

Body weights of pregnant rabbits dosed with CH-19 Sweet extract during gestation[a]

Dose (mL/kg/day)	Statistic[b]	Gestation days														Gain	
		0	3	6	8	10	12	14	16	18	19	22	24	26	28	6-19	19-28
0	Mean	3.31	3.42	3.48	3.50	3.53	3.55	3.57	3.62	3.63	3.64	3.69	3.71	3.75	3.79	0.16	0.15
	S.D.	0.16	0.16	0.17	0.16	0.16	0.17	0.17	0.17	0.20	0.20	0.20	0.18	0.18	0.21	0.14	0.08
0.25	Mean	3.29	3.42	3.47	3.48	3.51	3.53	3.57	3.59	3.62	3.62	3.68	3.70	3.74	3.77	0.15	0.14
	S.D.	0.16	0.18	0.18	0.17	0.17	0.18	0.17	0.16	0.17	0.17	0.19	0.18	0.19	0.20	0.08	0.09
0.5	Mean	3.30	3.38	3.44	3.46	3.49	3.51	3.54	3.58	3.60	3.62	3.67	3.70	3.72	3.77	0.18	0.15
	S.D.	0.15	0.17	0.17	0.16	0.17	0.17	0.16	0.17	0.17	0.17	0.18	0.17	0.17	0.17	0.06	0.07
1	Mean	3.31	3.43	3.48	3.48	3.50	3.53	3.56	3.60	3.62	3.61	3.66	3.69	3.74	3.78	0.13	0.17
	S.D.	0.13	0.13	0.12	0.11	0.12	0.13	0.14	0.15	0.14	0.16	0.14	0.13	0.14	0.13	0.12	0.10

[a]Data expressed as mean (kg) and standard deviation (*S.D.*): N = 17 to 22 rabbits/group
[b]No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 9

Food consumption by pregnant rabbits dosed with CH-19 Sweet extract during gestation[a]

Dose (mL/kg/day)	Statistic[b]	Gestation days													
		1	3	6	8	10	12	14	16	18	19	22	24	26	28
0	Mean	166	172	174	159	157	152	130	132	139	139	138	126	122	125
	S.D.	30	22	23	25	19	23	35	39	40	37	23	27	25	32
0.25	Mean	162	174	173	160	158	146	124	130	138	140	140	121	118	115
	S.D.	42	21	23	22	22	20	23	32	30	26	29	22	37	32
0.5	Mean	143	167	165	158	143	142	122	130	145	139	138	119	109	125
	S.D.	32	28	24	28	32	18	27	37	28	23	23	22	24	12
1	Mean	147	165	165	150	140	142	129	132	136	130	130	120	122	125
	S.D.	27	14	11	15	40	27	19	41	40	38	26	29	23	19

[a]Data expressed as mean (g) and standard deviation (*S.D.*): N=17 to 22 rabbits/group
[b]No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 10

Macroscopic observations in pregnant rabbits dosed with CH-19 Sweet extract[a]

Finding	Dose (mL/kg/day)			
	0	0.25	0.5	1
No. of dams examined	19	22	21	17
No. of dams with abnormal findings	0	0	0	0

[a]Data are expressed as frequency of observation

TABLE 11

Intrauterine parameters examined at cesarean section on gestation day 28
of pregnant rabbits dosed with CH-19 Sweet extract

	0 mL/kg/day	0.25 mL/kg/day	0.5 mL/kg/day	1 mL/kg/day
No. of pregnant animals examined				
Total	19	21	21	17
No. of corpora lutea				
Total	190	212	196	182
Mean±*SD*	10.0±1.6	10.1±1.7	9.3±2.0	10.7±2.6
No. of implantations				
Total	156	187	177	153
Mean±*SD*	8.2±2.8	8.9±2.1	8.4±2.3	9.0±2.8
Implantation index				
%, Mean±*SD*	83.1±25.0	88.1±15.1	91.4±17.3	84.5±18.8
No. of dead embryo or fetuses				
Total	21	13	10	16
%, Mean±*SD*	11.2±13.7	6.4±8.3	5.7±7.9	10.8±11.7
Macerated fetuses and dead fetuses				
Total	11	10	7	8
Resorbed embryo and placental remnant				
Total	10	3	3	8
No. of live fetuses				
Total	135	174	167	137
Mean±*SD*	7.1±2.4	8.3±2.0	8.0±2.3	8.1±2.7
Sex ratio				
Male:Female	66:69	80:94	82:85	77:60
Male ratio (%)[a]	0.47	0.47	0.48	0.55
Fetal body weight (g)				
Male (Mean±*SD*)	35.77±4.44	34.70±4.84	35.37±3.19	32.70±5.07
Female (Mean±*SD*)	34.00±3.65	33.69±4.29	33.06±3.17	32.60±6.06
No. of fetuses with external abnormalities				
Total	2[b]	0	1[c]	1[d]
%, Mean±*SD*	1.6±4.9	0.0±0.0	0.5±2.2	0.0±0.0
Placental weight (g)				
Mean±*SD*	3.31±0.54	3.19±0.60	3.33±0.47	3.02±0.61
Gross evaluation of placenta				
No. of dams with abnormal findings	0	0	0	0

