Subchronic safety evaluation of hot-water extract from thinned immature mangos (*Mangifera indica* ‘Irwin’): 90-days oral toxicity study in rats

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**ABSTRACT**

Thinned immature fruit of the mango tree (*Mangifera indica* ‘Irwin’) are handled as waste. In this study, we conducted a 90-days toxicity study in male and female Sprague Dawley rats to evaluate the safety of a hot-water extract of thinned immature mango fruits (TIMEx) administered by oral gavage at doses of 500, 1000 and 2500 mg/kg body weight/day. Treatment did not result in death or changes in the behavior or external appearance of the animals. No alterations were observed in hematological or serum chemical parameters, urinalysis, food consumption, body weight gain or organ weights at the end of the treatment period, with the exception of higher mean corpuscular volume in male rats that received high doses and lower serum creatine phosphokinase levels in female rats that received medium doses. Under the conditions of this study and based on the toxicological endpoints evaluated, the no-observed-adverse-effect level (NOAEL) for TIMEx was 2500 mg/kg/day. The findings indicate that TIMEx is safe for consumption and should be investigated as a candidate food.

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**1. Introduction**

Mango (*Mangifera indica*) is one of the most favored tropical fruits in the world. Global mango production reached about 40 million tons in 2018, an increase of 2.8 percent over 2017 [1]. In addition to dense sweetness, the fruit pulp has high nutritional value for vitamins, dietary fiber and diverse polyphenols [2-4]. Therefore, the mango is referred to as the “King of fruits” [5].

For many fruits, including mango, size is a major factor that determines their yield, marketability and price. An increasing number of fruit per tree correlates with decreases in mean fruit weight and in the proportion of fruit in the larger size grades [6,7]. The mango fruit develops on the tree starting with an immature stage (Fig. 1A). The average weight of this stage is <10 g and the color is green. The fruits then grow and reach the mature size, but the color is still green (mature/unripe stage; Fig. 1B). This stage is the typical harvest point for mangos that are to be exported. During transportation and distribution and finally with the consumer, unripe mangos progress to a ripe/read to eat stage. Mature/unripe mangos produce ethylene, a naturally occurring ripening hormone and ripen normally on their own [8]. When most of the fruits are thinned during the immature stage, the size and quality of the remaining mango fruits are increased (mature/unripe after thinning stage; Fig. 1C) [9]. Fruit that is harvested when immature will soften, but it will not develop a pleasing flavor indicating that the ripening process will not salvage immature mango fruit [8]. Therefore, most thinned immature mango fruits are handled as waste.

We have recently focused on thinned immature fruits (Fig. 1D) of the Irwin mango cultivar (*Mangifera indica* ‘Irwin’) as an unused natural resource. Important biochemical, physiological and structural changes that affect mainly nutritional and phytochemical composition and produce softening, aroma and flavor modification, and antioxidant capacity, occur during the development stage from unripe to ripe. For example, lipid content increases during ripening, particularly omega-3 and omega-6 fatty acids [3]. Additionally, the Brix value, which represents the dry substance content of squeeze solutions containing mainly sucrose and fructose [10], increases, and acidity, peel strength and pulp...
firmness decrease during ripening [11], while magnesium levels increase but phosphorus, potassium, calcium and sodium levels decrease with ripening [12]. However, there is insufficient information concerning the safety and eating characteristics of thinned immature mango fruits to enable their use as a natural resource. In this study, we therefore carried out subchronic safety evaluation of hot-water extract from thinned immature mango fruits, which can be consumed whole, body including the peel, flesh, and seed, using a 90-day safety study in rats.

2. Materials and methods

2.1. Experimental materials and preparations

2.1.1. Immature mango fruits

Immature Irwin mango fruits grown in Miyazaki, Japan, were collected by hand between the middle of February and the end of March 2019, and taxonomically identified on the basis of morphological characteristics by Mr. Kenta Hidaka (Star-Fruits Company, Ltd., Miyazaki, Japan). The weight and size of individual fruits were <25 g and < Φ3 cm (Fig. 1D). The fresh samples were immediately transported to the laboratory, and washed with tap water to remove dirt and dust. The cleaned whole fruits including peel, flesh and seed were rapidly frozen in liquid nitrogen, and then lyophilized using a freeze dryer (FDU-2110, Tokyo Rikakikai Co., Tokyo, Japan). Thinned immature mango powder was obtained using a Knife mill grindomix GM 200 (Verder Scientific Co., Tokyo, Japan), and stored away from light at 4 °C until extraction. All other reagents were of the highest grade available.

