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MUTAGENICITY OF KOJIC ACID PRODUCED FROM WILD TYPE *Aspergillus oryzae*

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ABSTRACT

Kojic acid (KA) is a multipurpose natural compound, commonly used in the food and cosmetics industry. It is produced by different types of molds especially by the species Aspergillus *oryzae*. In this study, we test the mutagenicity of local produced kojic acid PKA produced by the wild-type strain of A. *oryzae* as well as the standard commercially produced kojic acid SKA and ascorbic acid SAA for comparison to stop food manufacturers doubts about using KA. AMES test with Salmonella enterica ATCC 29629 strain TA1535 and S9 liver enzyme for metabolic activation of the tested compounds were utilized in this study by direct and indirect methods were used in the test. The study results showed that the tested PKA kojic acid had cannot induce reverse mutation in the strain ATCC 29629TA1535 used in the test in contrast with the positive control in direct and indirect methods, even where the tested acids were treated with S9 liver enzymes with or without pre-incubation for three hours at 37 °C hadn't given positive results on TA1535. The used concentration of 1% and 10% S9 liver enzymes hadn't metabolically activated the three acids. 6 mg/plate of KA inhibited the growth of TA1535. SAA gave the same negative results as PKA and SKA. In conclusion, the tested PKA produced by wild-type A. *oryzae* was not has mutagenic effect on bacterial strain TA1535 and gave the same effect as the commonly used as food additive SAA and SKA even when treated with S9 liver enzymes.

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INTRODUCTION

Kojic acid (KA) is one of the compounds that are naturally produced by certain types of molds (Ravirala *et al*.,2023). It is produced in various environments, the most important of which is an oriental fermented food, which uses the 'initiator' called koji in its production. This acid is found in many well-known and widely spread Japanese foods around the world. The use of KA in food manufacturing has increased due to its multiple and useful chemical functions for manufacturing processes and to maintain some important characteristics of products. It has been used as an anti-browning agent since it can prevent enzymatic browning in a range of foods such as lard, potatoes, apples, and white shrimp. It has proven its effectiveness as an anti-tanner by interfering with the oxygen consumption required for the action of the browning enzyme, as well as the ability of the acid to reduce the compound oxyquinones to diphenoles to prevent the formation of brown pigment in these foods

(Chen *et al*.,1991). Hasebe *et al*., (1995) proved that KA has two important activities against enzymatic browning, on the one hand, it gives a similar benefit to adding ascorbic acid as a base material for the action of the browning enzyme, and on the other hand, it gives similar results to the inhibitors of browning enzymes. The acid showed high efficiency during the production of green tea, as it had an effective role in inhibiting the browning enzymes, which cause major problems during production (Wang *et al*.,2003). The acid was also used to prevent discoloration of marine crustaceans, red meat and some fresh vegetables (Saleh *et al*.,2011), and to extend the storage period of vegetables and fruits, and prevent the staining of their surfaces (Zheng *et al*.,2008). Burdock *et al*., (2001) mentioned that KA has important functions besides its effect on harmful microorganisms such as its use as a food additive for beef to prevent the formation of storage flavour in addition to its use in frying pork to reduce the formation of some effective compounds harmful to health such as nitrosopyrrolidine along with its use in other manufacturing fields, such as accelerating the ripening of chile and a flavour enhancer for some compounds used in food processing (Ariff *et al*.,1997). It has been used as a bleaching agent and as an antioxidant in the cosmetics industry (Zieniuk *et al*.,2022). The research was directed to prove kojic acid's effectiveness as a multi-tasking food additive, especially as it is a natural product and is used in regulating obesity and reducing triglycerides in the blood (El-Korany *et al*.,2020). Recently, kA has been studied by linking it with packaging materials to perform part of its functions as an anti-microbial, as it has an inhibitory activity against some types of pathogenic microorganisms that cause food spoilage bacteria, such as Staphylococcus aureus and Escherichia coli (Liu *et al*.,2020), and it enhances competition between microorganisms and exhibits antibacterial activity that causes spoilage of duck meat such with, for instance, Pseudomonas spp. (Hou *et al*.,2021). Food risks are among the most important challenges facing food manufacturers, represented by diseases that can be transmitted by food. In addition, prolonging the shelf life of food by reducing the number of microorganisms that cause food spoilage and stopping the action of some undesirable enzymes in foods is an important objective. However, new challenges are facing specialists in this field, such as the adaptation of some microorganisms to the conditions of manufacturing processes and the emergence of resistant strains of E. coli O157:H7, Listeria monocytogenes and some strains of Salmonella spp. It increases the risk of diseases such as listeriosis, haemorrhagic colitis and salmonellosis. In addition, the emerging resistance of food spoilage microorganisms creates economic threats to the industry, such as spoilage of meat and its processed products, off-oddor emission, change of flavour, discoloration and changes in viscosity (Mathew *et al*.,2007 and Mith *et al*.,2014). The frequent consumption of foods rich in KA has spread over the ages, especially in East and Southeast Asian countries. The Japanese Ministry of Health has authorized the use of acid as a food additive for processed food products (Burdock *et al*.,2001). Bentley, (2006) pointed out that the presence of acid in many foods and drinks gives a clear idea of the safety of using this acid from a health point of view because some of these foods have been produced and consumed for several centuries and without showing any side effects or toxicity, especially soy sauce.The reported study aimed at elucidation the safety of