[a]Number of males / number of liveborn pups.
[b]Meningocele and sirenomelia, each 1 fetus
[c]Gastroschisis
[d]Club foot
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 12

Observations made during visceral examinations of fetuses in rabbits delivered by cesarean section from dams dosed with CH-19 Sweet extract[a]

	0 mL/kg/day	0.25 mL/kg/day	0.5 mL/kg/day	1 mL/kg/day
No. of dams	19	21	21	17
No. of fetuses				
Total	133	174	166	136
No. of dams with abnormality				
Total	2	6	3	2
No. of fetuses with abnormality				
Total	3	6	6	2
(%, Mean±*SD*)	(2.0±6.3)	(3.9±7.0)	(3.2±8.9)	(1.3±3.8)
Abnormal lung lobation (absent of accessory lobe)				
Total	2	0	4	2
(%, Mean±*SD*)	(1.3±3.9)	(0.0±0.0)	(2.0±7.4)	(1.3±3.8)
Ventricular septal defect (membranous)				
Total	0	1	0	0
(%, Mean±*SD*)	(0.0±0.0)	(0.5±2.4)	(0.0±0.0)	(0.0±0.0)
Right ventricle muscle defect				
Total	0	1	0	0
(%, Mean±*SD*)	(0.0±0.0)	(0.7±3.1)	(0.0±0.0)	(0.0±0.0)
Atrioventricular valve right defect				
Total	0	2	0	0
(%, Mean±*SD*)	(0.0±0.0)	(1.9±6.1)	(0.0±0.0)	(0.0±0.0)
Cor triloculare (atrioventricular aplasia and small left ventricular)				
Total	0	1	0	0
(%, Mean±*SD*)	(0.0±0.0)	(0.5±2.4)	(0.0±0.0)	(0.0±0.0)
Levocardia				
Total	1	0	0	0
(%, Mean±*SD*)	(0.7±2.9)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Narrowed aorta				
Total	0	1	0	0
(%, Mean±*SD*)	(0.0±0.0)	(0.5±2.2)	(0.0±0.0)	(0.0±0.0)
Malposition of right subclavian artery				
Total	1	0	0	0
(%, Mean±*SD*)	(0.7±2.9)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Abnormal liver lobation				
Total	0	1	0	0
(%, Mean±*SD*)	(0.0±0.0)	(0.5±2.4)	(0.0±0.0)	(0.0±0.0)
Absent of gallbladder				
Total	0	0	2	0
(%, Mean±*SD*)	(0.0±0.0)	(0.0±0.0)	(1.2±5.5)	(0.0±0.0)

[a]Data expressed as mean±*SD* (percentage).
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 12 (continued)

Observations made during visceral examinations of fetuses in rabbits delivered by cesarean section from dams dosed with CH-19 Sweet extract[a]

	0 mL/kg/day	0.25 mL/kg/day	0.5 mL/kg/day	1 mL/kg/day
No. of dams	19	21	21	17
No. of fetuses				
Total	133	174	166	136
No. of fetuses with variation				
Total	46	77	63	60
(%, Mean±*SD*)	(33.1±23.1)	(43.0±27.3)	(36.8±29.7)	(46.1±25.0)
Thymic remnant in neck				
Total	11	34	28	32
(%, Mean±*SD*)	(7.4±18.5)	(18.5±22.4)	(17.2±24.2)	(24.1±21.4)*
Supernumerary right coronary orifice				
Total	34	46	43	38
(%, Mean±*SD*)	(25.0±19.8)	(25.8±19.6)	(24.5±21.6)	(28.9±19.9)
Retrocaval ureter (right)				
Total	2	10	2	2
(%, Mean±*SD*)	(1.4±4.2)	(5.7±13.2)	(0.9±2.8)	(2.7±8.4)