2.1.2. Hot-water extraction

Hot-water extraction was conducted according to the modified method for making the hot-water extract from green tea leaf [13]. Briefly, thinned immature mango powder was mixed with ten volumes of water at 95 °C. After agitation for 10 min, filtrate was obtained via filtration through a 5 μm mesh (ADVANTEC No. 2, Toyo Roshi Kaisha, Tokyo, Japan), and then lyophilized using an FDU-2110 freeze dryer. The mango powder yield was 25–28 %. The lyophilized powder of hot-water extract from thinned immature mango (TIMEx) was stored away from light at 4 °C until used in animal experiments.

2.1.3. Preparation of test solutions

Before starting the experiment, we checked the solubility of TIMEx in deionized water, and found that the maximum solubility was about 250 mg/mL. Therefore, this concentration was employed as maximum concentration, and further dissolved at concentrations of 1000 and 500 mg/10 mL in deionized water according to the OECD 408 guideline “Repeated Dose 90-day Oral Toxicity Study in Rodents” [14] and our previous study [15]. After thorough mixing, solutions were administered to rats at 10 mL/kg body weight/day.

2.2. Animal experiments

2.2.1. Institutional approval of the study protocols

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miyazaki, Japan (No. 2019-015-01). This study was conducted in accordance with the Japanese Law for the Humane Treatment and Management of Animals (Law No. 105, 1973), which defined animal experimentation as the use of animals for scientific purposes with the consideration of the 3Rs.

2.2.2. Animals and treatments

The study protocol followed modified methods of our previous study [15] according to the OECD 408 guideline “Repeated Dose 90-day Oral Toxicity Study in Rodents” [14]. Briefly, thirty-two 4-week-old Sprague-Dawley rats of each sex (thirty-two males and thirty-two female) were obtained from Japan SLC (Shizuoka, Japan). In this study, groups of eight rats of each sex were employed as similar to the previous reports [16,17]. The animals were housed singly in polycarbonate cages (W270 mm × L440 mm × H187 mm) with paper bedding (Alpha-dri Certified, EPS Ekishin Co., Tokyo, Japan) at 23 ± 2 °C, with 55 ± 10 % humidity and a 12 h light/dark cycle (light period: 9:00 am to 9:00 pm) and with free access to laboratory chow (MF; Oriental Yeast Company, Tokyo, Japan) and deionized water. After 1 week of acclimatization, the
animals were randomly divided into four groups. Three groups were orally administered the TIMEx solution once daily during the middle of the light period. Each group received daily 2500 mg/10 mL/kg (high-dose), 1000 mg/10 mL/kg (medium-dose) or 500 mg/10 mL/kg (low-dose). The fourth group received 10 mL/kg of the vehicle (deionized water).

### 2.2.3. Clinical and physiological observations

All animals were observed twice daily for mortality, general condition, and clinical signs. Any abnormal findings were recorded with respect to symptom, extent, severity, and date of detection. Body weights were measured daily, immediately prior to administration, and food consumption was measured at least three times per week. Water consumption was measured during days 85–88. The effect on locomotor activity was also evaluated according to our previous report [18]. Briefly, on days 75–80, rats were placed in an open-field space (60 cm × 90 cm), and their locomotion and rearing frequency was observed in a 5-min period.

#### 2.2.4. Urinalysis

Each rat was housed individually in a metabolic cage (KN-647, Natsume Seisakusho Co., Tokyo, Japan) during days 78–87, and urine was collected over a period of 24 h. Urine volume was calculated using weight and density, which was analyzed by a urine specific gravity refractometer (MASTER-SURJM, Atago Co., Tokyo, Japan). The color and turbidity were evaluated visually. Urinary glucose, total protein, and creatinine levels were analyzed using a Dri-Chem 4000v chemistry analyzer (Fujifilm Co., Tokyo, Japan). Urinary pH was measured with a pH meter (LAQUAtwin pH-11B, HORIBA, Kyoto, Japan).