PKA and SKA specifically, its mutagenic activity in compared with the widely commercially used SAA as food additive.

MATERIALS AND METHODS

Kojic Acid and Ascorbic Acid

Standard kojic acid (SKA) from Rita Corporation (Ramsey, NJ, USA) and local produced kojic acid (PKA) produced from the mold Aspergillus *oryzae* isolate 2a obtained from a previous study by Ibrahim *et al*., (2017) were used, and standard ascorbic acid (SAA) from Sigma-Aldrich (St. Louis, MO, USA) was used to conduct the tests. The solutions (distilled water as a solvent) were prepared according to the company's instructions in with concentrations of (0.5,1,2,4,6 mg/plate).

AMES test

The test kit supplied by Presque Isle Culture (Erie, PA, USA) was used in three ways to complete the Ames test. The first was by adding the tested substance in the form of a solution to the plates. The second was by adding the tested materials after their treatment with S-9 liver enzymes, which were supplied by MOLTOX Molecular Toxicology, Inc (Boone, NC, USA), at a concentration of (1 and 10)%, respectively, incubation temperature of 35 ℃ for one hour, once and again without incubating period. And third, by adding acid crystals directly to the Petri dishes with grown Salmonella enterica ATCC 29629. The method was carried out according to the instructions of the supplier company, using distilled water as a negative control sample. The mutagenic compound potassium N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid was used as a positive control sample to compare the results in the reverse mutation of the used bacterial strain, along with the method of the supplier company.

RESULTS AND DISCUSSION

Growth rate

Figure 1 is depicting the addition of crystals of (PKA, SKA and SAA) to dishes inoculated with S. enterica ATCC 29629 by the direct method, which indicates that no mutagenic activity by the three acids, with no induction of the reverse mutation of bacterial strain compared with the positive control.

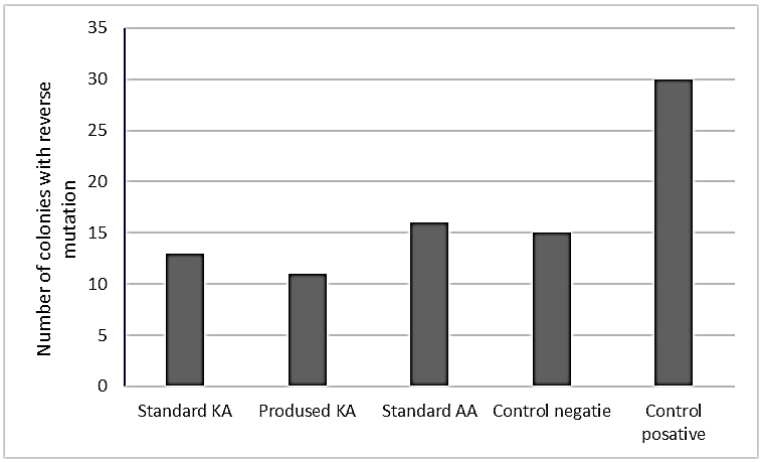


Figure 1: Direct inoculation method on dishes with acids crystals.

It is noted from Figure 2 that conducting the test by the indirect method and without treatment of acids with liver enzymes type S9 gave negative mutagenic results for each of the used three acids compared to the positive control sample. The three acids did not show the ability to cause reverse mutation in S. enterica ATCC 29629 used in the test when adding the acids in the form of a solutions without their treatment by liver enzymes S9.

