Report No: BP-A-230581~BP-A-230583EN

Test Report

Sample Name:	Silicone mixture
Client Name:	Dongguan City Kedo Silicone Material Co.,Ltd
Client Address:	Next to Fang Zhong 1st Road, Shilata Industrial zone,Liangbian Village, Liaobu Town, Dongguan City, Guangdong Province, China
Date of Issue:	2024.03.07

Shanghai WEIPU Testing Technology Group Co., LTD.

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Organization name: **Shanghai WEIPU Testing Technology Group Co., LTD.**

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Shanghai WEIPU Testing Technology Group Co., LTD.

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Task No.	BP-A-230581~BP-A-230583	Detection category	Commission test
Sample No.	BP-S-2300760	Sample source	Sent by client
Sample name	Silicone mixture	Batch number	/
Model	/	Sample number	1
Specification	/		
Manufacturer	Dongguan City Kedo Silicone Material Co.,Ltd		
Manufacturer address	/		
Client	Dongguan City Kedo Silicone Material Co.,Ltd		
Client address	Next to Fang Zhong 1st Road,Shilata Industrial zone,Liangbian Village,Liaobu Town,Dongguan City,Guangdong Province,China		
Receiving date	2023.12.29		
Test location	3 Floor, Building 7,166-1, Fengjin Road, Fengxian District, Shanghai.		
Test period	2023.12.29 to 2024.01.29		
Test item	1、Skin sensitization test; 2、Skin Irritation Test; 3、MTT cytotoxicity test;		
Test criterion	GB/T 16886.10-2017/ISO 10993-10:2010 GB/T 16886.5-2017/ISO 10993-5:2009		
Test conclusion	The test samples all meet the requirements of relevant standards. Date of issue 2024.03.07		
Implementation standard	ISO/IEC 17025:2017; RB/T214—2017		
Remarks	"N/A" in the report indicates that this item is not applicable, and "/" in the report indicates that this item is blank.		

Edited by	Checked by	Approved by (Authorized signatory)
Date: 2024.03.07	Date: 2024.03.07	Date: 2024.03.07

Test Lists

No.	Test item	Test criterion	Evaluation standard	Test results
1	Skin sensitization test	GB/T 16886.10-2017/ISO 10993-10:2010	The grades of 1 or greater in the test group generally indicate sensitization, provided grades of less than 1 are seen in control animals	The skin reaction grade was 0 in each period, and the positive provocation rate in the test group was 0%, and there was no allergic reaction
2	Skin Irritation Test	GB/T 16886.10-2017/ISO 10993-10:2010	The requirements of the test are met if the final test sample score is 1.0 or less	The final score of the test samples was 0, and the skin irritation was very minimal
3	MTT cytotoxicity test	GB/T 16886.5-2017/ISO 10993-5:2009	If the 100% extract viability is reduced to < 70% of the blank	The cell viability rate (%) of the 100% extract was 107.31%, and there was no potential toxicity

Catalogue

Skin sensitization test

1 Objective

The test was designed to evaluate the potential of a test article to cause skin sensitization using Guinea Pig Maximization Test.

2 Test method

Guinea Pig Maximization Test.

3 Test conclusion

Under the conditions of this experiment, the skin reaction grading of all animals in each period was grade 0, and the positive activation rate of the experimental group was 0%, indicating that the extract of the sample did not produce allergic reaction.

4 Test and control samples

4.1 Test samples

(The information in the form is provided by the client)

Sample name	Silicone mixture
Sterilization state	Non-sterilized
Sterilization methods	/
Sample material	/
Application	/

4.2 Control samples

Polar negative control sample：0.9%NaCl injection	
Manufacturer	Shandong Qidu Pharmaceutical Co., Ltd.
Specification	500mL/bottle
Batch No.	15B23040501
Non-polar negative control sample：Cottonseed oil	
Manufacturer	Shanghai Macklin Biochemical Co., Ltd.
Specification	13kg
Batch No.	C15691020
Positive control sample：2,4-Dichloronitrobenzene	
Manufacturer	TCI
Specification	25g/bottle
Batch No.	WT6CA-LZ

Concentration	Intradermal induction phase	0.8%
	Topical induction phase	0.8%
	Challenge phase	0.2%

5 Reagents and Instrument

5.1 Reagents

Name	Supplier
0.9%sodium chloride injection	Shandong Qidu Pharmaceutical Co., LTD.
Cottonseed oil	Shanghai Macklin Biochemical CO., LTD.

