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Research Article

A Study on the Possible Mutagenicity of Different Types of Plant Growth Regulators

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Abstract: It is known that most chemicals, which have been increasingly used since ancient times, have carcinogenic and mutagenic effects. As an alternative to these chemicals, natural compounds, such as plant growth regulators (PGRs) have been used. However, are the PGRs used to obtain efficient plants in agriculture as harmless as it is thought today when it is essential to access natural foods in the food and agriculture sectors? Therefore, this study investigates the mutagenic activity of two plant growth regulators (Kinetin, Chlormequat chloride (CCC)) using the Ames/*Salmonella* short-time test system. Experiments were performed in the presence (+ S9) or absence (-S9) of metabolic activation enzymes using TA 98 and TA 100 strains of *Salmonella typhimurium*. 5 non-cytotoxic doses for each test substance were investigated. The results were evaluated by comparing them with spontaneous control plates. According to the results, a 2500 µg/plate dose of Kinetin was to be found mutagenic on the strain TA 98 in the presence and in the absence of S9 enzyme, and on the strain TA 100 in the absence of the S9 enzyme. The other substance CCC did not cause mutagenic effects on the bacterial strains.

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1. INTRODUCTION

Parallel to the advancement of technology, the number of chemicals used in agriculture and food is increasing (Tomatis, 1979; Öncül, 2009). Therefore, the identification of substances capable of inducing mutations has become an important procedure in safety assessment (Mortelmans & Zeiger, 2000).

Plant growth regulators (PGRs) are organic compounds, other than nutrients, that modify plant physiological processes. Specific PGRs are used to modify crop growth rate and growth patterns during the various stages of development, from germination through harvest and post-harvest preservation (Harms, 1988). Concerns about the unconscious use of plant growth regulators are increasingly being expressed. These are plant growth regulators that act as synthetic auxins or plant hormones that alter plant metabolism. It is widely used as a herbicide in agriculture and forestry to control broadleaf weeds (Uysal, 2010).

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PGRs fall into five groups: auxins, gibberellins, cytokines, abscisic acid, and ethylene. In addition to these, brassinosteroids, polyamines, jasmonates, oligosaccharin, and salicylates are also considered in this class by a number of researchers (Basra, 2000; Morsünbül *et al*., 2010).

The cytokines discovered during the investigation of the factors that stimulate the division of plant cells (cytokinesis) in subsequent studies are effective in many physiological and developmental events, such as the transport of nutrients, apical dormancy, formation and activity of the stem apical meristem, leaf development, breakage of bud dormancy and seed germination. In addition, it has been determined that cytokines play a role in many light-controlled developments, such as chloroplast differentiation, autotrophic metabolism development, leaf, and cotyledon expansion (Akman & Darıcı, 1998; Tiaz & Zeiger, 2008).

Kinetin is a synthetic cytokine which is first discovered (Özen & Onay, 2007; Tiaz and Zeiger, 2008). The big difference with this substance compared to others is that it is essentially a by-product of DNA degradation by heat, and it affects growth, especially by promoting cell division. In this by-product, the adenosine is displaced from position 9 to position 6 on the ring (Tiaz & Zeiger, 2008). Kinetin also maintains the synthesis of protein and nucleic acid, thereby ensuring the longevity of cut flowers (Güleryüz, 1982; Westwood, 1993; Kaynak & Ersoy, 1997).

Chlormequat chloride (CCC) is a synthetic PGR that prevents the growth of plant height. Although CCC prevents elongation in grains, it is widely used to prevent lying in grains. It is also applied to increase the fruit set in grapes. Due to a belief that it has a carcinogenic effect, its use has been limited to ornamental plants (Sağlam, 1991; Kaynak & Ersoy, 1997).

The Ames Test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs because there is a high predictive value for carcinogenicity when a mutagenic response is obtained (McCann *et al.,* 1975; Zeiger *et al.,* 1990). In this work bacterial reverse mutation assay (Ames test) was used to evaluate the mutagenic potential of the two-plant regulator (Kinetin, Chlormequat Chloride) widely used as plant growth regulators.

