IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 10, Issue 4 Series 1 (July. – August. 2024), PP 10-19
www.iosrjournals.org

Assessment Of Genotoxicity And Chromosomal Stability Of Aloe Extract Complex Using An In Vitro Test

Dongmyong Kim[1,5†], Chaeyun Yang[2†] , Yeojin Lee[3], Seohyeon Hwang[4] Hyung-Kon Lee[1], Yong-Seong Kwon[1], And Yeon-Mea Choi[5]

1R&D Center KJMBIO Ltd, Korea
2Department Of Chemical & Biomolecular Engineering, KAIST, Korea
3Department Of Biomedical Engineering, UNIST, Korea
4Department Of Biological Sciences, KAIST, Korea
5kimjeongmoon Aloe Ltd, Korea
†: Contributed Equally

Abstract:
Aloe has long been valued for its medicinal properties, particularly its anti-inflammatory, antioxidant, and immunomodulatory effects. Despite its widespread application in cosmetics, dietary supplements, and pharmaceuticals, concerns regarding the genetic stability of aloe extract components require thorough scientific evaluation. This study aimed to investigate the genotoxic potential of a complex aloe extract containing quercetin and aloesin. Utilizing the reverse mutation test on specific bacterial strains (Salmonella typhimurium and Escherichia coli) and the chromosomal aberration test on Chinese hamster lung fibroblasts (CHL/IU cells), we assessed the mutagenicity and chromosomal stability of various concentrations of aloe extract. Results from the reverse mutation test indicated no significant increase in revertant colonies across all bacterial strains, both with and without metabolic activation. Similarly, the chromosomal aberration test showed no statistically significant rise in chromosomal abnormalities in CHL/IU cells treated with aloe extract. These findings suggest that aloe extract does not induce genetic mutations or chromosomal aberrations under the tested conditions, thereby supporting its genetic stability and safe use in multiple industrial applications.

Key Word*: Aloe extract, Genotoxcity, chromosomal stabiliy, mutagenicity,safety*

Date of Submission: 21-07-2024 Date of Acceptance: 31-07-2024

I. Introduction

Aloe is a plant that has been widely recognized for its medicinal properties since ancient times and contains a variety of bioactive compounds that have been reported to have various health benefits, including anti-inflammatory, antioxidant, and immunomodulatory effects.[1,2] Aloe extract in particular has been utilized in a variety of fields, including cosmetics, dietary supplements, and pharmaceuticals, and has been the subject of much research due to its potential usefulness. [3]

However, while existing research suggests that aloe components such as Aloin, Barbaloin, and Anthraquinone contribute to skin regeneration and wound healing,[3,4] the lack of systematic and scientific evaluation of genetic stability raises concerns about their use. Previous studies have reported that certain compounds in aloe may interact with the DNA of cells and cause genetic changes.[5-7].

Chromosomal aberrations include structural changes such as deletions, duplications, inversions, and translocations, which are important indicators of genotoxicity.[8] Genotoxicity is the ability of a substance to damage genetic information in cells and cause mutations, which can potentially lead to cancer.[9,10] Therefore, genotoxicity and gene mutations need to be thoroughly characterized using established scientific methods.

This study, first, using the reverse mutation test, a widely used method for assessing mutagenicity, we studied whether a complex aloe extract containing Quercetin and Aloesin induces genetic mutations using specific bacterial strains of *Salmonella typhimurium* and *Escherichia coli*. In a comprehensive analysis of chromosomal aberration types and frequencies, genotoxic effects of aloe extracts on CHL/IU cell line derived from Chinese hamster lung and cytogenetic analysis of chromosomal aberration types and frequencies were performed. Different concentrations were used to assess the frequency and type of chromosomal aberrations to determine if these extracts and other complexes are genotoxic. The aims is to confirm the genetic stability of aloe extract and other complexes and support their continued use in various products.

II. Material And Methods

Reverse Mutation Test

1) Materials

(1) Test substance

Aloe extract containing Quercetin: 0.48 mg/g, Aloesin: 0.06 mg/g in the form of off-white powder was supplied by Kim JeongMoon Aloe Life Science Research Institute.

(2) Negative control substance

Dimethyl sulfoxide (DMSO; Sigma-Aldrich Co, USA) was used

(3) Positive control substance

Positive controls were 2-Aminoanthracene(2-AA) and Benzo[a]pyrene(B[a]P) in the metabolically active assay and Sodium azide(SA), 2-Nitrofluorene(2-NF), 4-Nitroquinoline-1-oxide(4NQO), and Acridine Mutagen(ICR-191) in the non-metabolically active assay from Sigma-Aldrich Co. (USA) according to the OECD guideline TG 471.