5.2 Instrument

Name	Instrument ID
Electronic balance	WPE-TL0288
Clean bench	WPE-TL0127
Thermostatic water bath	WPE-TL0053

6 Test system

6.1 Test animal selection

The guinea pig is believed to be the most sensitive animal model for this type of study.

6.2 Test animal information

Species	guinea pig
Number	30
Sex	Male
Weight	310g~369g
Age	Early adulthood
Health condition	Healthy
Adaptation	5 days
Source	Shanghai Songjiang Chedundongwu Breeding Farm Co., Ltd., License number:SCXK（Shanghai）2022-0001，Quality Qualification Certificate number: 20220001001351

6.3 Feeding and management

Fodder	Shanghai Zhouyu Biotechnology Co., LTD., License number: Shanghai Feed Certificate (2021) 04027

Water	Tap water, free drinking water.
Environment	Common animal room 325, the temperature range of 18~29℃, humidity range of 40~70%.
Light	Control cycle light (12 hours on, 12 hours dark)
Veterinary	Give necessary veterinary attention
Raise	The animals were raised in accordance with the "Feeding and management procedures of the experimental animal guinea pig" in our Toxicology laboratory.
Pollutant	The feed provided and the possible contaminants in the water will not have a potential impact on the results of this experiment.
Certification body	The animal laboratory of this institution shall be certified by Shanghai Laboratory Animal Center, and the certifying authority: Shanghai Municipal Department of Science and Technology. Experimental animal use License No.: SYXK (Shanghai) 2021-0023, applicable to ordinary grade rabbits in general environment.
Welfare	The IACUC established by the Institute confirmed that the experiment used a minimum number of animals without affecting the test results, and relevant documents were developed to safeguard animal welfare.

7 Test procedure

7.1 Sample preparation

The extracts were prepared according to the method in the table below. After the extraction, the changes of the extracts were checked. The extracts were not centrifuged and etc. The pH was not adjusted. Blank control, negative control and positive control samples were prepared by the same method.

Table 7-1 Preparation of extracts

Extraction solvent	experimental stage	Actually sample	Sampling ratio	Solvent volume	Sampling condition	Whether the extract is clear	pH
0.9%NaCl injection	Intradermal induction phase	3.3043g	0.1g:1mL	33.04mL	70±1℃ 24±2h 60rpm	Yes	5.29
	Topical induction phase	1.2315g		12.31mL		Yes	5.52
	Challenge phase	1.2365g		12.36mL		Yes	5.91

Extraction solvent	experimental stage	Actually sample	Sampling ratio	Solvent volume	Sampling condition	Whether the extract is clear	pH
Cotton seed oil	Intradermal induction phase	3.0068g	0.1g：1mL	30.06mL	70±1℃ 24±2h 60rpm	Yes	4.00
	Topical induction phase	1.2797g		12.79mL		Yes	4.50
	Challenge phase	1.2560g		12.56mL		Yes	4.98