[a]Data expressed as mean±*SD* (percentage).
*p<0.05; **p<0.01 (significantly different from control group; Dunnett-type rank test)

TABLE 13

Observations made during skeletal examinations of fetuses in rabbits delivered by cesarean section from dams dosed with CH-19 Sweet extract[a]

	0 mL/kg/day	0.25 mL/kg/day	0.5 mL/kg/day	1 mL/kg/day
No. of dams	19	21	21	17
No. of fetuses				
Total	133	174	166	136
No. of dams with abnormality				
Total	4	1	1	4
No. of fetuses with abnormality				
Total	5	1	1	4
(%, Mean±*SD*)	(3.6±7.8)	(0.5±2.4)	(0.7±3.1)	(3.0±5.6)
Malposition of cervical vertebral body (with splitting)				
Total	1	0	0	0
(%, Mean±*SD*)	(0.7±2.9)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Misaligned cervical vertebral body				
Total	1	0	0	0
(%, Mean±*SD*)	(0.7±2.9)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Misshapen cervical vertebral body (small)				
Total	1	0	0	0
(%, Mean±*SD*)	(0.7±2.9)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Absent of rib				
Total	1	0	0	0
(%, Mean±*SD*)	(0.8±3.3)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Branched rib				
Total	1	0	0	1
(%, Mean±*SD*)	(0.8±3.3)	(0.0±0.0)	(0.0±0.0)	(0.8±3.5)
Fusion of rib				
Total	0	0	0	1
(%, Mean±*SD*)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)	(0.8±3.5)
Malposition of thoracic vertebral body				
Total	2	0	0	1
(%, Mean±*SD*)	(1.5±4.5)	(0.0±0.0)	(0.0±0.0)	(0.8±3.5)
Absence of thoracic vertebral body				
Total	0	0	0	1
(%, Mean±*SD*)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)	(0.8±3.5)
Misaligned thoracic vertebral body				
Total	1	0	0	1
(%, Mean±*SD*)	(0.8±3.3)	(0.0±0.0)	(0.0±0.0)	(0.8±3.5)
Misshapen thoracic vertebral body				
Total	0	0	0	1
(%, Mean±*SD*)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)	(0.8±3.5)
Misshapen thoracic vertebral body (small)				
Total	2	0	0	0
(%, Mean±*SD*)	(1.5±4.5)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Fusion of thoracic vertebral body				
Total	1	0	0	0
(%, Mean±*SD*)	(0.8±3.3)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Thoracic hemivertebra				
Total	0	0	0	1
(%, Mean±*SD*)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)	(0.8±3.5)

[a]Data expressed as mean±*SD* (percentage).
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 13 (continued)

Observations made during skeletal examinations of fetuses in rabbits delivered by cesarean section from dams dosed with CH-19 Sweet extract[a]

	0 mL/kg/day	0.25 mL/kg/day	0.5 mL/kg/day	1 mL/kg/day
No. of dams	19	21	21	17
No. of fetuses				
Total	133	174	166	136
No. of fetuses with abnormality				
Total	5	1	1	4
(%, Mean±*SD*)	(3.6±7.8)	(0.5±2.4)	(0.7±3.1)	(3.0±5.6)
Lumbar hemivertebra				
Total	0	0	0	1
(%, Mean±*SD*)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)	(0.8±3.5)
Fusion of sternebra				
Total	2	1	1	2
(%, Mean±*SD*)	(1.4±4.2)	(0.5±2.4)	(0.7±3.1)	(1.3±3.6)
Variations				
Unossified cervical centrum				
Total	0	0	0	2
(%, Mean±*SD*)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)	(1.0±2.8)
Cervical rib				
Total	1	2	0	0
(%, Mean±*SD*)	(0.7±2.9)	(0.7±3.4)	(0.0±0.0)	(0.0±0.0)
Supernumerary sternebra				
Total	1	0	0	0
(%, Mean±*SD*)	(0.6±2.5)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Splitting of sternebra				
Total	2	4	2	4
(%, Mean±*SD*)	(1.5±4.9)	(2.6±8.1)	(1.4±4.8)	(3.3±6.2)
Dissymmetric sternebra				
Total	1	0	0	0
(%, Mean±*SD*)	(0.8±3.3)	(0.0±0.0)	(0.0±0.0)	(0.0±0.0)
Unossified talus				
Total	0	2	3	6
(%, Mean±*SD*)	(0.0±0.0)	(1.4±6.2)	(1.4±4.5)	(3.5±6.1)*
No. of fetuses with 13th rib				
Total	90	124	120	112
(%, Mean±*SD*)	(66.2±35.9)	(73.0±27.0)	(73.1±29.8)	(81.3±22.8)