2. MATERIAL and METHODS

2.1. Material

The Kinetin that tested for mutagenicity is in the cytokinins group. The second substance is Chlormequat chloride (CCC), the other regulator that inhibits plant growth for the determination of mutagenicity. As the positive control mutagen, while 4-Nitro-o-phenylenediamine was used in the absence of S9 mix, 2-aminofluorene (2AF) was used in the presence of S9 mix for *S. typhimurium* TA 98 strains. As the positive control mutagen for *S. typhimurium* TA 100 strains, sodium azide (SA) in the absence of S9 mix, and 2AF was used in the presence of S9 mix (Table 1).

Table 1. Some chemical properties of the plant growth regulators and positive control examined.

Common Name	Chemical name	Chemical Formula	Purity (%)	Usage as	Reference
Kinetin	6-furfuryl-aminopurine	$C_{10}H_9N_5O$	≥98%	Plant growth regulator	Merck Inc. (2021a)
CCC	Chlormequat chloride	$C_5H_{13}Cl_2N$	100 %	Plant growth regulator	Merck Inc. (2021b)
SA	Sodium Azide	NaN_3	99.99%	Potent mutagen	Merck Inc. (2021c)
2AF	2-aminofluorene	$C_{13}H_{11}N$	98%	Potent mutagen	Merck Inc. (2021d)
4-NOP	4-Nitro-o-phenylenediamine	$C_6H_7N_3O_2$	98%	Potent mutagen	Merck Inc. (2021e)

2.1.1. *The test strains*

In the study were used TA 98 (his D 3052, rfa, Δuvr B, +R) and TA 100 (his G 46, rfa, Δuvr B, +R) strains were used, having been developed by in vitro mutations from LT2 wild strains of *S. typhimurium* by Ames et al. TA 98 strains determined the frameshift mutations and TA 100 strains determined the base-pair substitution mutations.

2.1.2. *The dose of test substance and preparation*

The plant growth regulators were weighed in a proportion and were dissolved in dimethyl sulfoxide (DMSO) and stored at room temperature. Doses of all the substances were prepared individually, and cytotoxic doses were determined. After determining the cytotoxic dose, 5 doses were selected for each item and mutagenesis experiments were applied to these doses. Non-cytotoxic doses of 2500-1000-250-25-2.5 µg/petri were chosen for the Kinetin and 1600-800-400-200-100 µg/petri for the CCC.

2.2. Methods

Preparation of stock cultures of *S. typhimurium* TA 98 and TA 100 strains, controlling the genetics of the bacteria, and an Ames/*Salmonella*/microsome test were conducted by the petri incorporation method, according to the procedure of Maron and Ames (Maron & Ames, 1983). In this method, two different agars, as Minimal Glucose Agar (MGA) and top agar, are used. The basis of the method is the counting of revertant colonies that grow between the upper layer (top agar) and the lower layer (MGA).

The metabolites of chemicals may also have mutagenic effects. Therefore, the S9 microsomal enzyme is also used to measure the mutagenic effects of the metabolites. In this manner, the determination of direct and indirect effects of mutagenic substances were studied. Experiments were performed both with and without an S9 mix in the two groups. Each dose tested using three Petri dishes and independent experiments were carried out at different times. The tests were repeated when needed. Positive controls, solvent control (DMSO), and spontaneous controls were also performed as parallel experiments. Spontaneous control petri dishes that only containing bacteria and histidine/biotin solution were used to calculate the number of colonies that automatically return from each strain. After incubation for 48-72 hours, histidine-dependent bacterial colony counts were taken from the average value of the results obtained. Standard deviation values were calculated for these results.

In the mutation testing, a rotating force of the test substance is measured from mutant strain to wild strains, whether with the original mutation. Therefore, the strains are controlled to see whether they have the genotype in terms of mutant characters.