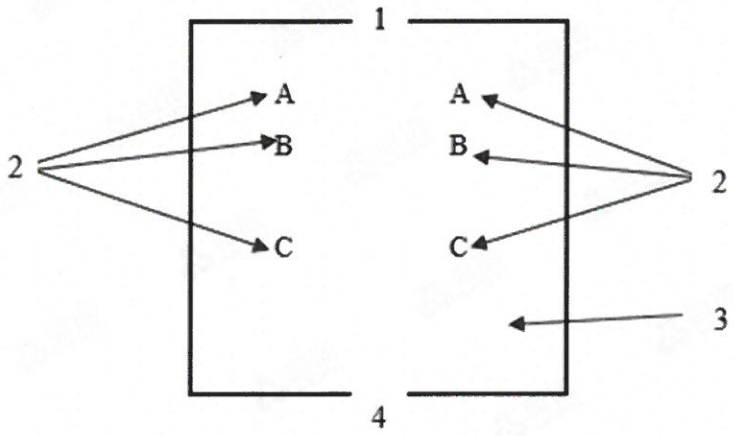
7.2 Test procedure

Before the experiment, the guinea pigs are marked and weighed. They are randomly divided into 0.9%NaCl injection test groups and control groups, Cottonseed oil test groups and control groups.

The skin reactions of guinea pigs are observed after 24h and 48h, and the body weight is recorded.

7.2.1 Intradermal induction phase

Make a pair of 0.1 mL intradermal injections of each of the following, into each animal, at the injection sites (e.g. sites A, B and C), as shown in Figure 1, in the clipped intrascapular region.



1——cranial end; 2——0.1mL intradermal injections; 3——clipped intrascapular region; 4——caudal end; A, B, C——injection sites

Figure 1 — Location of intradermal injection sites

Site A: A stable emulsifier mixed by injection of Freund's complete adjuvant with 0.9%NaCl injection and Cottonseed oil solvent in 50:50 (volume ratio) ratio..

Site B: The test sample (undiluted extract); inject the control animals

with the extraction vehicle/solvent alone.

Site C: The test sample at the concentration used at site B, emulsified in a 50:50 volume ratio stable emulsion of Freund's complete adjuvant and the solvent/extraction vehicle; inject the control animals with an emulsion of the blank liquid with adjuvant.

7.2.2 Topical induction phase

At 7d after completion of the intradermal induction phase, administer the test sample by topical application to the intrascapular region of each animal, using a patch of area approximately 8cm^2 (absorbent gauze) soaked with extract, so as to cover the intradermal injection sites. Use the concentration selected in the intradermal induction phase for site B. The concentration in Intradermal induction phase I did not produce irritation, animals were pretreated with 10% sodium dodecyl sulfate 24±2hours before the topical induction application. Secure the patches with an occlusive dressing. Remove the dressings and patches after 48±2h.

7.2.3 Challenge phase

At 14±1d after completion of the topical induction phase, challenge all test and control animals with the test sample. The local sticker is applied to the unbound site during the induction phase, and then covered with a layer of glass paper, and then fixed with no stimulating tape. After (24±2) h, remove the bandaging band and apply the fillet.

7.3 Observation

Observe the appearance of the challenge skin sites of the test and control animals (24±2) h and (48±2) h after removal of the dressings. Describe and grade the skin reactions for erythema and oedema according to the Magnusson and Kligman grading scale for each challenge site and at each time interval.

Table 7-1 Magnusson and Kligman scale

Patch test reaction	Grading scale
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and/or swelling	3

7.4 Evaluation standard

Magnusson and Kligman grades of 1 or greater in the test group generally indicate sensitization, provided grades of less than 1 are seen in control animals. If grades of 1 or greater are noted in control animals, then the

reactions of test animals which exceed the most severe reaction in control animals are presumed to be due to sensitization. If the response is equivocal, rechallenge is recommended to confirm the results from the first challenge. The outcome of the test is presented as the frequency of positive challenge results in test and control animals. Occasionally, the test group has a greater number of animals showing a response than the controls, although the intensity of the reaction is not greater than that exhibited by the controls. In these instances, a rechallenge can be necessary to define the response clearly. A rechallenge shall be carried out 1 week to 2 weeks after the first challenge. The method used shall be as described for the first challenge, using a naïve side on the animal.

8 Test result

The skin reaction grade and positive excitation rate of guinea pigs in this experiment are shown in Table 8-1, and the positive test results are shown in Table 8-2.