2) Test system

(1) Strains

The strains used in this study were obtained from Molecular toxicology Inc. (P.O. BOX 1189 BOONE, NC 28607, USA), which were purchased from Molecular toxicology Inc. and characterized and subcultured at ChemOn Nonclinical Research Center, and the histidine-requiring strains Salmonella typhimurium TA100, TA1535, TA98, TA1537 (Maron and Ames, 1983)[11] and the tryptophan-requiring strain Escherichia coli WP2 uvrA (Green and Muriel, 1976) were used. These strains were chosen because they are recommended in the MFDS and OECD guidelines mentioned above and have been demonstrated to be sensitive in detecting mutagenicity of various chemicals. For long-term storage, strains were frozen at -70°C or below in frozen vials with 90 µL of DMSO per 1 ml of culture.

(2) Media

- Liquid medium:

2.5% Oxoid Nutrient broth No. 2 was used for pre-culture of the strain

- Minimal glucose agar plate:

1.5% Bacto agar (Difco) with Vogel-Bonner medium E and 2% glucose was used, dispensed in 25 mL portions into Petri dish (90 x 15 mm, gamma-irradiated). For *E. coli*,[12,13]the same minimal medium as above with 0.1% tryptophan solution added at 0.25 mL/L was used.

- Top agar:

Prepared with 0.6% Bacto agar and 0.5%NaCl, 10 mL of 0.5 mm histidine-biotin solution per 100 mL was added to the top agar for Salmonella strains only.

- Master plate:

Master plates were prepared by thawing frozen strains and plating them on appropriate minimal media for 10 hours of incubation and refrigerated for subsequent use in the test. A portion of the culture was used for characterization, and only those strains that were characterized were used for testing.

(3) Preparation of metabolically active system

S9 used liver from male Sprague-Dawley rats induced with Aroclor-1254, protein content was 35.0 mg/mL, frozen at -15°C or below. Cofactor was used as Cofactor-1 (Wako Pure Chem. Ind., Ltd. (Japan)) and stored at -1~10°C. For 1 mL of S9 mix, 8 µmol $MgCl_2$, $6H_2O$, 33 µmol KCl, 5 µmol G-6-P, 4 µmol NADPH, 4 µmol NADH, 100 µmol sodium phosphate buffer (pH 7.4), and 0.3 mL S9 were used, and the prepared S9 mix was filled with ice.

(4) Test substance preparation

The test substance was used in the preparation at its weight without correction for content. An appropriate amount of the test substance was weighed and the highest concentration was made by adding a negative control substance, and each concentration group was prepared by diluting it with the same negative control substance. The test substance for treatment was prepared immediately before treatment. No analysis was performed on the test substance at this time.

(5) Preparation of Positive Controls

Positive control substance SA was prepared with sterile water (Daehan Pharmaceutical Industry Co., Ltd., manufacture no. A6M7F21), frozen and thawed. 2-AA, B[a]P, 2-NF, 4NQO, and ICR-191 were prepared in DMSO (Sigma-Aldrich Co., # 472301-500ML, manufacture no. SZBD0030V, ≥99.9%) and stored frozen, then thawed and used.

3) Test method

(1) Treatment concentration determination test

For the determination of the treatment concentration in this test, the results of the preliminary test were determined based on the same method as this test. Eight levels of concentration ranging from 5-5000 μg/plate were treated, and the plates were observed for precipitation during mixing with top agar and agglutination counting. As a result, the test groups were organized as shown in table 1, and three plates per concentration group, including negative and positive controls, were used in this test.

Table 1: Test group composition for a reverse mutation test

Strain	S9 mix	Concentration(μg/plate)					
TA strains	+ / -	15	50	150	500	1500	5000
WP2 *uvr*A	+ / -	15	50	150	500	1500	5000

(2) Main test

In this test, the test substance was treated by direct plate incorporation method. The test strain was inoculated into 20 mL of liquid medium (2.5% Oxoid Nutrient Broth No. 2) from the master plate, pre-culture for 10 hours in a shaking incubator (37 ± 2°C, 120 rpm), and the live bacterial count was calculated by measuring the absorbance at a wavelength of 600 nm, and then refrigerated until use in the test. Autoclaved top agar was dispensed in 2 mL aliquots into sterile tubes (12 x 75 mm) placed in a dry bath maintained at a temperature of 45 ± 2°C, followed by 0.5 mL of S9 mix for metabolic activator application and 0.5 mL of sodium-phosphate buffer, pH 7.4, 0.1 mL of bacterial broth, and 0.1 mL of test substance solution were added, mixed together, vortexed for 2-3 seconds with a vortex mixer, poured onto minimal glucose agar plates, and spread evenly by tilting in several directions to solidify.

The negative control was performed by adding 0.1 mL of Dimethylsulfoxide instead of the test substance solution, and the positive control was performed by adding the positive control solution in the same way. To confirm the sterility of the test substance and S9 mix, plates were prepared by mixing 0.1 mL of the highest concentration of the test substance and 0.5 mL of the S9 mix into 2 mL of top agar. After the top agar solidified at the end of the treatment, the plates were inverted and incubated at 37 ± 2°C for 50 ± 2 hours, and the returning mutant colonies were visually counted. [14,15]

4) Observation and measurement

(1) Observation of precipitation of test substance

During the test substance treatment, the test substance solution was mixed with top agar and observed for precipitation. If particles that can be seen with the naked eye are observed, it was judged as precipitation.