2.2.1. *Determination of the cytotoxic effects of the test substances*

Before starting the mutagenicity assay, the cytotoxic effect (LD_{50}) should be determined. The importance of determining the LD_{50} doses that can cause inhibition on test bacteria is that it will not result in a mutagenicity test. Primarily, containing the appropriate number of bacteria ($1\text{-}2x10^9$) of the overnight culture was made up to 10^{-6} dilution using a broth. To determine the cytotoxic effect it is always used in dilution 10^{-6} of culture. 0.1 ml overnight bacterial culture and 0.1 ml of various concentrations of the test substances were added to the top agar. After this, the mixture was spread over Nutrient Agar (NA) petri dishes, and the petri dishes were incubated at 37 ºC for 24 hours. At the end of incubation, the colonies were counted (Dean *et al.,* 1985). A comparison with the control dishes without the addition of chemicals toxic and nontoxic doses was determined. Less than half of the number of colonies in the control plates were considered of a cytotoxic dose.

2.2.2. *The Ames Test*

In the assay without S9, 0.3 ml of histidine/biotin solution, 0.1 ml of various doses of the test substance, and 0.1 ml of bacterial cultures that had been incubated for 12-16 hours were added to tubes containing 3 ml top agar at 45^0C. The tubes spread to the MGA Petri dishes. After allowing to freeze, the petri dishes were reversed and located in the incubator at 37 ^{o}C for incubation over 48-72 hours. After incubation a count of colonies was counted.

In spontaneous control, histidine/biotin solution and the bacterial culture were added in the top agar tubes, 0.1 ml of DMSO was added to the DMSO control, in addition to the histidine/biotin solution and the bacterial culture. In the positive control experiment, the histidine/biotin solution, bacteria culture, and the absolute mutagenic substances specific to the strain were added. Experiments were performed in five doses for the Kinetin and CCC substances, and for each dose three petri dishes were used.

An S9 mix was prepared fresh immediately before the S9 (+) experiment. After adding the 3 ml of top agar, the histidine-biotin solution, the bacterial culture, and the tested substances as mentioned in the S9(-) experiments, then 0.5 ml of ice-held S9 mix was added. The mixture uniformly spread on MGA petri dishes. When the agar solid was incubated in an incubator for 72 hours at 37 ^{o}C. Spontaneous, positive, and dimethyl sulfoxide controls were prepared by adding 0.5 ml of the S9 mix in the same manner. After incubation, the colonies were counted manually, and the results were analysed.

3. RESULTS / FINDINGS

For a substance to be called a mutagenic agent, the number of colonies counted in mutagenicity test results must be at least twice the number of spontaneous revertant colonies. 2-3 times that of spontaneous revertant number of suspected mutagen colonies, and three times more than those which are mutagenic. Which is less than twice the number of spontaneous revertant with the results being evaluated as non-mutagenic (Maron & Ames, 1983, Emig *et al.,* 1996).

3.1. Results of Salmonella/Microsome Mutagenicity Test

The plate incorporation test results for *S. typhimurium* TA 98 and TA 100 in the presence and absence of the S9 enzyme are given in Table 2. Considering Table 2, a 2500 µg/petri dose of Kinetin was found to be mutagen in tests conducted without the S9 on the TA 98 strain. Other doses of Kinetin were found to be non-mutagenic.

Table 2. The mean and standard deviation of the results of mutagenicity testing of 5 dose of the Kinetin.

KINETIN	TA 98		TA 100	
	S9(-)	S9(+)	S9 (-)	S9(+)
Spontaneous Control (SC)	32.5±2.5	56.5±3.5	168±26	188±13
DMSO Control (DC)	32.5±2.5	58.5±7.5	189±9	176.5±5.5
2,5 µg/ petri	23±0[a]	64.5±0.5 [a]	231±20 [a]	169±37 [a]
25 µg/ petri	38.5±0.5 [a]	75±3 [a]	235.5±31 .5 [a]	215.5±34.5[a]
250 µg/ petri	43.5±5.5 [a]	88.5±4.5 [a]	248±16 [a]	290.5±46.5[a]
1000 µg/ petri	56±1 [a]	86.5±4.5 [a]	252±10 [a]	328±70 [a]
2500 µg/ petri	694±437 [b]	788±352 [b]	247.5±17.5 [a]	834±206 [b]