Table 8-1 Skin sensitization reaction

Extraction solvent	Group	Animal No.	Weight before test (g)	Weight after test (g)	Topical induction phase grade	Challenge phase grade		positive activation rate
						24h	48h	
0.9% NaCl injection	Control	1101	317	407	0	0	0	0
		1102	317	412	0	0	0	
		1103	326	410	0	0	0	
		1104	330	421	0	0	0	
		1105	310	401	0	0	0	
	Sample	2101	349	418	0	0	0	0
		2102	322	416	0	0	0	
		2103	343	420	0	0	0	
		2104	350	423	0	0	0	
		2105	347	431	0	0	0	
		2106	336	415	0	0	0	
		2107	358	422	0	0	0	
		2108	369	429	0	0	0	
		2109	370	431	0	0	0	
		2110	322	423	0	0	0	

Extraction solvent	Group	Animal No.	Weight before test (g)	Weight after test (g)	Topical induction phase grade	Challenge phase grade		positive activation rate
						24h	48h	
Cottonseed oil	Control	3101	317	411	0	0	0	0
		3102	351	421	0	0	0	
		3103	344	425	0	0	0	
		3104	332	417	0	0	0	
		3105	347	426	0	0	0	
	Sample	4101	354	419	0	0	0	0
		4102	355	420	0	0	0	
		4103	319	423	0	0	0	
		4104	323	413	0	0	0	
		4105	311	417	0	0	0	
		4106	320	413	0	0	0	
		4107	352	430	0	0	0	
		4108	368	426	0	0	0	
		4109	323	408	0	0	0	
		4110	332	411	0	0	0	

Form 8-2 Skin sensitization reaction of positive control

Group	Animal No.	Weight before test (g)	Weight after test (g)	Challenge phase grade		positive activation rate
				24h	48h	
Positive control	2001	351.10±21.25	452.90±15.95	2	1	100
	2002			2	2	
	2003			1	1	
	2004			1	1	
	2005			2	2	
	2006			2	2	
	2007			1	2	
	2008			2	2	
	2009			2	2	
	2010			2	2	
Remarks	Quote: report of project WP-23071911-BC-01 Test date: 2023.08.10~2023.09.03					

9 Test conclusion

Under the conditions of this experiment, the skin reaction grading of all animals in each period was grade 0, and the positive activation rate of the experimental group was 0%, indicating that the extract of the sample did not produce allergic reaction.

10 Deviations

The test was carried out in strict accordance with the standard operating procedures, and no deviation affecting the validity of the test data occurred.

11 Record Preservation

All raw data and records related to this test and copies of the final report are kept in the archives。

Skin Irritation Test

1 Objective

The potential of medical device and materials to produce local skin stimulation response under experimental conditions was evaluated using relevant animal models.

2 Test method

Rabbit skin irritation test.

3 Test conclusion

Under this test condition, the irritation index of the test sample was 0, and no erythema and edema were observed, indicating that the sample polar extract had negligible irritation to the skin.

4 Test and control Samples

4.1 Test sample

The information is provided by the client.

Sample name	Silicone mixture
Sterilization state	Non-sterilized
Sterilization methods	/
Sample material	/
Application	/

4.2 Control sample

Blank control sample：0.9%sodium chloride injection	
Manufacturer	Shandong Qidu Pharmaceutical Co., Ltd.
Specification	500mL/bottle
Batch No.	15B23040501
Negative control sample：Cotton oil	
Manufacturer	Shanghai Macklin Biochemical Co., Ltd.
Specification	13kg
Batch No.	C14894162
Positive control sample: SLS	
Manufacturer	Adamas-beta

Specification	100g/bottle
Batch No.	P1880796

5 Reagents and Instrument

5.1 Reagents

Name	Supplier
0.9%sodium chloride injection	Shandong Qidu Pharmaceutical Co., Ltd.
Cotton oil	Shanghai Macklin Biochemical Co., Ltd.
SLS	Adamas-Beta

5.2 Instrument

Name	Instrument number
Electronic counting scale	WPE-TL0055
Clean bench	WPE-TL0127
Thermostatic water bath	WPE-TL0053
pH meter	WPE-TL0079
Electronic balance	WPE-TL0288

6 Test system

6.1 Selection of laboratory animals

Rabbit skin irritation test has been mentioned in GB/T 16886.10-2017, which proves that this method is the most sensitive method at present, and has been widely used in skin irritation evaluation of medical devices/materials.