(2) Colony counting

Colonies were also counted visually, and when counting colonies, the formation of the background lawn of the plates was checked compare with the negative control, and the occurrence of contamination or other abnormalities was checked. The counts were expressed as the mean ± standard deviation of the number of colonies obtained from three plates per concentration group and as a multiple of the increase over the negative control, and cytotoxicity was determined when the number of colonies decreased or increased to less than 50% of the negative control.

Chromosome Anomaly Test

1) Materials

(1) Test material

Same as for the reverse mutation test

(2) Negative control substance

Sterilized injectable water (Daihan Pharmaceutical Industry, Korea)

(3) Positive control substance

Benzo[a]pyrene (B[a]P) was used for metabolically active system and Ethylmethanesulfonate (EMS) was used for non-metabolically active system from Sigma-Aldrich Co. (USA) according to OECD guideline TG 473.

2) Test system

(1) Cell

The CHL/IU cell line derived from female Chinese hamster lung fibroblasts was used in this study. [16] This cell line has been widely used for genotoxicity testing of various chemicals and has been shown to be suitable for detection of chromosomal aberrations [17-19]. The cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) (Cat. No. CRL-1935). According to information provided by ATCC, this cell line has a chromosome modal number of 25 and a division cycle of approximately 15 hours. Based on the cell proliferation rate and karyotype of the cell line used in this test, the chromosome modal number is 25 and the division cycle is approximately 13.5 hours.

(2) Culture Method

The culture medium was prepared by adding sodium bicarbonate (2200 mg), L-glutamine (292 mg), and penicillin-streptomycin broth (Gibco-BRL #15140-122) to 1 liter of Minimum Essential Medium (Gibco-BRL #41500-034) powdered medium, and adjusting the total volume to 1000 mL with sterile sterile water. Filtered through a 0.2 μm membrane filter to which 100 mL of Fetal Bovine Serum (Gibco-BRL #16000-044) was added. Cells were cultured in cell culture flasks (75 cm^2 culture area, Falcon) at 37 ± 1°C in a thermostat incubator (Forma 311 and 3111) containing 5% carbon dioxide and saturated water vapor.[20] Cells were detached and passaged in 0.1% trypsin solution every 2-3 days. 6×10^4 cells per flask (Falcon) with a culture area of 25 cm^2 were seeded with 5 mL of culture medium and incubated for approximately 3 days. Two flasks were used per concentration group.

For long-term preservation of cells, cells were frozen in liquid nitrogen with 10% (v/v) dimethylsulfoxide added to suspension in culture medium containing 15% fetal bovine serum (FBS). Thawed and cultured cells were subjected to proliferation rate measurements, karyotyping, and examination for contamination with mycoplasma. Frozen cells were thawed and incubated for at least 7 days before being used for subsequent test material processing. The actively proliferating cells were trypsinized and the number of cells per mL was calculated using a Coulter counter model Z2 (Beckman Coulter), and based on this, 6×10^4 cells per 25 cm^2 flask were seeded in 5 mL of culture medium and incubated for approximately 3 days before treatment with the test substance.

(3) Preparation of metabolically active systems

S9 and Cofactor were prepared the same as in the reverse mutation test and the composition of the S9 mix is the same.

(4) Preparation of test substance and positive control substance

The test substance was prepared in the same manner as the reverse mutagenesis test, and the positive control B[a]P was prepared by dissolving in Dimethylsulfoxide (DMSO), and the crude solution was frozen at 50 °C or below, thawed immediately before treatment, and used in the test. EMS was prepared by dissolving in culture medium immediately prior to treatment.

3) Test method

(1) Treatment concentration determination test

The treatment concentration for this test was determined based on the results obtained in a preliminary test. In this test, the highest concentration was set at 5000 μg/mL, and four concentration groups of 0, 1250, 2500, and 5000 μg/ml were set, and the test groups were organized as shown in Table 2.

Table 2: Test group composition for a Chromosome Anomaly Test

Metabolic activation	Positive control and dose (μg./mL)	Treatment time-recovery time (hrs)	Dose of test article (μg./mL)
+	B[a]P 20	6-18	0, 1250, 2500, 5000
-	EMS 800	6-18	0, 1250, 2500, 5000
-	EMS 600	24-0	0, 1250, 2500, 5000

(2) Main test

In this test, cells were prepared by dividing them into series 1, 2, and 3, and all the culture medium in each flask was removed before the test substance treatment, and 2.2 mL of culture medium for the metabolically active group (series 1) and 4.5 mL for the untreated group (series 2 and 3) was dispensed and allowed to stand for at least 1 hour before the test substance treatment. The negative control was treated with the same amount of Sterilized injectable water only. The treatment solution composition is shown in the Table 3 below. For series 1 and series 2, the treatment solution containing the test article was removed from the flask approximately 6 hours after the start of treatment, and the cell layer was washed once with 5 mL of Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (CMF D-PBS), followed by 5 mL of fresh culture medium and continued incubation until the collection of Metaphase cells. Series 3 was processed without washing and continued until Metaphase cell collection.