Table 2. *Continues*

	Positive Controls (PC)			
4-Nitro-o- phenylenediamine (100 µg/ petri)	1457±497	-	-	-
Sodium Azide (100 µg/ petri)	-	-	862±216	-
2AF (100 µg/ petri)	-	658±39	-	688±13

a: negative, b: positive, c: suspected mutagen

As can be seen in the results in Table 3, all doses of the CCC are determined to be non-mutagenic in the presence and absence of the S9 enzyme. Chemical dose-response curves of bacteria were tested with the test substances shown in the following chart.

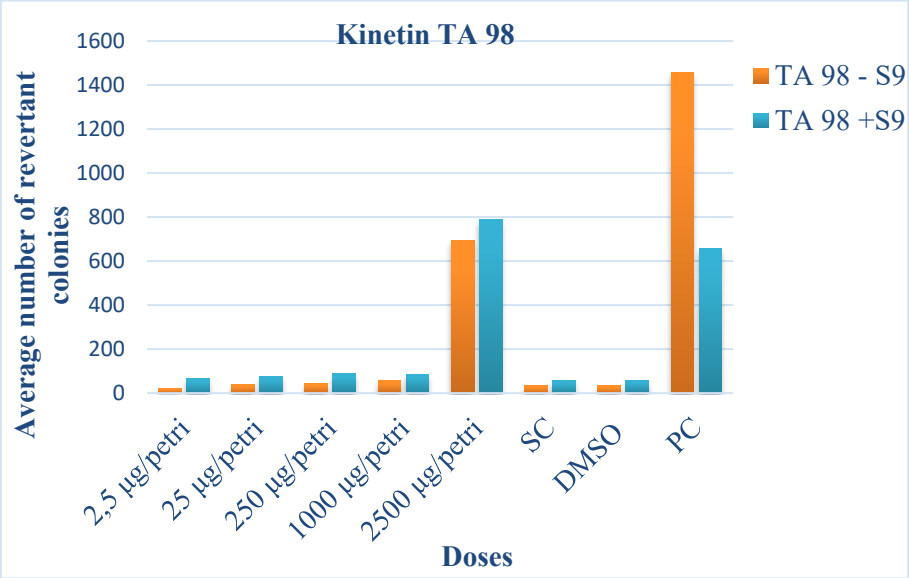
Table 3. The mean and standard deviation of the results of mutagenicity testing of 5 dose of CCC.

CCC	TA 98		TA 100	
	S9 (-)	S9(+)	S9 (-)	S9(+)
Spontaneous Control (SC)	32±2	59±8	130.5±7.5	142±13
DMSO Control (DC)	27.5±2.5	60.5±0.5	143±7	154.5±32.5
100 µg/ petri	38±7[a]	35.5±4.5 [a]	136±24 [a]	128.5±2.5 [a]
200 µg/ petri	28.5±9.5 [a]	67.5±1.5[a]	103.5±4.5 [a]	150.5±3.5[a]
400 µg/ petri	38.5±18.5 [a]	65±22 [a]	155.5±4.5 [a]	183.5±24.5[a]
800 µg/ petri	36±2 [a]	87±2 [a]	136±8 [a]	186±33 [a]
1600 µg/ petri	49±1 [a]	86.5±3.5 [a]	166.5±16.5[a]	182±21 [a]
	Positive Controls (PC)			
4 Nitro-o- phenylenediamine (100 µg/ petri)	643±317	-	-	-
Sodium azide (100 µg/ petri)	-	-	646.5±22.5	-
2AF (100 µg/ petri)	-	535±25	-	434±30

a: negative, b: positive, c: suspected mutagen

As can be seen in Figure 1, when the mutagenic activity of the Kinetin was investigated in the presence and absence of the S9 enzyme on the TA 98 strain, it was found that the number of colonies obtained was more than twice the number of spontaneous colonies, and it was determined that a 2500 µg/petri dose of Kinetin was mutagenic against the TA98 strain.