### Table 1

| TIMEx (mg/kg body weight/day) | Control | 500 | 1000 | 2500 | Control | 500 | 1000 | 2500 |
|------------------------------|---------|-----|------|------|---------|-----|------|------|
| Males (n = 8)                |         |     |      |      | Females (n = 8)          |     |      |      |
| days                         | 0       | 10  | 20   | 30   | 40      | 50  | 60   | 70   | 80   | 90     | 0   | 10   | 20   | 30   | 40    | 50  | 60   | 70   | 80   | 90    |
|                             | 182.5 ± 11.4 | 219.9 ± 12.9 | 292.2 ± 20.2 | 355.4 ± 30.3 | 398.6 ± 38.3 | 429.0 ± 44.1 | 456.5 ± 50.3 | 483.4 ± 53.9 | 503.0 ± 55.5 | 518.5 ± 53.5 |
|                             | 183.0 ± 15.4 | 220.2 ± 20.4 | 298.7 ± 24.7 | 360.9 ± 28.5 | 409.6 ± 31.1 | 441.0 ± 30.6 | 465.9 ± 27.6 | 492.2 ± 29.7 | 513.5 ± 32.5 | 530.9 ± 32.5 |
|                             | 182.7 ± 9.4 | 223.0 ± 12.3 | 299.0 ± 18.5 | 355.5 ± 28.2 | 402.2 ± 36.0 | 428.8 ± 38.6 | 452.8 ± 42.4 | 476.6 ± 45.6 | 497.1 ± 22.6 | 517.2 ± 47.1 |
|                             | 183.8 ± 10.6 | 222.4 ± 15.4 | 291.0 ± 18.2 | 357.6 ± 22.6 | 402.0 ± 26.9 | 430.4 ± 29.9 | 451.1 ± 31.7 | 478.9 ± 32.0 | 499.9 ± 31.4 | 517.6 ± 34.8 |
|                             | 123.8 ± 10.6 | 143.3 ± 12.6 | 178.9 ± 16.1 | 207.5 ± 18.4 | 227.4 ± 19.6 | 243.0 ± 21.1 | 253.9 ± 22.3 | 261.7 ± 22.2 | 268.6 ± 21.2 | 276.1 ± 22.4 |
|                             | 123.5 ± 7.3 | 144.2 ± 7.2 | 177.5 ± 10.1 | 208.6 ± 14.2 | 228.7 ± 19.0 | 242.9 ± 20.8 | 253.2 ± 19.0 | 259.9 ± 23.2 | 270.6 ± 22.8 | 274.1 ± 20.0 |
|                             | 123.5 ± 6.6 | 145.9 ± 10.1 | 180.6 ± 17.0 | 208.8 ± 21.3 | 230.6 ± 25.6 | 245.1 ± 29.4 | 254.6 ± 32.2 | 264.2 ± 35.3 | 271.3 ± 36.4 | 277.5 ± 34.6 |

TIMEx hot-water extract from thinning immature mango. All values represent the mean (in grams) ± S.D. (n = 8). No significant differences were found between control and treated rats (P < 0.05, Tukey Kramer test).

### 2.2.5. Hematology and blood chemistry

After administration of TIMEx for 90 days, the rats were fasted for 12 h, and blood samples were taken from the abdominal vein under anesthesia with isoflurane (2.5%). A 2 mL aliquot was added to a K-EDTA Venoject tube (VP-DK052K05, Terumo Medical Corp., Tokyo, Japan) and allowed to stand at room temperature for 30 min. Hematological parameters were then analyzed using Celltac MEK-6500 (Nihon Kohden Co., Tokyo, Japan). Next, another aliquot of blood (6 mL) was added to a Venoject tube containing a procagulant (VP-AL076 K, Terumo Medical Corp.). After standing for 30 min at room temperature, the serum fraction was obtained by centrifugation (1200 × g, 10 min, room temperature) and stored at −80 °C until analysis. Serum biochemical parameters listed in Table 4 were analyzed using a Dri-Chem 4000v chemistry analyzer.