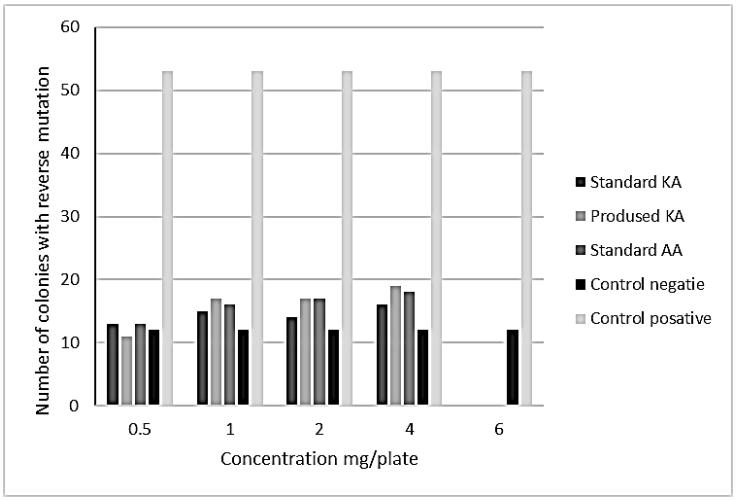
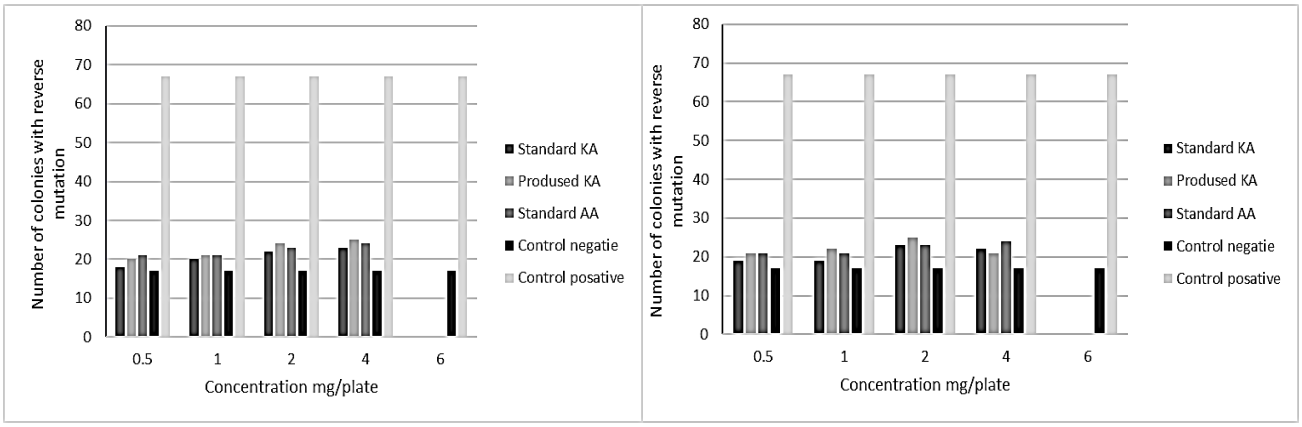


Figure 2: Indirect method without treatment of acids with liver enzymes S9.

Figure 3 a and b show the results of the effect of the three acids on the strain S. enterica ATCC 29629 after treatment with liver enzymes at a concentration of 1 and 10% and without using prior incubation, which stimulated the emergence of reverse mutations in the strain.



(a) **(b)**

Figure 3: (a) Indirect method with treatment with liver enzymes S9 at a concentration of 1% without prior incubation. (b) Indirect method with treatment with liver enzymes S9 at a concentration of 10% without prior incubation.

Figure 4a and b show the effect of the acids used in S. enterica ATCC 29629 after they were treated with liver enzymes S9 and pre-incubated at 37 degrees for one hour, which did not give a stimulating effect to the reverse mutation in the used strain compared with the positive control sample.

From the above three sets of experiments, it is clear that the three acids didn't have mutagenic effect in the Ames test and that treatment with liver enzymes with or without incubation did not contribute to a change in this characteristic. The researcher

Wei *et al.,* (1991) indicated the possibility of kojic acid in producing mutants of Salmonella bacteria of strain TA100 and strain TA98 by direct casting or pre-incubation decanting treated with S9 liver enzymes. As some mutagenic substances activate their effectiveness on causing reverse mutations in the test after being treated with liver enzymes, for example, aflatoxin has no mutagenic ability when added to cells in its normal form, while after treatment with liver enzymes it led to a chemical change in its composition that yielded to its activation and thus becomes mutagenic (Alonso-Jauregui *et al.,*2022). From the test results, it is clear that PKA and SKA were not able to cause reverse mutations and the same as SAA, which proves the safety of its use in various food processing fields. The results showed that the use of 6 mg applied to the SKA and PKA probably led to the killing of cells of S. enterica ATCC 29629, which is a normal effect of kojic acid as it has an anti-microbiological effect, especially against Gram-negative bacteria, which is consistent with Ibrahim *et al.,* (2017) who concluded that KA has an inhibitory and fatal effect on Salmonella, especially when high concentrations are used.