For sample preparation, approximately 22 hours after the start of test substance treatment, all flasks were treated with colchicine solution (50 μL) (final concentration 1 μM) and Metaphase cells were collected by shaking after 2 hours. The cultures containing metaphase cells were centrifuged, mixed with 75 mM KCl solution, fixed (methyl alcohol:glacial acetic acid=3:1 v/v) and air-dried to prepare chromosome samples and stained with 5% Giemsa solution. Samples were prepared in duplicate for each flask. After the collection of metaphase cells, the cells remaining in the flasks were detached and counted with trypsinization solution to calculate the RCC value, which was utilized as a measure of cytotoxicity.

Table 3: Test substance treatment solution composition table according to series

Series	Medium+Aloe extract Complex	S9 mix	Final volume
1	2.2 mL + 0.3 mL	0.5 mL	3.0 mL
2	4.5 mL + 0.5 mL	-	5.0 mL
3	4.5 mL + 0.5 mL	-	5.0 mL

4) Observation and measurement

(1) Counting of chromosomal aberrations

The morphology and counting of chromosome anomaly were performed according to the 'Atlas of Chromosome Phases (1988)' of the MMS of the Japan Environmental Mutagen Society (JEMS). One out of two samples from each flask was selected and coded, and chromosomal aberrations were counted at a magnification of 1000x for 100 metaphase cells.

For structural abnormalities, metaphase with a centromere number of 23-27 was observed for the presence or absence of mitotic figures and chromosome aberrations, and if chromosome aberrations were observed, the type and number of abnormalities and their location on the slide were recorded. Abnormalities were categorized into chromosomal breaks and exchanges and chromosomal breaks and exchanges, and cases with and without gaps were noted. Chromosomal abnormalities with 10 or more chromosomal aberrations (including gaps, multiple aberrations) or fragmentation were categorized as 'other' and counted as one abnormality.

In the numerical abnormalities, 100 metaphases with or without abnormalities were counted and categorized into diploid (23 - 36 motifs), polyploid (37≤ motifs), and endoreduplication according to the number of motifs, and the number was recorded.

(2) Statistical processing and judgment

Statistical analysis of the test results was performed using the SPSS (ver. 10.1K) program. First, metaphase cell with at least one structural abnormality were categorized as abnormal metaphase cell and applied to statistical analysis, and metaphase cell with only GAP were excluded. Data from the negative control and treatment groups were compared by Fisher's exact test, and statistical significance was determined when the *P* value was less than 0.05. Dose correlation was tested by linear-by-linear association using the Chi-square test when the frequency of abnormal metaphase cell was significantly increased in the test substance treatment group.

Numerical abnormalities were evaluated in the same way as structural abnormalities for the sum of metaphases with a mobilization number of 37 or higher and metaphases with nuclear internalization.

The determination of negative and positive controls was considered positive if the frequency of metaphases with chromosomal aberrations in the test article treated group was statistically significant and increased in a dose-dependent manner compared to the negative control, or if there was a reproducible positive test in one or more dose steps. However, statistical significance was not the sole basis for a positive test result; biological relevance, frequency of abnormal metaphases, and degree of cytostatic (cytotoxic) effects were also considered.

III. Result

Dose determination in the reverse mutation test

In preliminary testing at concentrations of 5-5000 µg/plate, no precipitation was observed at all concentrations, and no cytotoxicity was observed on plates upon counting. No substantial increase in colony counts was observed for any strain. When the preparations were mixed with top agar, no turbidity or sedimentation was observed at any concentration. No precipitation or other abnormalities were observed on all plates during colony counting, and no colonization due to microbial contamination was seen on plates used to confirm sterility of the highest concentration of the test substance and S9 mix.

Observation of reverse mutation colony counts (main test)

In this study, the returning colony counts were similar to the negative control or increased but did not exceed 2-fold in all doses and in four *Salmonella typhimurium* TA100, TA1535, TA98, and TA1537, regardless of whether metabolic activation was applied in the complex with aloe extract. No dose-dependent increase was observed. However, when compared to the positive control, a clear increase was observed in all strains, well above 2-fold compared to the negative control. (Table 4, 5)

In four strains of *Salmonella typhimurium*, TA100, TA1535, TA98, and TA1537, with and without metabolic activator, no increase in colonies was observed with increasing concentrations of the test substance, and no cytotoxicity was observed.

In *E. coli* WP2 *uvr*A, there was no increase in the number of colonies with increasing concentrations of the test substance, both with and without metabolic activity, and no cytotoxicity. On the other hand, all positive controls gave clear positive results.