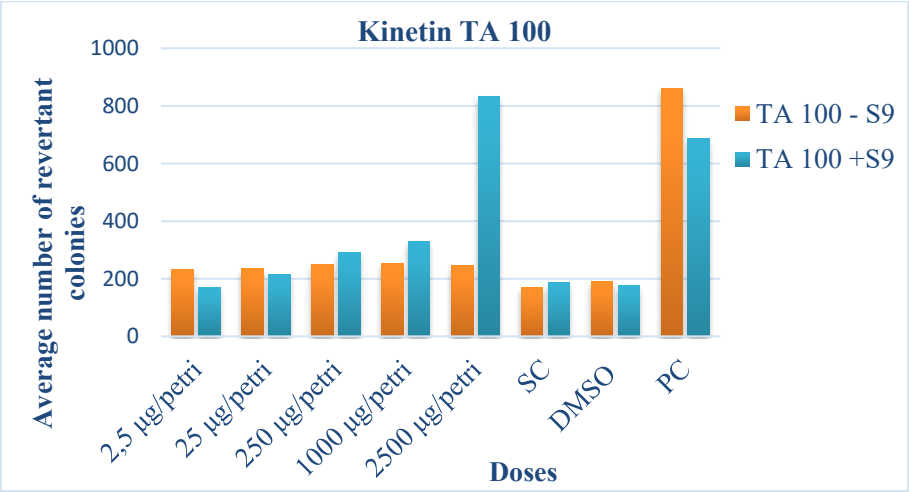
Figure 1. The dose-response chart of Kinetin in the experiments with or without S9 on the TA 98.



*Doses of SC,PC and DMSO controls are 100 µg/petri

As can be seen in Figure 2, when the mutagenic activity of the Kinetin substance was investigated in the presence and absence of the S9 enzyme on the the TA100 strain, since the number of colonies obtained in the presence of the S9 enzyme was determined to be more than twice the number of spontaneous colonies, it was determined that a 2500 µg/petri dose of Kinetin was mutagenic against the TA100 strain.

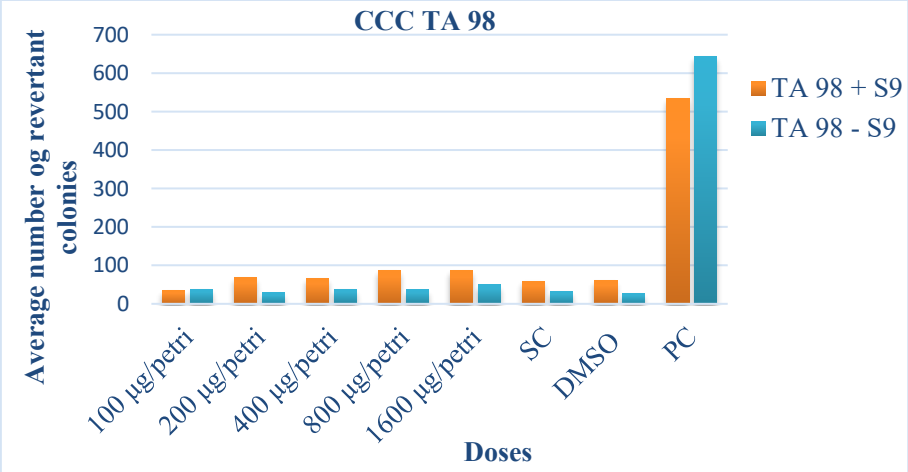
Figure 2. The dose-response chart of Kinetin in the experiments with or without S9 on the TA 100.