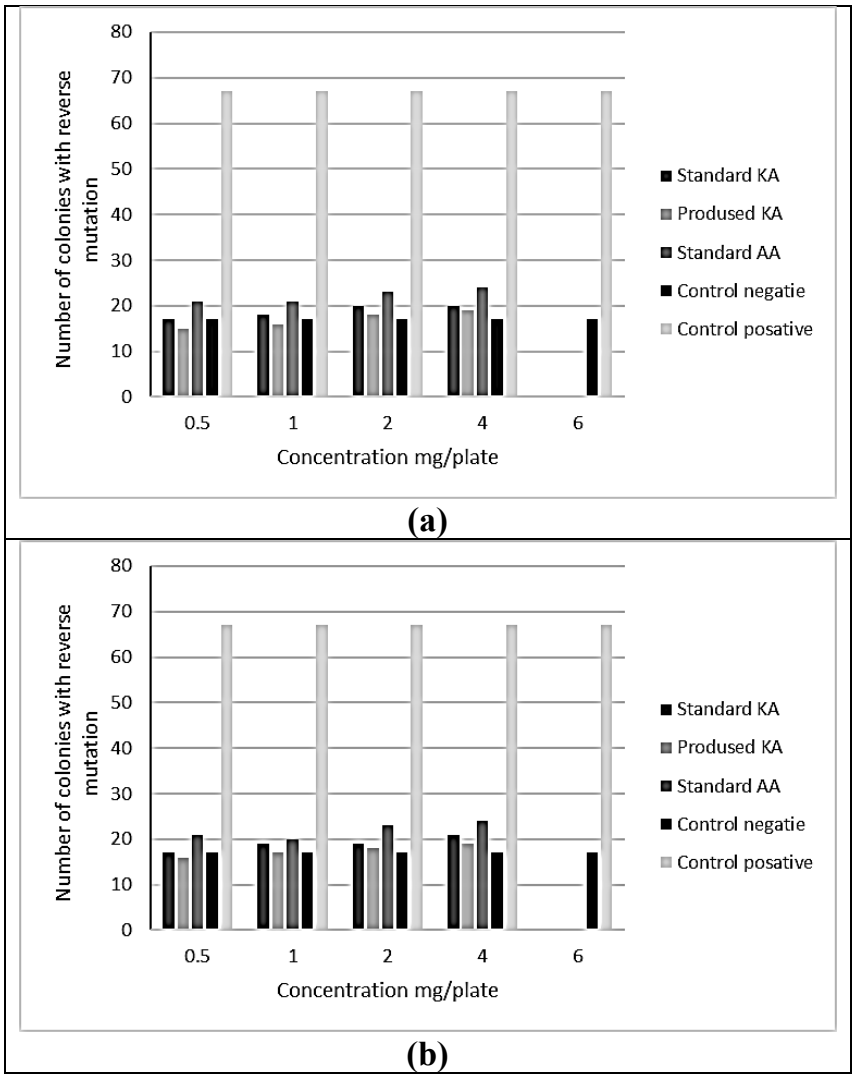


Figure 4: (a) Indirect method with treatment with liver enzymes S9 at a concentration of 1% with pre-incubation for an hour at a temperature of 37°C. (b) Indirect method with treatment with liver enzymes S9 at a concentration of 10% with pre-incubation for an hour at a temperature of 37°C.