#### 2.2.6. Necropsy and organ weights

After blood collection, the following organs and tissues were evaluated macroscopically and any abnormalities were recorded: Adrenal gland, duodenum, epididymis, eyes, heart, ileum, jejunum, kidneys, liver, lungs, ovaries, prostate, pancreas, skeletal muscle, skin, spleen, stomach, urinary bladder, uterus, testes, thymus, and thyroid. The following organs and tissues were weighed: Adrenal gland, brain, carcass, heart, kidney, liver, lung, spleen, thymus, thyroid, mesenteric visceral fat, testis, seminal vesicle, ovary and uterus.

#### 2.2.7. Determination of serum cytokine levels

Serum cytokine levels were analyzed using a multiplex biometric enzyme-linked immunosorbent assay according to the manufacturer’s instructions (Rat Cytokine/Chemokine 9-Panel, RECYTMAG-65 K, Millicore, Billerica, MA, USA), for the simultaneous detection and quantitation of interleukin (IL) 4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-18,
### Table 3
Hematological parameters of rats administered hot-water extract from unripe mango fruits for 90 days.

|                | Males                                     | Females                                   |
|----------------|-------------------------------------------|-------------------------------------------|
|                | Control (n = 8)                           | TIMEx (mg/kg body weight/day)              |
|                | 500 (n = 8)                               | 1000 (n = 8)                              | 2500 (n = 7) |
|                |                                            |                                            |              |
| WBC (10³/µL)  | 6.51 ± 0.80                               | 8.13 ± 0.45                               | 7.28 ± 0.65  | 7.76 ± 0.20  |
| RBC (10⁶/µL)  | 8.25 ± 0.25                               | 8.32 ± 0.12                               | 8.33 ± 0.05  | 8.16 ± 0.13  |
| HGB (g/dL)    | 13.9 ± 0.2                                | 13.9 ± 0.2                                | 14.1 ± 0.1   | 14.1 ± 0.2   |
| HCT (%)       | 38.7 ± 1.2                                | 39.0 ± 0.6                                | 39.8 ± 0.3   | 39.5 ± 0.7   |
| MCV (fl)      | 46.9 ± 0.3                                | 46.8 ± 0.3                                | 47.8 ± 0.4   | 48.4 ± 0.2*  |
| MCH (pg)      | 16.9 ± 0.2                                | 16.7 ± 0.2                                | 16.9 ± 0.2   | 17.3 ± 0.1   |
| MCHC (g/dL)   | 36.0 ± 0.4                                | 35.7 ± 0.2                                | 35.4 ± 0.2   | 35.8 ± 0.2   |
| RDW (%)       | 120.0 ± 0.1                               | 120.0 ± 0.1                               | 122.0 ± 0.7  | 120.0 ± 0.3  |
| RDW-CV (%)    | 6.4 ± 0.06                                | 6.20 ± 0.06                               | 6.3 ± 0.02   | 6.19 ± 0.05  |
| PCT (%)       | 15.5 ± 0.4                                | 14.8 ± 0.2                                | 15.1 ± 0.1   | 15.2 ± 0.1   |