*Doses of SC,PC and DMSO controls are 100 µg/petri

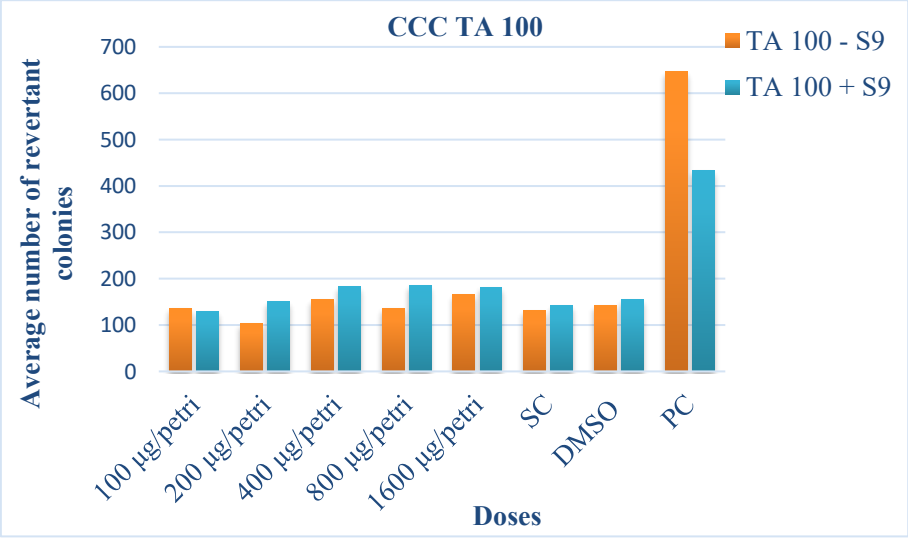
When Figures 3-4 are examined, the count of colonies of the experiments of the CCC in the presence of and absence of the S9 enzyme were two times less or similar to the number of colonies of spontaneous control. Therefore CCC was evaulated as a "non-mutagenic agent". According to the numbers achieved, the CCC was not mutagenic on the TA 98 and TA 100 strains. 2500 µg/petri of Kinetin was identified as mutagenic on the TA 98 and TA 100 strains.

Figure 3. The dose-response chart of the CCC in the experiments with or without S9 on the TA 98.



*Doses of SC,PC and DMSO controls are 100 µg/petri

Figure 4. The dose-response chart of the CCC in the experiments with or without S9 on the TA 100.



*Doses of SC,PC and DMSO controls are 100 µg/petri

4. DISCUSSION and CONCLUSION

There are several of synthetic and natural substances with unknown biological effects in our environment (Bağcı, 1985; Forman & Ames, 1991). In fact, any chemicals that do not show directly can create genetic damage in humans (Ames, 1985). Small amounts of various chemicals in our environment can cause mutations or cancer today (Dökmeci, 1988; Vural, 1984). One of the most important issues in the application of PGR is the user level that will not harm the humans, the environment, or plants and harvest before a certain time of these substances (Halloran & Kasım, 2002). In terms of acute toxicity, the effects of PGR are not immediately lethal, but the experimental studies show that they may cause harmful effects for carcinogenic, immune-toxic, reproductive, and so on, if there is long-term exposure to these substances (Çetinkaya & Baydan, 2006). For all these reasons, in our research, we aim to investigate two plant growth regulators frequently used in agriculture that have potential mutagenic effects and whether they are carcinogenic.

The Ames Test is a widely accepted and simple test that can be used to determine the mutagenic and antimutagenic potential of various chemicals, pesticides and hormones, foods,

drugs, and so on that can cause gene mutations and which have a high predictive value for in vitro carcinogenicity (Zeiger *et al.*, 1990; Pillco & de la Peña, 2014). Therefore, in this study, the incorporation the Ames Test using *S. typhimurium* TA98 and TA100 was employed to assess the mutagenic activity of Kinetin and CCC in the presence or absence of metabolic activation with an S9 fraction.