The five strains used in the test had a live count of 1.84-2.95 x 10^9 (TA strain) and 3.20 x 10^9 (*E. coli*) CFU/mL based on absorbance at wavelength 600 nm, and all plates had more than 0.5 x 10^8 CFU of treated live bacteria per plate.

Table 4: Reverse mutagenicity assay results in the presence of S9 mix

Strain	Chemical treated	Dose(*µg*/plate)	Colonies/plate			Mean ± S.D.
TA100	Complex of aloe extract	0	122	139	132	131 ± 9
		15	133	139	110	127 ±1 5
		50	130	116	139	128 ± 12
		150	130	122	144	132 ± 11
		500	132	131	113	125 ± 11
		1500	113	121	124	119 ± 6
		5000	127	118	133	126 ± 8
	2-AA	1.0	928	900	904	911 ± 15
TA1535	Complex of aloe extract	0	9	9	12	10 ± 2
		15	10	5	10	8 ± 3
		50	9	8	9	9 ± 1
		150	12	10	10	11 ± 1
		500	10	14	9	11 ± 3
		1500	9	9	5	8 ± 2
		5000	3	8	8	6 ± 3
	2-AA	2.0	190	188	184	187 ± 3
TA98	Complex of aloe extract	0	38	39	33	37 ± 3
		15	32	30	38	33 ± 4
		50	21	39	33	31 ± 9
		150	40	38	36	38 ± 2
		500	31	34	30	32 ± 2
		1500	36	33	40	36 ± 4
		5000	36	36	35	36 ± 1
	B[a]P	1.0	210	222	248	227 ± 19
TA1537	Test article	0	10	12	10	1 ± 11
		15	4	10	8	7 ± 3
		50	12	6	14	11 ± 4
		150	6	12	11	10 ± 3
		500	8	6	8	7 ± 1
		1500	9	15	9	11 ± 3
		5000	10	8	6	8 ± 2
	2-AA	1.0	166	177	159	167 ± 9
E. coli WP2 *uvr*A	Complex of aloe extract	0	14	22	24	20 ± 5
		15	28	25	20	24 ± 4
		50	20	18	23	20 ± 3
		150	16	11	19	15 ± 4
		500	22	18	21	20 ± 2
		1500	18	18	23	20 ± 3

		5000	21	23	29	24 ± 4
	2-AA	6.0	96	94	90	93 ± 3

Table 5: Reverse mutagenicity assay results in the absence of S9 mix

Strain	Chemical treated	Dose(μg/plate)	Colonies/plate			Mean ± S.D.
TA100	Complex of aloe extract	0	118	112	109	113 ± 5
		15	122	122	107	117 ± 9
		50	130	122	101	118 ± 15
		150	121	119	116	119 ± 3
		500	110	122	128	120 ± 9
		1500	121	115	115	117 ± 3
		5000	92	98	113	101 ± 11
	2-AA	1.0	560	542	555	552 ± 9
TA1535	Complex of aloe extract	0	8	9	10	9 ± 1
		15	6	9	7	7 ± 2
		50	6	6	9	7 ± 2
		150	9	8	10	9 ± 1
		500	10	7	9	9 ± 2
		1500	8	11	9	9 ± 2
		5000	7	6	10	8 ± 2
	2-AA	2.0	355	332	370	352 ± 19
TA98	Complex of aloe extract	0	31	33	37	34 ± 3
		15	25	32	26	28 ± 4
		50	32	38	33	34 ± 3
		150	37	31	38	35 ± 4
		500	27	33	31	30 ± 3
		1500	37	28	32	32 ± 5
		5000	42	37	34	38 ± 4
	B[a]P	1.0	280	230	236	249 ± 27
TA1537	Test article	0	8	10	12	10 ± 2
		15	8	12	8	9 ± 2
		50	10	15	13	13 ±3
		150	12	12	10	11 ± 1
		500	14	16	12	14 ± 2
		1500	8	8	12	9 ± 2
		5000	10	14	6	10 ± 4
	2-AA	1.0	130	101	104	112 ± 16
E. coli WP2 *uvr*A	Complex of aloe extract	0	21	15	14	17 ± 4
		15	18	18	14	17 ± 2
		50	13	13	20	15 ± 4
		150	18	23	17	19 ± 3
		500	17	21	23	20 ± 3
		1500	16	20	25	20 ± 5
		5000	16	16	19	17 ± 2
	2-AA	6.0	127	154	181	154 ± 27

Frequency of anomalous metaphase in the metabolically active group in the chromosomal aberration test

The frequency of abnormal metaphases with numerical abnormalities was 0.0, 0.0, 0.0, 0.0, and 0.5 in the negative control(0), 1250, 2500, and 5000 μg/mL, respectively, while the frequency of metaphases with structural abnormalities was 17.0 in the positive control. (P<0.01) The sum of the frequencies of polyploidy and endoreduplication in both the negative and positive controls was zero, and the sum of the frequencies of polyploidy and endoreduplication in the test substance-treated groups in the complex with aloe extract was observed to be zero at all concentrations. (Table 6)