6.2 Laboratory animal informatics

Species	New Zealand Rabbits
Quantity	6
Gender	Male
Weight	2.222~2.315kg
Age	Early adulthood
Health	Healthy, absent and not pregnant
Adaptive phase	5 Days

Animal origin	Shanghai Songjiang Chedun Laboratory Animal Breeding Farm Co., Ltd.，License number: SCXK（Shanghai）2022-0001，Quality Qualification Certificate number: 20220001001341

6.3 Feeding and management

Fodder	Laboratory rabbit maintenance feed，Shanghai Zhouyu Biotechnology Co., Ltd.，License number: Shanghai feeding certificate（2021）04027.
Water	Tap water, free drinking water.
Cage	All instruments are sterilized in a pulsating vacuum sterilizer at 121°C for 30min.
Environment	Conventional animal room 322 the temperature range of 16~26℃, humidity range of 40~70%.
Light	Control cycle light (12 hours on, 12 hours dark)
Veterinary	Give necessary veterinary attention
Raise	The animals were raised in accordance with the "Feeding and management procedures of the experimental animal rabbits" in our Toxicology laboratory.
Pollutant	The feed provided and the possible contaminants in the water will not have a potential impact on the results of this experiment.
Certification body	The animal laboratory of this institution shall be certified by Shanghai Laboratory Animal Center, and the certifying authority: Shanghai Municipal Department of Science and Technology. Experimental animal use License No.: SYXK (Shanghai) 2021-0023, applicable to ordinary grade rabbits in general environment.
Welfare	The Institutional Animal Care and Use Committee established by the Institute confirmed that the experiment used a minimum number of animals without affecting the test results, and relevant documents were developed to safeguard animal welfare.

7 Test content

7.1 Test sample and control preparation

Under aseptic operation, take the sample, the extracts were prepared according to the method in the table below. After the extraction, the changes of the extracts were checked. The extracts were not centrifuged and etc. The pH was not adjusted.

Table 11-1 Preparation of extracts

Extraction solvent	Actually sample	Sampling ratio	Solvent volume	Sampling condition	Whether the extract is clear	pH
0.9%sodium chloride injection	3.3043g	0.1g：1mL	33.04mL	70±1℃ 24±2h 60rpm	Yes	5.29
Cotton oil	3.0068g		33.06mL		Yes	4.00

7.2 Test procedure

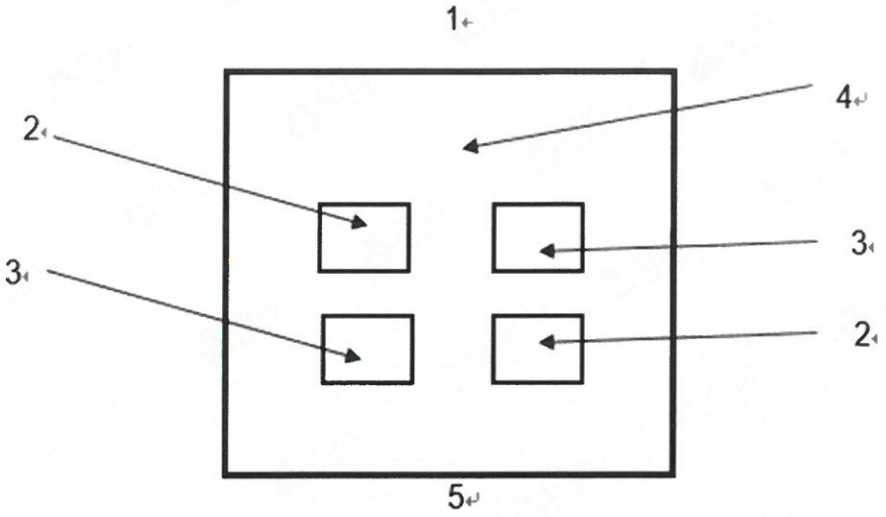
Marking and weighing should be performed before drug administration. The marking site is shown in Figure 1.

