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ACUTE AND SUBCHRONIC TOXICITY STUDY OF TUD-RAK-KA-SAI-PUU RECIPE IN RATS

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Abstract

Acute and subchronic toxicities of Tud-Rak-Ka-Sai-Puu (TR) recipe were studied in male and female rats. After 14 days of a single oral administration of test substance (5,000 mg/kg body weight), measurement of the body and organs weights, necropsy and health monitoring were performed. No signs and differences in the weights and behavior were observed relative to the control rats, suggesting that TR recipe in the dose of 5,000 mg/kg body weight does not produce acute toxicity. The subchronic toxicity was determined by oral feeding in male and female rats daily with the test substance at 2, 20, 200 and 2,000 mg/kg body weight for 90 days. No defects of animal behavior were observed in the test groups. Both test and control groups (on the 90th day) as well as the satellite group (on the 118th day) were analyzed by measuring their final body and organ weights, taking necropsy, and examining hematology, blood clinical chemistry, and microanatomy. These results together with the information of signs, behavior and health monitoring can lead to a conclusion that an oral administration of TR recipe at 2, 20, 200 and 2,000 mg/kg body weight for 90 days did not cause subchronic toxicity.

Key words: Acute toxicity, Subchronic toxicity, Tud-Rak-Ka-Sai-Puu Recipe

Introduction

According to the Thai ancient textbook, “Ka-Sai” means physical deterioration, thin shape because of consistent chronic diseases or illnesses. In the other meaning of folk healer, Ka-Sai dyes, sticks, sweeps, and ties tightly. If Ka-Sai has stuck, tied tightly at any organs, it indicates that those organs are dysfunctional, wasted and pale. In accordance with the theory of modern medicine, “Ka-Sai-Puu” is caused by gastritis, more acid in stomach, blocked alimentary canal, intestine inflammation, and appendicitis (Subcharoen et al., 2001).

From Thai traditional medicine perspective, Ka-Sai-Puu disease is hyperacidity syndrome. This syndrome is a very common dietary disorder as a consequence of the increase of acidity in the stomach. Hyperacidity results from irregular and stressful lifestyle in conjunction with an unhealthful and irregular diet. Nowadays, the modern stressful lifestyle and stress on acidic food items like carbonated and alcoholic beverages encourage excess production of stomach acids leading to these common stomach discomforts (http://www.speedyremedies.com/home-remedies-for-hyperacidity-and-heartburn.html). Tud-Rak-Ka-Sai-Puu (TR) recipe has long been prescribed in Thai traditional folk medicine. Tud-Rak means cure or relieve the symptom. So, Tu-Rakh-Ke-Sai-Pu recipe is used for relieving the symptoms of hyperacidity syndrome. This recipe is composed of thirteen herbs, and their traditional uses of these plants are shown in Table 1. Although TR recipe has long been used, the toxicity effects of this recipe have never been evaluated. The present study is aimed to assess the adverse effects related to different doses in order to find the acceptably safe level of the TR recipe in rats by determining both oral acute and subchronic toxicities.

Materials and Methods

Plant material and preparation of the extract

All plant materials of TR recipe were obtained and identified by Thai Herbal Product Center, Department for Development of Thai Traditional Medicine, Ministry of Public Health. The preparation of the extract is in accordance with the traditional method, by mixing of each powder of plants as shown in Table 2. Then, the mixture was combined with 1,350 g of coconut sugar and 1,000 g of juice from *C. nucifera*. Next, the mixture was simmered until it was absolutely blended together in a sticky paste. The paste was kept at 4-5°C and was suspended in distilled water before feeding to the animal.

