Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 4, No. 2, 73-81, 2014

Evaluation of Mutagenic Potentials of Some Food Additives by Ames Test

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Received: 24 October 2013 Accepted: 20 May 2014

ABSTRACT: Some chemicals used by human such as food additives are mutagenic and mutagenicity effects analysis of them is very important because of considerable consumption by man. One of the most common methods used in the recognition of the chemicals' mutagenicity is Salmonella Typhimurium Reverse Mutation (Ames) test. The purpose of the present study is mutagenicity effects analysis of sodium nitrite, boric acid and various kinds of borax. Bacterial strains in this study are TA98 and TA100 which are derived from *Salmonella Typhimurium* by creation of several mutations in Histidin operon and their dependency on this chemical; also, by creation of *rfa* and UVrB mutations and pKM101 plasmid addition. Sodium nitrite, boric acid and three kinds of borax were selected for the test and various dilutions of them were provided. Performing Ames test, revertant colonies average of TA98 and TA100 strains were calculated for each dilution separately as the result of the above chemicals' function; then, according to the achieved A value, dose - response curve was depicted. The results of present study demonstrate non-mutagenic effects of sodium nitrite, boric acid and three kinds of borax through Ames test, by TA98 and TA100 strains. According to the wide consumption of these food additives in food industry, analysis of their mutagenic effects is very important and we suggest more investigation using different test to approve the results.

Keywords: *Ames test, Boric Acid, Borax, Mutagenicity, Sodium Nitrite*.

Introduction

Food additives consist of compounds of several chemical components that are used in each process of food production, conversion, storage and packaging besides the foods' main ingredients. These chemicals are utilized in the food spoilage prevention and food taste maintenance during preservation, microorganism growth prevention, color intensity, physical modification and stabilization of the product, and product appearance improvement (Food WHO, 2010). Nowadays, wide range of chemicals containing food preservatives, flavors, sweeteners, concentrating materials, and emulsifiers, etc. are utilized in food industry as common food additives (Growther *et al*., 2009; Appendini Hotchkiss, 2002). Some food additives are mutagenic which their identification, function and chemical structure have great importance because of their wide usage by individuals. On the other hand, mutagenic chemicals have specific roles in carcinogenicity; therefore, their mutagenicity recognition in carcinogenicity prediction is of great significance. Preservatives are numerated as one of the most used additives in food industry; in addition of food health preservation, they prolong the food maintenance and reduce the sale problems (Sugimura, 2006; Shelby, 1988; Young, 2002; Ashby Tennant, 1988).

Nitrite compounds especially sodium nitrite is a prevalent preservative used in cured meat preparation. Sodium nitrite

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besides the disinfectant property in low amounts is used for color, odor, and flavor creation (Sindelar Milkowski, 2012; Tenovuo, 2006). On the other hand, this chemical is used to prevent the growth of microorganism, creat a pink colour and provide a good taste, microorganism growth specially *Clostridium Botulinum*, of the cured meat might be regarded as an example (Hartman, 2006; Vittozzi, 1992). In contrast, nitrite compounds consist of several biologic and toxic properties such as vasodilatory, lowering blood pressure, methemoglobin formation, nitrosamines formation and tumor creation along with other chemicals (Reinik, 2007). The most important harmful effect of nitrites is nitrosamine and nitrosamide formations in presence of amines types II, III and amides. Mutagenicity and carcinogenicity effects of nitrosamines and nitroamides are reported through different studies which according to them, wide researches about nitrite's application and the amount in foods seem necessary (Lundberg *et al*., 2004; Woods, 1994).

Boric acid and its derivatives like borax (Na2B401) have disinfectant effects that have same influence on the growth of yeasts and somehow on bacteria and molds, therefore they are used as preservatives in medicines and foods (See *et al*., 2010). Boric acid is utilized as a disinfectant additive (in maximum concentration of 4 g/l) in foods like caviar, meat and dairy products. Studies have shown that the constant and low amount usage of boric acid and its derivatives lead to chronic poisoning (See *et al*., 2010; Ku *et al*., 1993). Several reports have been published about the poisonous property of this chemical in animals' reproductive system. In addition, various studies have been performed with regard to carcinogenicity of this chemical among animals, but valid evidences have not been achieved (Qureshi *et al*., 2001; Bridges, 1980). According to the wide consumption of sodium nitrite, boric acid and borax in food industry, analysis of their mutagenicity has extraordinary importance. Generally, each of various methods have been applied to recognize that mutagenicity effects contain its specific advantages and disadvantages (Wakabayashi, 1992). Among them, Salmonella Typhimurium Reverse Mutation (Ames) test that is one of the most prevalent methods used by the majority of the researches. In this study, the direct analysis of mutagenic effects of the mentioned chemicals have been performed through Ames test by TA98 and TA100 strains (Kayraldiz *et al*., 2006). According to the wide consumption of sodium nitrite, boric acid and borax in food industry, analysis of their mutagenicity is quite important. Generally, each of various methods have been applied to recognize the effects and advantages and disadvantages. Among them, Salmonella Typhimurium Reverse Mutation (Ames) test is one of the most prevalent methods that the majority of the researches use it. In this study, the mutagenic effects of the mentioned chemicals have been performed through Ames test by TA98 and TA100 strains (Kayraldiz *et al*., 2006; Qureshi et al., 2001).