|                | 500 (n = 8)                               | 1000 (n = 8)                              | 2500 (n = 8) |
|                |                                            |                                            |              |
| Control (n = 8)|                                            |                                            |              |
|                | 500 (n = 8)                               | 1000 (n = 8)                              | 2500 (n = 7) |
|                |                                            |                                            |              |
| WBC (10³/µL)  | 5.18 ± 1.03                               | 5.53 ± 1.63                               | 4.78 ± 0.75  | 5.88 ± 1.34  |
| RBC (10⁶/µL)  | 7.73 ± 0.40                               | 7.45 ± 0.17                               | 7.44 ± 0.56  | 7.50 ± 0.29  |
| HGB (g/dL)    | 14.7 ± 0.7                                | 14.4 ± 0.4                                | 14.2 ± 1.1   | 14.3 ± 0.2   |
| HCT (%)       | 38.8 ± 2.1                                | 39.5 ± 0.8                                | 39.2 ± 2.9   | 39.8 ± 1.1   |
| MCV (fl)      | 52.9 ± 0.8                                | 53.0 ± 1.4                                | 52.7 ± 0.8   | 53.1 ± 1.6   |
| MCH (pg)      | 19.1 ± 0.4                                | 19.3 ± 0.5                                | 19.1 ± 0.3   | 19.1 ± 0.6   |
| MCHC (g/dL)   | 36.1 ± 0.8                                | 36.4 ± 0.4                                | 36.2 ± 0.5   | 35.9 ± 0.7   |
| RDW (%)       | 12.5 ± 0.4                                | 12.3 ± 0.8                                | 12.3 ± 0.5   | 12.4 ± 0.6   |
| RDW-CV (%)    | 6.0 ± 0.26                                | 6.04 ± 0.24                               | 5.99 ± 0.23  | 5.88 ± 0.19  |
| PCT (%)       | 15.4 ± 0.3                                | 15.4 ± 0.4                                | 15.5 ± 0.3   | 15.4 ± 0.2   |

### Table 4
Serum biochemistry parameters of rats administered hot-water extract from unripe mango fruits for 90 days.

|                | Males                                     | Females                                   |
|----------------|-------------------------------------------|-------------------------------------------|
|                | Control (n = 8)                           | TIMEx (mg/kg body weight/day)              |
|                | 500 (n = 8)                               | 1000 (n = 8)                              |
|                |                                            | 2500 (n = 7)                              |
|                |                                            |                                            |
| ALT (U/L)     | 32.3 ± 3.6                                | 36.9 ± 4.8                                | 38.3 ± 10.5  | 32.0 ± 4.3   |
| AST (U/L)     | 51.4 ± 10.9                               | 63.6 ± 10.1                               | 72.5 ± 30.7  | 49.6 ± 3.5   |
| Alkaline phosphatase (U/L) | 511 ± 95                                | 605 ± 228                                | 549 ± 165    | 531 ± 153    |
| Amylase (kU/L) | 3.2 ± 0.6                                 | 3.4 ± 0.7                                 | 3.5 ± 0.8    | 2.7 ± 0.5    |
| Creatine phosphokinase (U/L) | 134 ± 16                               | 118 ± 21                                 | 141 ± 37     | 164 ± 32     |
| Cholinesterase (U/L) | 0.04 ± 0.08                             | 0.47 ± 0.05                               | 0.50 ± 0.08  | 0.53 ± 0.05  |
| Total bilirubin (mg/dL) | 0.13 ± 0.07                            | 0.10 ± 0.04                               | 0.11 ± 0.04  | 0.16 ± 0.07  |
| Uric acid (mg/dL) | 0.79 ± 0.16                              | 0.67 ± 0.06                               | 1.90 ± 0.43  | 1.63 ± 0.07  |
| Glucose (mg/dL) | 195 ± 18                                 | 198 ± 13                                 | 213 ± 31     | 201 ± 16     |
| Blood urea nitrogen (mg/dL) | 19.5 ± 1.4                           | 18.8 ± 2.0                                | 19.0 ± 4.6   | 20.1 ± 2.3   |
| Creatinine (mg/dL) | 0.34 ± 0.17                             | 0.42 ± 0.26                               | 0.36 ± 0.19  | 0.33 ± 0.22  |
| Total cholesterol (mg/dL) | 66 ± 13                                 | 58 ± 7                                   | 65 ± 14      | 71 ± 20      |
| HDL cholesterol (mg/dL) | 186 ± 4.9                              | 17.2 ± 2.1                                | 17.6 ± 3.9   | 18.6 ± 6.2   |
| Triglycerides (mg/dL) | 137 ± 57                                | 123 ± 24                                 | 133 ± 49     | 114 ± 40     |
| Albumin (g/dL) | 3.5 ± 0.2                                 | 3.4 ± 0.1                                | 3.5 ± 0.3    | 3.3 ± 0.3    |
| Total protein (g/dL) | 6.4 ± 0.2                               | 6.4 ± 0.2                                | 6.4 ± 0.5    | 6.2 ± 0.5    |
| Magnesium (mg/dL) | 10.5 ± 0.5                              | 10.5 ± 0.4                                | 10.7 ± 0.2   | 10.6 ± 0.4   |
| Sodium (mEq/L) | 13.0 ± 0.2                               | 13.0 ± 0.2                                | 13.0 ± 0.2   | 13.0 ± 0.3   |