Table 1: Composition of plants in TR recipe, part used and their pharmacological effects

Species	Plant part	Traditional use	References
Angelica sylvestris Linn. (Koad-So, Umbelliferae)	Root	Antipyretic, cough remedy, anti-asthmatic, cardiotonic	Saralamp et al., 1992
Atractylodes lancea (Thunb) DC. (Koad-Kamao, Compositae)	Rhizome	Carminative	Saralamp et al., 1992
Croton tiglium Linn. (Purging croton, Euphrobiaceae).	Fruit and seed	Hydragogue purgative	Saralamp et al., 1992
Cuminum cyminum Linn. (Cumin, Umbelliferae)	Seed	Carminative, expectorant, treatment of leukorrhea	Saralamp et al., 1992
Diospyros decandra Lour. (Chan khao, Ebenaceae)	Wood	Antipyretic, blood tonic, anti-thirsty	Bunyapraphatsara and Chokechaijaroenporn, 1996
Dracaena loureiri Gagnep. (Chan Pha, Agavaceae)	Wood	Antipyretic, blood tonic, anti-thirsty	Bunyapraphatsara and Chokechaijaroenporn, 2000
Elettaria cardamomum Maton (Krawaan thet or Cardamom, Zingiberaceae)	Dried fruit and seed	Carminative, expectorant	Bunyapraphatsara and Chokechaijaroenporn, 1996
Gloriosa superba Linn. (Climbing Lily, Liliaceae)	Rhizome	Analgesic	Saralamp et al., 1992
Levisticum officinale Koch. (Lovage, Umbelliferae)	Root	Antipyretic, cough remedy, carminative, blood tonic	Saralamp et al., 1992
Ludisia discolor (Ker Gawl.) A. Rich (Jewel orchid**,** Orchidaceae)	Rhizome	Antipyretic, carminative, appitizer	Bunyapraphatsara and Chokechaijaroenporn, 2000
Nigella sativa Linn. (Black cumin, Ranunculaceae)	Seed	Carminative, expectorant, anti-emetic, blood tonic	Saralamp et al., 1992
Plumbago indica Linn. (Rose-colored Leadwort, Plumbaginaceae)	Root	Carminative, emmenagogue, treatment of hemmorrhoids	Saralamp et al., 1992
Syzygium aromatlcum (L.) Merr & Perry (Clove, Myrtaceae)	Dried flower	Carminative, stomachic, antidiarrheal	Saralamp et al., 1992

Table 2: Amount of each plant powder in TR recipe

Plant materials	Weight of each plants (gram)
- *A. sylvestris* (root), *A. lancea* (rhizome), *L. officinale* (root and rhizome), *N. sativa* (fruit and seed), *C. cyminum* (fruit and seed), *S. aromatlcum* (flower), *D. decandra* (wood), *D. loureiri* (wood), *E. cardamomum* (dry fruit)	60
- *L. discolor* (rhizome), *P. indica* (root) and *G. superba* (rhizome)	120
- *C. tiglium* (fruit and seed)	1,500

Experimental animals

Male and female Sprague-Dawley rats (120-160 g) were obtained from the National Laboratory Animal Center, Nakorn Pathom, Thailand. All animals were kept in the room maintained under environmentally controlled conditions of 24 ± 1°C and 12 h light-12 h dark cycle. Rats were deprived of food except water 16-18 hour prior the experiments. All experimental protocols were approved by the Animal Ethics Committee of Faculty of Medicine, Thammasat University (No. 0003/2005).

Test substance administration

According to OECD guideline (2001), the volume should not exceed 1 ml/100 g body weight, except in the case of aqueous solution where 2 ml/100 g body weight may be used. In acute toxicity study, TR recipe was suspended in distilled water at the concentration of 1,000 mg/ml and orally administered in an equivalent volume of 0.5 ml/100 g body weight. For subchronic toxicity, test substance was suspended in distilled water at the concentration of 1, 10, 100, and 1,000 mg/ml and orally administered in an equivalent volume of 0.2 ml/100 g body weight. Control groups received distilled water in the same route of administration and same volume.

Acute toxicity study

The acute oral toxicity was performed following the protocol of World Health Organization (WHO) guideline (WHO, 2000) and the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD, 2001), which the limit test at one dose level of 5,000 mg/kg was used in this study. Adult rats of both sexes were used and divided into two groups as control and test group (ten male and ten female). Control group received distilled water, while the test group received TR recipe at a dose of 5,000 mg/kg body weight. The animals were observed for the appearance of signs of toxicity over 14 days. On the 15^{th} day, all rats were fasted overnight, and then sacrificed with the overdose of thiopental sodium (50 mg/kg, intraperitoneally). Next, the vital organs including heart, lungs, livers, kidneys, spleen, adrenals, sex organs and brain were weighted and grossly examined.