Materials and Methods

- *Chemical Compounds*

Agar agar, L-histidine, chloridric acid, D-glucose monohydrate (C6H12O6, H2O), dimethyl sulfocside (DMSO), nutrient broth, methyl methane sulfonate (MMS), sodium nitrite (Merck Chemical Company, Germany), boric acid (BDH, UK) and borax in three different variations (made in India, exist in Iran's markets).

- *Bacterial Strains*

The bacterial strains of this study are TA98 and TA100 which are derived out of Salmonella Typhimurium through several mutations in Histidin Operon and their dependency on this chemical; also, through rfa mutation (sensitivity to crystal violet), UVrB and PKM101 plasmid addition (R

factor). Using TA98 strain, frame shift mutations and using TA100 strain, base pair substitutions mutations could be identified. These two strains have high sensitivity to the mutagenic chemicals and many mutagens could be recognized through these two strains.

- *Genotype Strains Assays*

Bacterial strains have been tested at the beginning and during the assay according to the related mutations. Considering the TA98 and TA100 strains, Histidin dependency, *rfa* mutation, UVrB mutation, and PKM101 Plasmid tests have been analyzed.

- *Histidine requirement*

The media consisting of bacteria were incubated for 12h at 37ºC. Then, 0.1 ml of this media was added to histidine and biotion culture (minimal medium having a little histidine and biotin), then 0.1 ml S. typhimurium TA100 was added to biotin medium as control plate. All plates were incubated for 48h at 37ºC.

- *Rfa mutation*

A 150 µl sample of the overnight bacterial culture was inoculated in 2.5 ml of melted and cooled top agar and spread over an agar nutrient plate. Four disks dipped in crystal violet was later placed on every plates and after 18 hours period, a bright zone was observed around the disk, that is an indication of the lack of cell growth due to the *rfa* mutation. Therefore Sensitivity to crystal violet was tested in this test.

- *UVrB mutation*

This test is used to corroborate UV sensitivity. After culturing the bacteria on the plate, a half of one was covered with aluminum foil, and it was exposed to UV radiation for 6-10 seconds in 30 cm distance. The plate was then incubated for 18 h at 37ºC.

- *R-factor assay*

This test is used to indicate resistance factor against ampicillin. The absence of the zone of growth inhibition around the disk was an indication of ampR and a sign for the presence of the R-factor in the bacterial strain.

- *Various Dilutions Preparations of Sodium Nitrite, Boric Acid and Borax*

Sodium nitrite with molecular weight of 96 and purity of 99 percent has been selected for the test. By solving of 50, 100, 250, 500, 750, 1000, 1250, and 1500 mg of sodium nitrite in distilled water, the needed concentrations were prepared and then, by using autoclave at 121˚C, and 15 pounds pressure for 15 minutes, the samples were sterilized.

Boric acid with molecular weight of 61.84 and purity of 99.50 percent was selected and dilutions were made using 50, 100, 250, 500, 750, 1000, 1250, and 1500mg of the chemical dissolved in distilled water and then sterilized in an autoclave.

In this study, three types of borax with purity of 99 percent were selected and various dilutions were prepared.

- *Mutagenicity Assays of the Samples through Ames test*

Mediums and solutions were prepared according to Ames test (Boido, 1980). Under sterilized conditions from each bacterial strain, a loop was inoculated into nutrient broth. In order to have homogeneous environment mediums were incubated in 37 ˚C shaker incubator. The top Agar was melted through water bath 24 hours later; then 2 ml of it was transferred into each tube. For the negative and positive controls, distilled water and 10 µl of MMS which was solved in 400 µl of DMSO were used, consecutively. In the next step, 0.1 ml of the strains was added to all of the tubes. Each tube's contents were mixed by shaker and transferred to the plates containing the

minimum medium. After top Agar soften on the plates, they were preserved in 37˚C for 48-72 hours. Ultimately, these plates were taken out of Incubator after 48 hours and the number of the revertant colonies was counted by colony counter. Each experiment was performed twice more in order to see the reproducibility of the experiments.