**TIMEx hot-water extract from thinning immature mango. All values represent the mean ± S.D. HCT, hematocrit; HGB, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; PCT, plateletcrit; PDW, platelet distribution wide; PLT, platelet; RBC, red blood cell; RDWCV, red cell distribution CV; RDWSD, red cell distribution SD; WBC, white blood cell. 1One data was missing, because one blood sample was coagulated before analysis. *Significantly different vs Control group (P < 0.05, Tukey Kramer test).**

**3. Results**

### 3.1. Mortality and clinical signs

TIMEx was orally administered daily for 90 days. The treatment appeared to be well-tolerated. No rat died during the exposure period, and no clinical signs such as diarrhea, hair loss or aberrant activity (locomotion and resting frequency) were observed (data not shown).

### 3.2. Body weight and food consumption

Body weight gain and food consumption did not differ among the
3.3. Hematology and blood chemistry

Mean corpuscular volume (MCV) significantly increased in male rats in the high-dose group (Table 3). This change was not observed in female rats in any group. Assessment of serum biochemistry revealed significant changes in creatine phosphokinase activity in female rats in the medium-dose group (Table 4). This change was not observed in male rats in any group. Other hematology and blood chemistry parameters were not altered in either sex in any group at the end of the 90-day administrated period.

3.4. Urinalysis

No significant variations in pH, total protein or glucose levels were found by urinalysis in any treatment group (Table 5).

Table 5

| Treatment | TIMEx (mg/kg body weight/day) | pH | Total protein (g/g creatinine) | Glucose (g/g creatinine) |
|-----------|-------------------------------|----|--------------------------------|-------------------------|
| Control   | ---                           | 7.8 ± 0.4 | 2.27 ± 0.89 | 0.11 ± 0.06 |
| 500       |                               | 7.7 ± 0.2 | 2.18 ± 0.30 | 0.11 ± 0.02 |
| 1000      |                               | 7.8 ± 0.4 | 2.46 ± 0.49 | 0.12 ± 0.04 |
| 2500      |                               | 7.7 ± 0.4 | 2.10 ± 0.26 | 0.11 ± 0.04 |
| 31.5      |                               | 7.3 ± 0.4 | 2.13 ± 0.13 | 0.14 ± 0.04 |
| 100       |                               | 7.5 ± 0.3 | 1.70 ± 0.73 | 0.12 ± 0.04 |
| 250       |                               | 7.6 ± 0.5 | 2.34 ± 1.17 | 0.12 ± 0.04 |

TIMEx-hex water extract from thinning immature mango. All values represent the mean ± S.D. (n = 8). No significant differences were found between control and treated rats (P < 0.05, Tukey Kramer test).

Table 6

| Treatment | TIMEx (mg/kg body weight/day) | pH | Total protein (g/g creatinine) | Glucose (g/g creatinine) |
|-----------|-------------------------------|----|--------------------------------|-------------------------|
| Control   | ---                           | 7.8 ± 0.4 | 2.27 ± 0.89 | 0.11 ± 0.06 |
| 500       |                               | 7.7 ± 0.2 | 2.18 ± 0.30 | 0.11 ± 0.02 |
| 1000      |                               | 7.8 ± 0.4 | 2.46 ± 0.49 | 0.12 ± 0.04 |
| 2500      |                               | 7.7 ± 0.4 | 2.10 ± 0.26 | 0.11 ± 0.04 |
| 31.5      |                               | 7.3 ± 0.4 | 2.13 ± 0.13 | 0.14 ± 0.04 |
| 100       |                               | 7.5 ± 0.3 | 1.70 ± 0.73 | 0.12 ± 0.04 |
| 250       |                               | 7.6 ± 0.5 | 2.34 ± 1.17 | 0.12 ± 0.04 |

TIMEx-hex water extract from thinning immature mango. All values represent the mean ± S.D. (n = 8). No significant differences were found between control and treated rats (P < 0.05, Tukey Kramer test).
3.5. Necropsy

No visible alterations were associated with TIMEx treatment, with the exception of sporadic findings, including self-injury to the tail in one control male rat. Such injury was not observed in any other groups, including female rats (data not shown).