Subchronic toxicity study

According to Thai Traditional Medicine, the usage of TR recipe is in pill form at the dose of 18.75 mg/kg, the average weight of Thai adult is about 50 kg. For evaluation of subchronic toxicity, the animal dosage of the extract for rats can be calculated, following the method of Reagan-Shaw et al. (2008). The dosage of TR recipe for rat is 115.625 mg/kg. According to OECD (1998), at least three dose levels and a concurrent control should be used. So, TR recipe at the doses of 2, 20, 200 and 2,000 mg/kg were set for the experiment. The method was conducted according to the WHO guideline (WHO, 2000) and the OECD guideline (OECD, 1981). TR recipe at the doses of 2, 20, 200 and 2,000 mg/kg body weight was administered to rats (ten males, ten females) once daily over 90 days, but the control group received vehicle under the same experimental condition. The satellite group was orally treated with the extract at daily dose of 2,000 mg/kg/day for 90 days, and no further treatment for the following 28 days to determine the reversibility of toxic effects. Animals were observed during the test period for body weight, clinical signs of toxicity and mortality. At the end of the period of extract administration, all rats were fasted for 16 h and anesthetized with thiopental sodium (50 mg/kg, intraperitoneally). Blood was collected from the common carotid artery for hematological studies (complete blood count, red blood cell count, platelet count and red cell indices). The serum was tested for the clinical blood chemistry such as the concentrations of glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, total bilirubin, direct bilirubin, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT) and alkaline phosphatase (ALP).
The positions, shapes, sizes and colors of internal organs were evaluated. Heart, lungs, thymus, livers, kidneys, spleen, adrenals, small intestine, stomach and duodenum, muscle with sciatic nerve, thoracic spines, brain, eyes, sex organs, uterus and epididymis were removed from all rats for visually detection of gross lesions and weighed to determine relative organ weights. All tissues were fixed in 10% nature buffered formalin solution. After routine processing, the paraffin sections of each tissue were cut at 5 μm thickness and stained with haematoxylin and eosin for histopathological examination.

Statistical analysis

Results were expressed as mean ± standard error of mean (S.E.M.). Statistical significance was evaluated using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. The data obtained from acute toxicity studies were analyzed by Student's paired *t*-test. *P* values less than 0.05 were considered significant.

Results

Acute toxicity

In both male and female animals, neither signs of toxicity nor death among the rats were observed during 14 days of the acute toxicity experimental period after administration of a single oral dose of the TR Recipe at a single dose of 5,000 mg/kg. Toxicity evaluation was further carried out by observing body weight gain and internal organs' weights. The body weight gain of the male extract-treated rats on the 7^{th} day was slightly decreased, yet no significant change in the body weight gain was detected on the 14^{th} day. There were no difference in gross and weight examinations of the internal organs (data not shown). These results suggest that the TR recipe is not toxic after an acute exposure.

Subchronic toxicity

None of animals receiving the TR recipe at the dose of 2, 20, 200 and 2,000 mg/kg/day daily over 90 days exhibited any abnormal parameters such as animal behaviors, toxic signs, body weight gain. The weights of some internal organs such as lung, liver, kidney and spleen of both male and female rats were found to be statistically different from those of the treated and the control groups (data not shown). The histological examination of the lung, heart, liver, spleen, adrenal grand, kidney, thymus, stomach and duodenum, small intestinal, ovary, uterus, testis, epididymis, muscle and nerve, thoracic spine, eyes and brain was normal in both the control and the treated groups. Significant differences of red blood cell, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and platelet count were observed for female and male rats receiving TR recipe relative to the control group (Table 3 and 4). Moreover, the administration of TR recipe at a dose of 2, 20 and 200 mg/kg daily over 90 days of the female rats resulted in the differential white blood cell count values statistically higher than the control groups (Table 5). In addition, the clinical blood chemistry values of female and male rats were statistically different from that of the control group (Table 6 and 7). However, in our study, observed difference did not appear to be related to the treatment since the values remained within the limits of normal biological variation.