About 4-24h before the experiment, the hair on both sides of the spine of the experimental animals was cut off, without damaging the epidermis, and the hair removal range was about 10cm×15cm.

The 2.5cm×2.5cm gauze was applied to the skin on both sides of the rabbit spine after fully soaking the sample extract, and then fixed. The other side of the skin was covered with a gauze with the infiltrating medium as a control. The application time is 6 hours.

After the end of the contact period, remove the applied patch, mark the contact site with persistent ink, remove the residual subject with warm water, and wipe it dry carefully.

The skin conditions at the contact sites were observed and recorded at (1±0.1) h, (24±2) h, (48±2) h and (72±2) h after the removal of subjects, and the skin reaction score was performed according to Table 1. If there is a persistent injury, it is necessary to extend the observation period to evaluate the reversibility or irreversibility of the injury, but the extension period should not exceed 14 days.



Description: 1 - head; 2 - test site; 3 - control site; 4 - the back area where hair is removed; 5 - Tail.

Figure 1 Skin application site

Table 7-2 Skin irritation response score

Reaction	Irritation score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Well-defined oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond exposure area)	4
Maximal possible score for irritation	8
Other adverse changes at the skin sites shall be recorded and reported	

7.3 Evaluation

After 72 hours, the total erythema and edema scores caused by (24±2) h, (48±2) h, and (72±2) h for each animal were added, and the total scores were divided by 6 (two test/observation sites, three time points) to calculate the primary irritation index for an animal. The primary stimulation index score of each animal was added and then divided by the total number of animals (3) to obtain the stimulation index of the test sample. The primary stimulus score was obtained by subtracting the blank control index from the primary stimulus index.

Table 7-3 Types of skin irritation index

Mean score	Response category
0 ~ 0.4	negligible
0.5 ~ 1.9	slight
2.0 ~ 4.9	moderate
5.0 ~ 8.0	severe

8 Test results

8.1 Sample results

After the removal of subjects, the skin irritation response score at each time point was 0, as shown in Table 8-1, and the animal irritation index was also 0, as shown in Table 8-2

Table 8-1 Observation results of skin irritation test

Extraction medium	Animal number	Irritation score											
		24h				48h				72h			
		Sample		Contrast		Sample		Contrast		Sample		Contrast	
		Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema
0.9%sodium chloride injection	111	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0
	112	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0
	113	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0

Extraction medium	Animal number	Irritation score											
		24h				48h				72h			
		Sample		Contrast		Sample		Contrast		Sample		Contrast	
		Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema
Cotton oil	111	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0
	112	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0
	113	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0

Table 8-2 Summary of Skin irritation index

Extraction medium	Animal number	Test point average integral	Mean integral of control points	Score gap	Samples stimulation index	Response type
0.9%sodium chloride injection	111	0	0	0	0	negligible
	112	0		0		
	113	0		0		
Cotton oil	111	0	0	0	0	negligible
	112	0		0		
	113	0		0		

8.2 Positive test result

In the positive tests numbered SHA56-23083011-JC-01 from 2023.08.18.to 2023.09.01 the primary stimulus score was 4.83 for polar extracts and 4.94 for non-polar extracts. The positive test result indicates that the test result is credible. The indices are shown in Table 8-3.