Shibuya *et al.,* (1982) concluded that although weak mutagenicity was obtained in the TA98, TA1535 and TA100 bacterial strains used in the AMES test when KA was added directly without treatment with S9 liver enzymes, it gave a negative result in the eukaryotic cell mutagenicity tests, thus, it was considered that KA did not have the property of genetic mutation in eukaryotic cells. Wei *et al.,* (1991) also concluded that KA had a mutagenic effect on prokaryotic cells of the bacterial strains TA98 and TA100 in AMES test by plate-incorporation and pre-incubation method with or without treatment with liver enzymes type S9 and stated that despite the emergence of the acid's mutagenic ability, no cases of human poisoning with acid were recorded as a result of consuming types of foods rich in it. The researcher Nohynek *et al.,*(2004) also found out that the use of high concentrations of KA equal to or exceeding 1 mg/plate was mutagenic for bacterial strains TA98, TA100, TA1535 and TA102 except for strain TA1537, and that treating the two strains TA98 and TA100 with acid for three hours before adding them to the dishes gave negative results for mutagenesis furthermore he indicated that the positive results of the ability of the acid to cause reverse mutations for the strains used may be due to the presence of quantities of amino acids with the used acid or due to the acid's structural similarity with a group of chemical compounds used in the food industry, such as maltol, which stimulated the occurrence of reverse mutations in the strains used in the AMES test. Which is consistent with what our research dealt with using ascorbic acid, which has great similarity in chemical composition, with the exception of one carbon atom, which gave similar results to the effect of kojic acid in the different cases of the AMES test. Burdock *et al.,* (2001) mentioned that no cases of human poisoning have been recorded as a result of consuming foods containing kojic acid, such as fermented foods. Several studies indicated that the consumption of acid in proportions similar to its concentration in fermented foods does not affect the health of consumers, as most of these fermented foods have been widely consumed throughout the ages and have been known as healthy foods (El-Kady *et al.,*2014). Although the Japanese government does not believe in the possibility of the danger of consuming kojic acid within reasonable proportions, it stressed the precautionary check on the percentages of acid added to processed foods, so the Japanese Ministry of Health issued warnings against excessive consumption of acid and adding it in higher concentrations than its presence in naturally fermented foods (Burdock *et al.,* 2001, Blumenthal *et al.,*2014 and Ma & Chao,2014). Yang and Zhang, (2007) referred that the concentrations of kojic acid in fermented foods reached 5 mg/ml in sushi products and 1.5 mg/ml in vinegar. Burdock *et al.,* (2001) stated that the koji starter used in the production of miso and soy can produce kojic acid at rates up to 24 mg / g of fermented food.

CONCLUSIONS

The results showed that the tested PKA produced by wild-type A. *oryzae* has no mutagenic effect on bacterial strain TA1535 and provided similar effect as the commonly used as food additive (SAA and SKA) even when treated with S9 liver enzymes.

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CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest with the publication of this work.

القدرة التطفيرية لحامض الكوجيك المنتج من السلالة البرية *Aspergillus oryzae*

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مختبر تعزيز الصحة الطبيعية / كلية العلوم البيئية والبايولوجية / جامعة روتجرز الولاية / نيوجرسي 08901 / الولايات المتحدة الامريكية [4]

ان حامض الكوجيك هو مركب طبيعي متعدد الأغراض، شاع استخدامه في صناعة الأغذية ومستحضرات التجميل. يتم إنتاجه بواسطة أنواع مختلفة من القوالب خاصة من الأنواع *oryzae* Aspergillus. تم في هذه الدراسة باختبار الطفرات الوراثية لحامض الكوجيك المنتج محليًا PKA والذي تم انتاجه من سلالة من النوع البري من *oryzae* .A وكذلك حامض الكوجيك القياسي المنتج تجاريًا SKA وحامض الأسكوربيك SAA للمقارنة لإيقاف شك مصنعي المواد الغذائية حول استخدام KA. تم استخدام اختبار AMES مع Salmonella enterica ATCC 29629 من سلالة TA1535 و S9 من إنزيم الكبد للتنشيط الأيضي للمركبات المختبرة في هذه الدراسة بالطرق المباشرة وغير المباشرة المستخدمة في الاختبار. أظهرت نتائج الدراسة أن حامض الكوجيك PKA لا يمكنه إحداث طفرة عكسية في سلالة TA1535 ATCC 29629 المستخدمة في الاختبار بالمقارنة مع عينة السيطرة الإيجابية بالطرق المباشرة وغير المباشرة ، حتى عندما تم معاملة الأحماض المختبرة بإنزيمات الكبد S9 بدون أو مع الحضن المسبق لمدة ثلاث ساعات عند 37 درجة مئوية لم تعط نتائج إيجابية على TA1535. لم ينشط إنزيم الكبد S9 بتركيز 1٪ و 10٪ القدرة على احداث الطفرة الراجعة للأحماض الثلاثة. 6 ملغ / لوحة من KA أعاقت نمو TA1535. أعطت SAA نفس النتائج السلبية مثل PKA و SKA. استخلص من الدراسة ان PKA المنتجة من النوع البري *oryzae* .A ليس له تأثير مطفر على السلالة البكتيرية TA1535 وأعطت تاثيرا مشابها لما موجود في المضافات الغذائية مثل SAA و SKA وحتى بعد المعاملة بانزيمات الكبد.

الكلمات المفتاحية: حامض الكوجيك، الطفرات، اختبار AMES.

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