Table 3: Hematological values of male rats in subchronic toxicity of TR recipe

	Control	TR recipe				
		2 mg/kg	20 mg/kg	200 mg/kg	2,000 mg/kg [a]	2,000 mg/kg [b]
Red blood cells ($x10^6$/µl)	8.29 ± 0.13	8.07 ± 0.11	8.12 ± 0.05	8.00 ± 0.07	7.93 ± 0.10*	8.15 ± 0.19
Hemoglobin (g/dl)	16.00 ± 0.28	15.61 ± 0.18	15.78 ± 0.14	15.78 ± 0.14	15.65 ± 0.18	15.83 ± 0.25
Hematocrit (%)	49.40 ± 0.80	48.10 ± 0.64	48.40 ± 0.40	47.60 ± 0.45	47.10 ± 0.61*	48.80 ± 0.88
Mean corpuscular volume (fl)	59.43 ± 0.21	59.52 ± 0.27	59.54 ± 0.29	59.45 ± 0.26	59.12 ± 0.34	59.92 ± 0.78
Mean corpuscular hemoglobin (pg)	19.29 ± 0.11	19.34 ± 0.13	19.43 ± 0.15	19.72 ± 0.13	19.72 ± 0.16	19.47 ± 0.31
Mean corpuscular hemoglobin concentration (g/dl)	32.44 ± 0.12	32.50 ± 0.11	32.65 ± 0.16	33.19 ± 0.15*	33.39 ± 0.18*	32.49 ± 0.17
Platelet ($x10^5$/µl)	8.00 ± 0.31	8.65 ± 0.38	8.20 ± 0.36	8.45 ± 0.30	7.66 ± 0.78	7.47 ± 0.76

Values are expressed as mean ± S.E.M., n = 10

a: A group was treated with TR recipe at 2,000 mg/kg/day for 90 days.

b: A satellite group was with TR recipe at 2,000 mg/kg/day for 90 days followed by no treatment for 28 days.

* Significantly different from control, $p<0.05$.

Table 4: Differential white blood cell count values of rats in subchronic toxicity of TR recipe

	Control	TR recipe				
		2 mg/kg	20 mg/kg	200 mg/kg	2,000 mg/kg [a]	2,000 mg/kg [b]
Female						
White blood cells ($x10^3$/µl)	3.35 ± 0.31	3.42 ± 0.27	3.03 ± 0.35	5.39 ± 0.85*	3.20 ± 0.26	3.95 ± 0.98
Neutrophil (%)	13.80 ± 2.44	10.70 ± 1.76	12.40 ± 1.78	23.10 ± 5.61*	15.20 ± 1.70	15.20 ± 2.19
Lymphocyte (%)	80.20 ± 2.71	81.10 ± 1.71	79.90 ± 1.61	70.20 ± 5.43*	77.70 ± 1.44	78.40 ± 2.75
Monocyte (%)	3.90 ± 0.48	5.00 ± 0.54	5.40 ± 0.40*	5.60 ± 0.68*	5.10 ± 0.54	3.80 ± 0.44
Eosinophil (%)	2.10 ± 0.31	3.20 ± 0.42*	2.30 ± 0.37	1.00 ± 0.41	1.80 ± 0.31	2.60 ± 0.50
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Male						
White blood cells ($x10^3$/µl)	4.39 ± 0.33	4.76 ± 0.32	4.53 ± 0.36	4.71 ± 0.37	5.02 ± 0.43	5.34 ± 0.76
Neutrophil (%)	12.50 ± 1.19	11.50 ± 1.26	10.70 ± 0.99	15.80 ± 3.38	13.80 ± 3.03	14.20 ± 1.58
Lymphocyte (%)	79.10 ± 1.47	81.30 ± 1.13	82.40 ± 1.32	77.70 ± 2.90	79.40 ± 2.55	79.60 ± 1.79
Monocyte (%)	6.20 ± 0.93	5.30 ± 0.60	5.00 ± 0.87	4.70 ± 0.80	4.60 ± 1.09	4.30 ± 0.33
Eosinophil (%)	2.20 ± 0.33	1.90 ± 0.38	1.90 ± 0.28	1.90 ± 0.55	2.00 ± 0.44	1.90 ± 0.35
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values are expressed as mean ± S.E.M., n = 10

a: A group was treated with TR recipe at 2,000 mg/kg/day for 90 days.

b: A satellite group was with TR recipe at 2,000 mg/kg/day for 90 days followed by no treatment for 28 days.