Table 8-3 Summary of positive Skin irritation index

Animal number	Test point average integral	Mean integral of control points	Score gap	Samples stimulation index	Response type	Animal number
111	4.83	4.83	0	0	4.83	moderate
112	4.83		0			moderate
113	4.83		0			moderate
111	5.00	4.94	0	0	4.94	moderate
112	5.00		0			moderate
113	4.83		0			moderate

9 Test conclusion

Under this test condition, the irritation index of the test sample was 0, and no erythema and edema were observed, indicating that the sample polar extract had negligible irritation to the skin.

10 Test deviation declaration

The test was carried out in strict accordance with the standard operating procedures, and no deviation affecting the validity of the test data occurred.

11 Records retention

All raw data and records related to this experiment and copies of the final report are kept in the archives。

MTT cytotoxicity test

1 Objective

The biological response to L-929 cells was evaluated by *in vitro* cytotoxicity test.

2 Test method

MTT cytotoxicity test

3 Test conclusion

The cell viability of test sample extract was 107.31%, and the cell morphology grade of test sample extract was grade 0, the sample extract had no potential toxic effect on L-929 cells.

4 Test and control samples

4.1 Test samples

The information in the form is provided by the client

Sample name	Silicone mixture
Sterilization state	Non-sterilized
Sterilization methods	Ultraviolet irradiation for 30min
Sample material	/
Application	/

4.2 Control samples

Negative control sample：HDPE	
Manufacturer	USP
Specification	Three-piece pack
Batch No.	R149K0
Preservation condition	Room temperature
Positive control sample：DMSO	
Manufacturer	Sinopharm Chemical Reagent Co., Ltd.
Specification	500mL/bottle
Batch No.	20230324
Blank control sample：The MEM medium contained 10% FBS	

5 Reagents and Instrument

5.1 Reagents

Name	Supplier
FBS	damas life®
MEM medium (100IU/mLPNC，100ug Streptomycin)	Bio-Channel
Trypsin (EDTA) solution	Gibco
PBS	Biosharp
MTT	Beyotime
IPA	Sinopharm Chemical Reagent Co., Ltd.

5.2 Instrument

Name	Instrument ID
CO_2 incubator	WPE-TL0077
Biological microscope	WPE-TL0139
Clean bench	WPE-TL0125
Centrifuge	WPE-TL0279
Constant temperature incubator	WPE-TL0275
Electronic scales	WPE-TL0242
ELISA Reader	WPE-TL0170
pH meter	WPE-TL0079

6 Test system

Cloning L929 is standard recommended cell line, and this cell comes from Cell Bank/Stem Cell Bank, Chinese Academy of Sciences.

Contact of the test sample with the test system via an extract solution (The MEM medium contained 10% FBS) is considered the optimal route of administration and is the recommended method in standard.

7 Test procedure

7.1 Sterilization

Petri dishes, porous culture plates, pipette tips, samples (if necessary), and other utensils that may be used in the test are sterilized by high pressure steam prior to the test.

7.2 Sample preparation

Samples were sterilized by UV for 30 min before preparation.

Under aseptic operation, the extracts were prepared according to the method in the table below. After the extraction, the changes of the extracts were checked. The extracts were not centrifuged and etc. The pH was not adjusted. Blank control, negative control and positive control samples were prepared by the same method.

Table 7-1 Preparation of extracts

Extraction solvent	Actually sample	Sampling ratio	Solvent volume	Sampling condition	Whether the extract is clear	pH
MEM medium containing 10%FBS	3.7979g	0.1g: 1mL	37.97mL	37±1℃ 24±2h 60rpm	Yes	8.5

7.3 Test procedure

The test procedure is sterile operation.

L929 monolayer cells cultured in 10% FBS MEM medium for 48 h to 72 h were liquefied with enzyme liquid (trypsin / EDTA).

The cells are then resuspended in culture medium and the cell suspension is adjusted at a density of 1×10^5 cells/mL.

Using a multichannel pipette, dispense 100 µl culture medium only (blank) into the peripheral wells of a 96-well tissue culture microtitre plate. In the remaining wells, dispense 100 µl of a cell suspension of 1×10^5 cells/mL. Set blank (left and right 2 groups), negative control, positive control, sample group, each group has 6 parallel wells.