As stated by Ames (1973), spontaneous conversion of bacterial strains from a his- to a his+ state is possible within certain limits (20-50 revertant/plate for TA 98; 75-200 revertant/plate for TA 100). As a result of our research, for the TA98 strain, the mean number of spontaneous colonies was found to be 56.5±3.5 and a 59±8 revertant/plate in the presence of the S9 enzyme; the other hand spontaneous colonies were found to be 32.5±2.5 revertant/plate, 32±2 revertant/plate, and 41.5±6.5 revertant/plate were found in the absence of the S9 enzyme. For the TA 100 strain, the mean number of spontaneous colonies was 188±13 revertant/plate and 142±13 revertant/plate in the presence of the S9 enzyme and, on the other hand 134±4 revertant/plate, 168±26 revertant/plate, 130.5±7.5 revertant/plate, and 125.5±13.5 revertant/plate were found in the absence of the S9 enzyme.

At the start of our research, cytotoxic doses of Kinetin were determined. Since the number of colonies obtained from the petri dishes in which kinetin was applied at a dose of 10000 µg/petri, was below half the number of colonies obtained in the control petri dish, it was determined as cytotoxic. Therefore, 2.5-25-250-1000-2500 µg/petri doses of the kinetin substance were started in the study.

According to data obtained, a 2500 µg/ petri dose of Kinetin was evaluated as mutagenic for TA 98 strains in the presence and absence of the S9 enzyme. In experiments conducted in the presence of the S9 enzyme, a 2500 µg/petri dose of Kinetin for TA 100 strains was evaluated as mutagenic. Therefore, Kinetin was seen to be effective as mutagenic as frameshift and point mutations. The mutagenic effect that was initially not determined, but determined after the addition of the S9 enzyme for TA 100 strains, can be formed due to the resulting intermediate compound of the tested substance being metabolized by enzymes.

Yeşilada (2000) investigated the effect of Kinetin on mutant wing spots induced by mutagen EMS using the somatic mutation and recombination test of *Drosophila melanogaster* in a study. The 10^{-3} M concentration of Kinetin decreased the number of double spots, while the 10^{-4} M concentration caused an increase in the number of all types of spots. The change in the effect of Kinetin at high concentrations may be due to its mutagenic effect. In our study, while no mutagenic effect was observed at 2.5, 25, 250, 1000 µg/Petri doses of Kinetin, a significant difference was observed in terms of mutagenicity at a 2500 µg/Petri dose.

Kappas (1983) investigated the genotoxic activity of Kinetin (6-furfuryl-aminopurine) on *Aspergillus nidulans*. According to the test results, Kinetin did not increase the somatic separation frequency of *A. nidulans*. He also stated that S9 did not increase the effect of Kinetin in tests using the metabolic activation technique. However, in our study, Kinetin was not found to be mutagenic in the absence of the S9 enzyme on the TA 100 strain, while it was observed to be mutagenic in the presence of the S9 enzyme.

All the doses of CCC were evaluated as non-mutagenic in the presence and absence of the S9 enzyme in our research. When a literature review is conducted, mutagenicity data related to the the CCC substance, no genotoxic or mutagenic effect was found. However, in a study conducted by Sussmuth and Lingens (1976), while CCC did not show mutagenic effects on the *E. coli* K12 strain, valine sensitive in 0.2 to 0.5 M concentration and of a pH between 5 and 8, showed mutagenic effects at pH 9. In addition, it was found that maternal fertility may be affected at a high dosage of chlormequat in certain rodents (EFSA, 2008). For these reasons, it

could be thought that CCC does not cause frameshift and base-pair change mutations, while living things can produce different effects.

In terms of human health, the use of a combination of several short-time test systems instead of a single test system makes the results more reliable in determining the mutagenic properties of chemical compounds (Ennever & Rosenkranz, F 1986; Hofnung & Quillardet, 1986; McDaniels, 1990; Quillardet & Hofnung, 1988). In order for the test substances to be specified as mutagen or non-mutagen with certainty, these substances should be evaluated using different test systems, the results should be compared, and a reliable conclusion should be reached in this way.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Authors are expected to present author contributions statement to their manuscript such as. **Sevilay Yapici**: Investigation, Resources, Visualization, Formal Analysis, and Writing - original draft. **Guven Uraz**: Methodology, Supervision, and Validation. **Ebru Beyzi**: Investigation, Methodology, Supervision, and Validation.

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