[a]Data expressed as mean±*SD* (percentage).
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.

TABLE 13 (continued)

Observations made during skeletal examinations of fetuses in rabbits delivered by cesarean section from dams dosed with CH-19 Sweet extract[a]

	0 mL/kg/day	0.25 mL/kg/day	0.5 mL/kg/day	1 mL/kg/day
No. of dams	19	21	21	17
No. of fetuses				
Total	133	174	166	136
Progress of ossification				
No. of ossified sternebrae				
1st [Total (%, Mean±*SD*)]	131 (100.0±0.0)	174 (100.0±0.0)	166 (100.0±0.0)	136 (100.0±0.0)
2nd [Total (%, Mean±*SD*)]	131 (100.0±0.0)	174 (100.0±0.0)	166 (100.0±0.0)	136 (100.0±0.0)
3rd [Total (%, Mean±*SD*)]	131 (100.0±0.0)	174 (100.0±0.0)	166 (100.0±0.0)	136 (100.0±0.0)
4th [Total (%, Mean±*SD*)]	131 (100.0±0.0)	174 (100.0±0.0)	166 (100.0±0.0)	136 (100.0±0.0)
5th [Total (%, Mean±*SD*)]	99 (73.9±34.0)	143 (83.2±19.8)	145 (86.8±17.6)	111 (79.6±24.5)
6th [Total (%, Mean±*SD*)]	119 (92.4±16.0)	156 (90.5±21.9)	152 (91.0±17.1)	116 (83.5±30.8)
No. of ossified forelimbs (right)				
Metacarpal phalanges (Mean±*SD*)	4.87±0.20	4.77±0.25	4.90±0.13	4.68±0.34
Proximal phalanges (Mean±*SD*)	4.99±0.03	5.00±0.02	5.00±0.00	4.99±0.03
Medial phalanges (Mean±*SD*)	3.81±0.26	3.87±0.18	3.71±0.28	3.79±0.23
Distal phalanges (Mean±*SD*)	5.00±0.00	5.00±0.00	5.00±0.00	4.99±0.02
No. of ossified forelimbs (left)				
Metacarpal phalanges (Mean±*SD*)	4.88±0.22	4.80±0.25	4.90±0.13	4.70±0.31
Proximal phalanges (Mean±*SD*)	4.99±0.03	5.00±0.02	5.00±0.00	4.99±0.03
Medial phalanges (Mean±*SD*)	3.79±0.28	3.87±0.20	3.71±0.27	3.77±0.25
Distal phalanges (Mean±*SD*)	5.00±0.00	5.00±0.00	5.00±0.00	4.98±0.05
No. of ossified hindlimbs (right)				
Metacarpal phalanges (Mean±*SD*)	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
Proximal phalanges (Mean±*SD*)	4.00±0.00	4.00±0.00	4.00±0.00	3.99±0.02
Medial phalanges (Mean±*SD*)	3.95±0.12	3.99±0.05	4.00±0.00	3.98±0.04
Distal phalanges (Mean±*SD*)	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
No. of ossified hindlimbs (left)				
Metacarpal phalanges (Mean±*SD*)	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
Proximal phalanges (Mean±*SD*)	4.00±0.00	4.00±0.00	4.00±0.00	3.99±0.02
Middle phalanges (Mean±*SD*)	3.95±0.12	3.99±0.05	4.00±0.00	3.98±0.04
Distal phalanges (Mean±*SD*)	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
No. of ossified sacral and caudal vertebrae				
Mean±*SD*	19.59±0.51	19.62±0.36	19.59±0.34	19.51±0.33

[a]Data expressed as mean±*SD* (percentage).
No significant differences were observed between the control and any treated groups using Dunnett's test or Dunnett-type rank test.