* Significantly different from control, $p<0.05$.

Discussion

In acute toxicity test at the dose of 5,000 mg/kg, all rats did not exhibit signs of toxicity and mortality after a single oral administration of TR recipe. The body weight gain and internal organs' weights were next observed since a decrease or increase in both parameters would indicate the presence of toxicity. According to the OECD guideline for testing of chemicals (OECD, 2001), the results of acute toxicity suggested that the TR recipe is fairly nontoxic.

Results of the subchronic toxicity showed that oral administration of TR recipe over 90 days did not cause mortality, behavioral changes and body weight gain whereas the weights of some internal organs of both male and female rats were found to be statistically different from those of the treated and the control groups. However, these results may be due to variation of the size and/or weight of animals' organs (Lillie et al., 1996; Carol, 1995).

Table 5: Clinical blood chemistry values of female rats in subchronic toxicity of TR recipe

	Control	TR recipe				
		2 mg/kg	20 mg/kg	200 mg/kg	2,000 mg/kg [a]	2,000 mg/kg [b]
Glucose (mg/dl)	96.10 ± 3.77	87.90 ± 6.98	90.40 ± 3.49	98.80 ± 6.87	105.00 ± 4.84	107.60 ± 3.84
BUN (mg/dl)	19.70 ± 0.87	19.50 ± 0.86	20.70 ± 0.71	23.20 ± 1.17*	20.20 ± 0.92	22.10 ± 1.29
Creatinine (mg/dl)	0.37 ± 0.02	0.37 ± 0.02	0.33 ± 0.02	0.36 ± 0.02	0.33 ± 0.02	0.44 ± 0.02*
Total protein (g/dl)	5.37 ± 0.12	5.33 ± 0.12	5.37 ± 0.10	5.74 ± 0.15*	5.65 ± 0.15	5.61 ± 0.12
Albumin (g/dl)	2.94 ± 0.06	2.97 ± 0.08	2.91 ± 0.03	3.01 ± 0.10	3.00 ± 0.07	2.85 ± 0.06
Total bilirubin (mg/dl)	0.35 ± 0.04	0.57 ± 0.12	0.28 ± 0.06	0.62 ± 0.25	0.28 ± 0.06	0.47 ± 0.11
Direct bilirubin (mg/dl)	0.26 ± 0.04	0.51 ± 0.11	0.19 ± 0.05	0.53 ± 0.22	0.23 ± 0.05	0.32 ± 0.10
SGOT (U/l)	192.90 ± 13.52	231.90 ± 16.56	173.00 ± 14.94	212.60 ± 11.83	192.40 ± 18.73	201.20 ± 19.38
SGPT (U/l)	33.60 ± 1.80	35.60 ± 1.63	34.40 ± 1.27	38.70 ± 6.45	31.89 ± 1.19	48.00 ± 10.03*
ALP (U/l)	39.60 ± 2.43	39.20 ± 4.70	42.90 ± 2.30	46.50 ± 5.25	41.30 ± 2.53	36.90 ± 2.59

Values are expressed as mean ± S.E.M., n = 10

a: A group was treated with TR recipe at 2,000 mg/kg/day for 90 days.

b: A satellite group was with TR recipe at 2,000 mg/kg/day for 90 days followed by no treatment for 28 days.

* Significantly different from control, $p<0.05$.