Table 6: Chromosome aberration test in the presence of S9 mix-6 hour treatment

Dose(μg/ml)	No. Cells examined(mean)	Aberrations						PP+ER	No. aberrant metaphase		RCC
		csb	cse	ctb	cte	others	Gaps		+ Gaps	- Gaps	
0	100	0	0	0	0	0	0	0	0	0	100
	100	0	0	0	0	0	0	0	0	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	
1250	100	0	0	0	0	0	0	0	0	0	101
	100	0	0	0	0	0	0	0	0	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	
2500	100	0	0	0	0	0	0	0	0	0	102
	100	0	0	0	0	0	0	0	0	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	

5000	100	0	0	0	0	0	0	0	0	0	91
	100	0	0	0	1	0	1	0	2	1	
	(100)	(0.0)	(0.0)	(0.0)	(0.5)	(0.0)	(0.5)	(0.0)	(1.0)	(0.5)	
20 B[a]P	100	0	0	4	16	0	2	0	16	14	71
	100	0	0	10	20	0	1	0	20	20	
	(100)	(0.0)	(0.0)	(7.0)	(18.0)	(0.0)	(1.5)	(0.0)	(18.0)	(17.0)	

Test article: Complex of aloe extract
a) 6-hour treatment - 18-hour recovery.
b) Inclusive/exclusive gaps, means of duplicate cultures. 100 metaphases were examined per culture.
PP: Polyploid ER: Endoreduplication
B[a]P: Benzo[a]pyrene (positive control article)
RCC: Relative Cell Counts = (Cell count of treated flask / Cell count of control flask) x 100 (%)
Gaps: Chromosome type + Chromatid type gaps
csb: Chromosome type deletions cse: Chromosome type exchanges
ctb: Chromatid type deletions
cte: Chromatid type exchanges
Other: Metaphases with more than 10 aberrations (including gaps) or with chromosome fragmentation

Frequency of adverse intermediate phase in the untreated group

The frequency of abnormal metaphase in the 6 h treatment group without metabolite was 1.0, 0.0, 0.5 and 0.0 for the negative control (0), 1250 2500 and 5000 µg/mL treatments, respectively, while the frequency of metaphase with structural abnormalities was 23.5 in the positive control (EMS). ($P < 0.0.1$) In the negative control and all concentrations of the aloe extract complex, the sum of the polyploid and endoreduplication frequencies was zero. (Table 7)

The frequency of anaphase in the 24-hour treatment group was 0.0 in the negative control and all test article treatments, compared to 26.0 in the positive control. ($P < 0.0.1$) The frequency of mesophase with water phase was 0.0 in the negative control and all test article treatments. (Table 8)

Table 7: Chromosome aberration test in the absence of S9 mix-6hour treatment

Dose(µg/ml)	No. Cells examined(mean)	Aberrations						PP+ER	No. aberrant metaphase		RCC
		csb	cse	ctb	cte	others	Gaps		+ Gaps	- Gaps	
0	100	0	0	0	1	0	1	0	2	1	100
	100	0	0	1	0	0	2	0	3	1	
	(100)	(0.0)	(0.0)	(0.5)	(0.5)	(0.0)	(1.5)	(0.0)	(2.5)	(1.0)	
1250	100	0	0	0	0	0	3	0	0	0	103
	100	0	0	0	0	0	0	0	0	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(1.5)	(0.0)	(0.0)	(0.0)	
2500	100	0	0	1	0	0	0	0	1	1	107
	100	0	0	0	0	0	0	0	0	0	
	(100)	(0.0)	(0.0)	(0.5)	(0.0)	(0.0)	(0.0)	(0.0)	(0.5)	(0.5)	
5000	100	0	0	0	0	0	0	0	0	0	104
	100	0	0	0	0	0	1	0	1	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.5)	(0.0)	(0.5)	(0.0)	
800 EMS	100	0	0	6	30	1	1	0	24	24	78
	100	0	0	4	22	0	5	0	26	23	
	(100)	(0.0)	(0.0)	(5.0)	(26.0)	(0.5)	(3.0)	(0.0)	(25.0)	(23.5)	

Table 8: Chromosome aberration test in the absence of S9 mix-24 hour treatment

Dose(µg/ml)	No. Cells examined(mean)	Aberrations						PP+ER	No. aberrant metaphase		RCC
		csb	cse	ctb	cte	others	Gaps		+ Gaps	- Gaps	
0	100	0	0	0	0	0	0	0	0	0	100
	100	0	0	0	0	0	0	0	0	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	
1250	100	0	0	0	0	0	0	0	0	0	102
	100	0	0	0	0	0	0	0	0	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	
2500	100	0	0	0	0	0	0	0	0	0	97
	100	0	0	0	0	0	0	0	0	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	
5000	100	0	0	0	0	0	0	0	0	0	102