Incubate cells for 24 h (5 % CO_2, 37 °C, 90 % humidity) so that cells form a half-confluent monolayer.

After 24 h incubation, aspirate culture medium from the cells. Per well, add 100 µl of treatment medium containing either the appropriate concentration of sample extract, or the negative control, or the positive control, or blank control. Four different sample extraction concentrations (100%, 50%, 25%, 12.5%) were tested.

Incubate cells for 24 h (5 % CO_2, 37 °C, 90 % humidity).

After 24h of testing, the plate and cell morphology were examined under an inverted biommicroscope, and the changes in cell morphology due to cytotoxicity of the sample extract was recorded.

After the examination of the plates, carefully remove the culture medium from the plates. 50 µl of the MTT solution is then added to each test well and the plates are further incubated for 2 h in the incubator at 37°C. Then the MTT solution is discanted and 100 µl of isopropanol are added in each well. Sway this plate and subsequently transfer it to a microplate

reader equipped with a 570nm filter to read the absorbance (reference wavelength 650nm).

7.4 Data analysis

Compared with blank group, cell survival rate was calculated by following formula.

$$\text{Viab.}\ (\%) = \frac{100 \times \text{OD}_{570e}}{\text{OD}_{570b}}$$

where: OD_{570e}——is the mean value of the measured optical density of the 100 % extracts of the test sample.

OD_{570b}——is the mean value of the measured optical density of the blanks.

7.5 Qualitative evaluation

According to standard, A useful way to grade test samples is given in Table 7-2.

Table 7-2 Qualitative morphological grading of cytotoxicity of extracts

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth
1	Slight	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50 % growth inhibition observable.
3	Moderate	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completly destroyed, but more than 50 % growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

7.6 Quality check

If the average OD_{570} of the blank $\geqslant$ 0.2, the test meets the acceptance criteria.

A test meets acceptance criteria if the 96-well plate left side (row 2) and the right side (row 11) mean of the blanks do not differ by more than 15 % from the mean of all blanks.

7.7 Presentation of results

The lower the Viab. % value, the higher the cytotoxic potential of the test

item is.

If viability is reduced to < 70% of the blank, it has a cytotoxic potential. The 50% extract of the test sample should have at least the same or a higher viability than vv; otherwise the test should be repeated.

8 Test result

The qualitative morphological classification of cytotoxicity of extracts from different groups was shown in Table 8-1. Viab. (100%) was shown in Table 8-2.

Table 8-1 Qualitative morphological classification of cytotoxicity of extracts from different groups

Group	Cell morphology observation	Grade
Blank control	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth	0
Positive control	Nearly complete or complete destruction of the cell layers.	4
Negative control	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth	0
Sample solution (100%)	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth	0
Sample solution (50%)	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth	0
Sample solution (25%)	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth	0
Sample solution (12.5%)	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth	0

Table 8-2 Optical Density and Viab.%

Group	Average Optical Density	Viab.(%)
Blank control	0.4003±0.0409	100.00
Positive control	0.0773±0.0107	19.32
Negative control	0.4297±0.0199	107.35
Sample solution (100%)	0.4295±0.0376	107.31
Sample solution (50%)	0.4333±0.0293	108.27
Sample solution (25%)	0.4028±0.0323	100.65
Sample solution (12.5%)	0.3968±0.0247	99.14

9 Test conclusion

The cell viability of test sample extract was 107.31% the cell morphology grade of test sample extract was grade 0, the sample extract had no potential toxic effect on L-929 cells.

10 Deviations

The test was carried out in strict accordance with the standard operating procedures, and no deviation affecting the validity of the test data occurred.

11 Record Preservation

All raw data and records related to this test and copies of the final report are kept in the archives.

Shanghai WEIPU Testing Technology Group Co., LTD.

Test report photo page

Photos and descriptions

Test component description
Random sampling
Model, specification or other description
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***** End of report *****