Table 6: Clinical blood chemistry values of male rats in subchronic toxicity of TR recipe

	Control±	TR recipe				
		2 mg/kg	20 mg/kg	200 mg/kg	2,000 mg/kg [a]	2,000 mg/kg [b]
Glucose (mg/dl)	117.40 ± 3.83	112.30 ± 4.22	105.60 ± 5.97	104.50 ± 4.40	107.20 ± 6.81	117.50 ± 4.11
BUN (mg/dl)	18.30 ± 0.56	19.20 ± 1.01	18.50 ± 0.97	17.70 ± 0.79	20.00 ± 0.91	20.30 ± 1.00
Creatinine (mg/dl)	0.28 ± 0.01	0.27 ± 0.01	0.32 ± 0.02	0.27 ± 0.01	0.27 ± 0.02	0.34 ± 0.02*
Total protein (g/dl)	5.09 ± 0.07	5.20 ± 0.07	5.19 ± 0.07	4.62 ± 0.46	5.32 ± 0.07	5.48 ± 0.11
Albumin (g/dl)	2.72 ± 0.02	2.69 ± 0.03	2.74 ± 0.03	2.68 ± 0.04	2.80 ± 0.05	2.77 ± 0.07
Total bilirubin (mg/dl)	0.15 ± 0.02	0.14 ± 0.02	0.22 ± 0.04	0.14 ± 0.02	0.19 ± 0.04	0.27 ± 0.03*
Direct bilirubin (mg/dl)	0.10 ± 0.04	0.09 ± 0.03	0.14 ± 0.05	0.05 ± 0.02	0.13 ± 0.05	0.18 ± 0.03
SGOT (U/l)	145.90 ± 11.70	149.10 ± 8.52	156.80 ± 13.57	146.30 ± 11.48	154.50 ± 13.07	162.60 ± 19.28
SGPT (U/l)	39.80 ± 0.80	42.30 ± 3.49	40.00 ± 2.80	41.50 ± 1.97	39.10 ± 1.61	39.10 ± 4.06
ALP (U/l)	62.70 ± 3.32	63.00 ± 1.79	60.70 ± 2.51	61.10 ± 2.06	66.10 ± 3.44	57.10 ± 3.07

Values are expressed as mean ± S.E.M., n = 10

a: A group was treated with TR recipe at 2,000 mg/kg/day for 90 days.

b: A satellite group was with TR recipe at 2,000 mg/kg/day for 90 days followed by no treatment for 28 days.

* Significantly different from control, $p<0.05$.

The changes in the hematological system have a high predictive value for human toxicity, when the data are translated from animal studies (Olson et al., 2000). The hemoglobin, hematocrit and these red blood cell indices were helpful in the differential diagnosis of anemia (Voigt, 2000), yet the gross examinations of skin, eye and mucous membrane did not show any clinical defect. The elevation of the differential white blood cell count indicates the strengthening of the organism defense which suggests the ability of test substance to boost the immune system through increasing the population of defensive white blood cells

(Atsamo et al., 2011). In this study, some of the hematological values (red blood cell, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and platelet) and the differential white blood cell count values of rats were slightly different from those of the control group. However, the changes in all of the parameters were possibly not related to the TR treatment because these values lay within the normal range of the normal biological variation of the animals (Inala et al., 2002; Feldman et al., 2000).

The clinical blood chemistry values were used to analyze kidney, liver and pancreas functions. Liver is the main organ in the detoxification and metabolism of chemicals. The important enzyme in liver is SGOT and SGPT, especially SGPT is a better indicator of liver injury than SGOT (Ozer et al., 2010; Mehta et al., 2009). Normally, if the clinical blood chemistry values differ more or less than one fold from the normal values, abnormality of kidney, liver and pancreas's function should be noted (Angkhasirisa et al., 2002; Barry, 1995; Caisey and King, 1980; Sacher and McPherson, 1991a, 1991b). In both female and male groups, some clinical blood chemistry values such as creatinine, BUN, SGPT, total protein and total bilirubin were statistically different from that of the control group. In this study, the observed difference was less than one fold, suggesting normal function of the organs. Moreover, the histological examination of the lung, heart, liver, spleen, adrenal grand, kidney, thymus, stomach and duodenum, small intestinal, ovary, uterus, testis, epididymis, muscle and nerve, thoracic spine, eyes and brain were normal in both the control and the treated groups.

In conclusion, TR recipe does not cause oral acute and subchronic toxicities in rats. An additional study in chronic toxicity evaluation is needed to determine the long-term safety of the extract.

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