	100	0	0	0	0	0	1	0	1	0	
	(100)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.5)	(0.0)	(0.5)	(0.0)	
600 EMS	100	0	0	7	17	1	6	0	26	23	
	100	0	0	11	30	0	4	0	31	29	77
	(100)	(0.0)	(0.0)	(9.0)	(23.5)	(0.5)	(5.0)	(0.0)	(28.5)	(26.0)	

IV. Discussion

Reverse Mutation Test

Based on the preliminary experimental results, the highest dose for this test was 5,000 μg/plate, and the following six dose groups (12, 50, 150, 1500, and 5000 μg/plate) were established. In addition, a negative control group and a positive control group were established.

In this test, the frequency of cells with chromosomal structure abnormalities in the negative control group was within the range of historical control data (Table 9) and within 95% of historical control data. In the positive control group, the frequency of cells with chromosomal structure abnormalities was within the range of the historical control data, and a statistically significant increase was confirmed compared to the negative control group. In addition, more than 300 Metaphase during division cells were observed per dose of the control and test substances, and more than three readable phases were obtained, confirming that the test was conducted under appropriate conditions. For all test strains, there was no increase in the mean colony count in the test substance treatment group with or without the metabolic activator, which did not meet the criteria for a positive test result. Therefore, it was concluded that the aloe extract complex was not mutagenic to the test strains used under these test conditions.

Table 9: Historical Control Data of Dimethylsulfoxide(negative control)

Historical negative control(Dimethylsulfoxide) values of revertant colonies					
Strain	S9 mix	Range		Mean±S.D.	No. of plates
		Min	Max		
TA100	+	95	198	147±26	162
	-	88	207	143±24	162
TA1535	+	6	29	14±4	153
	-	8	33	15±4	153
TA98	+	15	46	30±6	159
	-	13	40	23±6	159
TA1537	+	4	28	14±4	156
	-	4	25	11±4	156
WP2 *uvr*A	+	13	39	25±6	159
	-	10	39	21±6	156

Table 10: Historical Control Data of Positive control

Historical positive control values of revertant colonies					
Strain	S9 mix	Range		Mean±S.D.	No. of plates
		Min	Max		
TA100	+	360	988	680±117	228
	-	180	820	464±93	429
TA1535	+	53	484	154±66	411
	-	62	648	293±90	411
TA98	+	95	521	228±87	273
	-	192	468	328±58	165
TA1537	+	49	711	164±75	420
	-	41	724	233±107	318
WP2 *uvr*A	+	69	298	130±39	219
	-	68	424	186±68	417

Chromosomal Anomaly Test

To determine the mutagenicity of aloe extract and other complexes, chromosomal aberration test was performed as an indicator of mutagenicity. For this purpose, Chinese hamster lung cells (CHL) were used to perform the chromosomal aberration test with (+S) and without (-S) metabolic activity system (S9 mix). The results showed that there was no visible turbidity in all concentration groups of the formulated test substance, indicating that there was no precipitation. Based on the above results, counting of chromosomal aberrations showed no statistically significant increase in the frequency of aberrant metaphases in any of the test article treated groups compared to the negative control. Therefore, it was concluded that Aloe vera extract did not cause statistically significant chromosomal aberrations in CHL/IU cells. Counting of chromosome translocations from 100 metaphases was possible in all flasks of at least three concentrations of the test article treatments. In the negative control group, the frequency of metaphase cells with structural abnormalities of chromosomes, excluding gaps, was less than 5%, while in the positive control group this frequency was greater

than 10%. The clear increase in the number of abnormal metaphases in the B[a]P-treated group provided evidence for the activity of S9 mix, thus fulfilling the validity of the test.

V. Conclusion

The reverse mutation test and chromosomal aberration test confirmed that the complex, including aloe extract, did not cause statistically significant genetic and chromosomal aberrations, further confirming the anti-inflammatory, antimicrobial, and antioxidant benefits of aloe in addition to previous studies that have demonstrated its anti-inflammatory, antibacterial, and antioxidant properties, and providing scientific evidence of its genetic stability. These findings could be used as a basis to promote the use of aloe extract in various industries, including dietary supplements, cosmetics, and pharmaceuticals.