3.6. Organ weights

There were no significant differences in absolute and relative organ weights among sexes and treatment groups, although visceral fat weights in both male and female rats tended to decrease as the TIMEx dose increased (Table 6).

3.7. Serum cytokine levels

Fig. 2 shows the serum levels of various cytokines in male and female rats following a 90-day TIMEx treatment at a dose of 2500 mg/kg body weight/day. Notable alterations were not observed in both sexes.

4. Discussion

The size and quality of mango fruits are increased if a large number of fruits is thinned during the immature stage [9]. However, thinned immature mangos do not undergo ripening [8], making them unsuitable for consumption as raw fruits. Therefore, they are usually handled as waste. However, we have considered the possibility of processed food materials from thinned immature mangos, for example hot-water extraction to produce a tea. We have focused on the practical values of this unused natural resource; however, safety information for the resource was insufficient. Therefore, we conducted the present study to evaluate the effects of consuming TIMEx. Firstly, the acute oral toxicity test was investigated according to the OECD 423 guideline [19]. Administration of a single dose of 2500 mg TIMEx/kg body weight to male Sprague-Dawley rats did not affect body weight gain or food consumption and did not cause diarrhea or loss of hair during 14 days of observation (data not shown). Subsequently, the repeated dose 90-day oral toxicity study was carried out. There were no deaths or changes in behavior or external appearance among the rats dosed daily with TIMEx at 500 mg/kg body weight/day (low dose), 1000 mg/kg body weight/day (medium dose) and 2500 mg/kg body weight/day (high dose) for 90 days. No significant alterations in hematological or serum chemical parameters, urinalysis, food consumption, body weight gain, or absolute and relative organ weights were noted in any of the dose groups, with a few exceptions. Higher MCV was noted in male rats in the high-dose group. MCV is a measure of the average volume or size of a red blood cell, and changes with average red blood cell (RBC) size [20]. Chronic alcohol consumption increases MCV and MCHC compared with control patients [22]. In our results, neither MCH nor MCHC were changed by the administration of
any TIMEx concentration. On the other hand, similar results obtained in 
this study, in which were higher MCV, equilibrium MCH and MCHC, 
but not toxic. The serum biochemistry showed lower creatine phosphokinase (CPK) activity in female rats in the medium-dose group. When muscle damage occurs, muscle cells release CPK into the blood; therefore, CPK is an accurate indicator of muscle damage [26] and abnormal values of CPK occur in a variety of extracardiac disorders [27]. This suggests that lower CPK activity observed in this study might be because of immobi-

lity. However, the locomotor activity was not different between the 
vehicle and medium-dose groups in female rats. Additionally, AST and ALT activities, which are typical biomarkers for hepatic injury, change concomitantly with CPK after muscle injury [28]. However, we did not observe any changes in serum AST or ALT activities in any group. Furthermore, the CPK findings in female rats in the medium-dose group did not indicate a dose-response relationship, and hence were sporadic but not toxic.

5. Conclusions

A 90-day TIMEx treatment was well-tolerated by Sprague Dawley rats. No significant changes in clinical signs, hematopoiesis, 
the next logical step is to design studies to evaluate if TIMEx administration 
the online version.

In addition, blood levels of anemia-related inflammatory cytokines, including IFN-γ, TNF-α, IL-6 and IL-10 [24,25], were 
not affected by the daily administration of 2500 mg TIMEx/kg body 
weight in both sexes. Therefore, we concluded these effects were sporo-
dic, but not toxic. In the next logical step, it is necessary to design a study to evaluate if TIMEx administration improves certain health conditions or prevents the onset of adverse health conditions, including obesity, diabetes and metabolic disorders. The authors thank Miss. Rio Uragami, Miss. Ayano Furutani, and 
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