References

[1]. Sánchez, Marta ; González-Burgos, Elena ; Iglesias, Irene ; Gómez-Serranillos, M. Pilar, Pharmacological Update Properties Of Aloe Vera And Its Major Active Constituents, Molecules (Basel, Switzerland), 2020-03, Vol.25 (6), P.1324, Article 1324

[2]. Darzi, Saeedeh ; Paul, Kallyanashis ; Leitan, Shanilka ; Werkmeister, Jerome A. ; Mukherjee, Shayanti, Immunobiology And Application Of Aloe Vera-Based Scaffolds In Tissue Engineering, International Journal Of Molecular Sciences, 2021-02, Vol.22 (4), P.1-19, Article 1708

[3]. Mariana Chelu , Adina Magdalena Musuc , Monica Popa And Jose Calderon Moreno, Aloe Vera-Based Hydrogels For Wound Healing: Properties And Therapeutic Effects, Gels 2023, 9(7), 539

[4]. Kloosterman, Wigard P. ; Hochstenbach, Ron, Deciphering The Pathogenic Consequences Of Chromosomal Aberrations In Human Genetic Disease, Molecular Cytogenetics, 2014-12, Vol.7 (1), P.100-100, Article 100

[5]. Yakkala, Lakshmi Madhuri ; Somanjeri, Nithya ; Begum, Shaheen, A Validated Stability-Indicating Rp-Hplc Method For The Simultaneous Estimation Of Aloe Emodin, Aloin, And Emodin In A Marketed Formulation Of Aloe Vera, Journal Of Analytical Chemistry (New York, N.Y.), 2024, Vol.79 (6), P.808-812

[6]. Sadiq, Uzma ; Gill, Harsharn ; Chandrapala, Jayani, Temperature And Ph Stability Of Anthraquinones From Native Aloe Vera Gel, Spray-Dried And Freeze-Dried Aloe Vera Powders During Storage, Foods, 2022-05, Vol.11 (11), P.1613

[7]. Unaydin-Akyildiz, Aysenur ; Yanikoglu, Rabia Sare ; Gulec, Meltem ; Alim-Toraman, Gulbahar Ozge ; Kuran, Ebru Didem ; Atasoy, Sezen ; Olgun, Abdullah ; Topcu, Gulacti, Emodin And Aloe-Emodin, Two Potential Molecules In Regulating Cell Migration Of Skin Cells Through The Map Kinase Pathway And Affecting Caenorhabditis Elegans Thermotolerance, Bmc Molecular And Cell Biology, 2023-07, Vol.24 (1), P.23-23, Article 23

[8]. Dey, Atreya ; Shi, Guang ; Takaki, Ryota ; Thirumalai, D., Structural Changes In Chromosomes Driven By Multiple Condensin Motors During Mitosis, Cell Reports (Cambridge), 2023-04, Vol.42 (4), P.112348-112348, Article 112348

[9]. Hüseyin Yılmaz, Tuğçe Kalefetoğlu Macar, Oksal Macar, Kültiğin Çavuşoğlu, Emine Yalçın, Dna Fragmentation, Chromosomal Aberrations, And Multi-Toxic Efects Induced By Nickel And The Modulation Of Ni-Induced Damage By Pomegranate Seed Extract In Allium Cepa L, Environmental Science And Pollution Research (2023) 30:110826–110840

[10]. Jangra, Alka ; Sharma, Garima ; Sihag, Sonia ; Chhokar, Vinod, The Dark Side Of Miracle Plant-Aloe Vera: A Review, Molecular Biology Reports, 2022-06, Vol.49 (6), P.5029-5040

[11]. Maron, Dm And Ames, Bn (1983): Revised Methods For The Salmonella Mutagenicity Test, Mutat, Res., 113:173-215

[12]. Green, Mhl And Muriel, Wj (1976): Mutagen Testing Using Trp+ Reversion In Escherichia Coli., Mutat. Res., 38:3-32

[13]. Green, Mhl (1984) Mutagen Testing Using Trp+ Reversion In Escherichia Coli In Kilbey, Bj, Legator, M, Nichols, W And Ramel. C (Eds.). Handbook Of Mutagenicity Test Procedeures. Second Edition, P.161-187, Elsevier Science Publishers Bv, Amsterdam.

[14]. Ames Test (Bacterial Reverse Mutation Test): Why, When, And How To Use

[15]. Animal Cell Culture Guide, Atcc

[16]. Koyama, H.. T. Utakoji, And T. Ono (1970): A New Cell Line Derived From Newborn Chinese Hamster Lung Tissue, Gann, 61:161-167

[17]. Ji Hye Hwang, Chul Jung, Jaseung Ku, Evaluation Of Genotoxicity Of Cp Pharmacopuncture Using An In Vitro Chromosome Aberration Test In Chinese Hamster Lung Cell, J Physiol & Pathol Korean Med 34(6):355~361, 2020

[18]. Jeong-Won Kim, Hyunjun Kim, Hyunjin Park, Ji-Soo Yoon, Myeong-Il Kim, Je-Won Ko, Tae-Won Kim, Repeated Oral Dose Toxicity And Genotoxicity Of A Standardized Quisqualis Indica Extract, Toxicol Res. (2022) 38:577–589

[19]. Kruhlak, Michael J; Sousa, Alioscka, Nanoimaging : Methods And Protocols, New York : Humana Press, 2013 A.

[20]. Fundamental Techniques In Cell Culture Laboratory Handbook 3rd Edition, Sigma-Aidrich