- *Statistical analysis*

The number of revertant colonies for each concentration and control sample colonies was counted in at least 15 plates and the averages were calculated. A value was calculated by the colonies number for each concentration divided to the average of control colonies number. Finally, using average and standard deviation, SEM was evaluated for each concentration.

Results and Discussion

- *The Effects of Various Concentrations of Sodium Nitrite, Boric Acid and Borax Types using Ames test*

The effects of sodium nitrite on each concentration, the average of revertant colonies number for TA98 and TA100 strain was calculated and A value was obtained and SEM and figure 1 were drawn as the results (Figure 1 and Table 1).

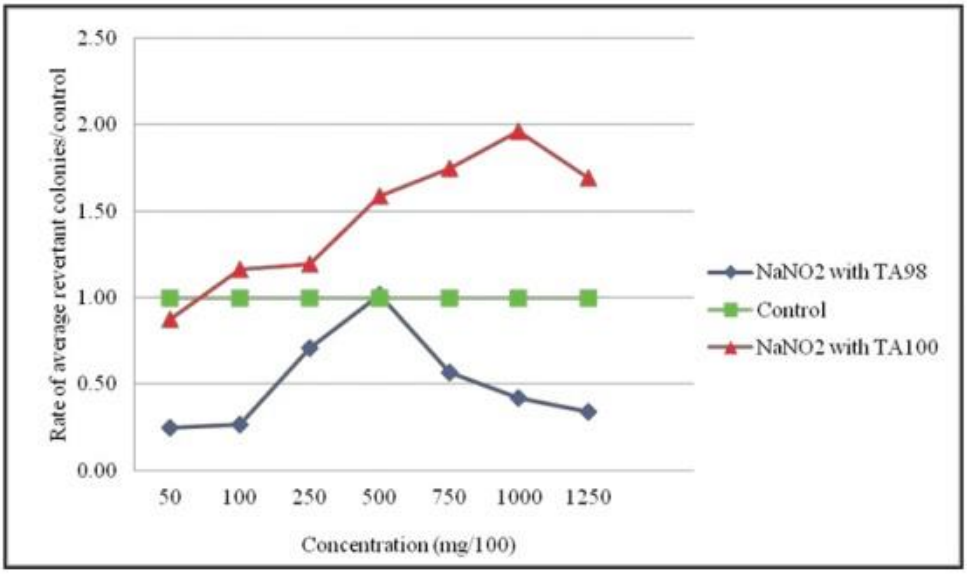

Fig.1. The effect of sodium nitrite different concentrations on TA98 and TA100 strains

Table 1. The reversion of TA98 and TA100 strains colonies by different concentrations of sodium nitrite Performance

A*	The average number of reverse colonies, TA100 strain	A*	The average number of reverse colonies, TA98 strain	Sample concentration, mg/100
0.877	69.33±5.70	0.25	6.02±1.32	50
1.164	91.86±3.35	0.266	6.93±1.12	100
1.194	94.26±6.27	0.71	18.46±2.38	250
1.588	125.46±8.46	1.016	26.4±2.88	500
1.747	138±5.8	0.57	15±1.24	750
1.962	155±7.12	0.42	11.3±1.20	1000
1.692	133.3±6.74	0.34	9±1.43	1250

*A value was calculated by the number of colonies division of each concentration to the number of controlled colonies average

The results of the effects of various concentrations of boric acid and borax TA98 and TA100 were obtained and SEM and the average of revertant colonies number for

each concentration was determined (Figures 2 and 3).

Increasing sodium nitrite concentration between 50 to 500 mg/100, the revertant colonies number with TA98 strain increased while there was a decrease for higher concentration. For the selected concentrations of sodium nitrite with TA100 test, increasing the chemical concentrations from 50 to 1000 mg/100g the revertant colonies number elevated; but A value in various concentrations was 1.96 approximately. For the selected concentrations of boric acid with TA98 strain, the revertant colonies number at 500 mg/100g concentration has increased as compared to 250 mg/100g concentration but at higher concentrations, the ratio has decreased. The effect of 250, 500, 750, and 100 mg/100g concentrations follow Dose-Response curve in comparison with the average of control colonies number (Table 2, Table 3).

Figures 2 and 3 present the effect of boric acid and different borax concentrations on TA98 and TA100 strains.

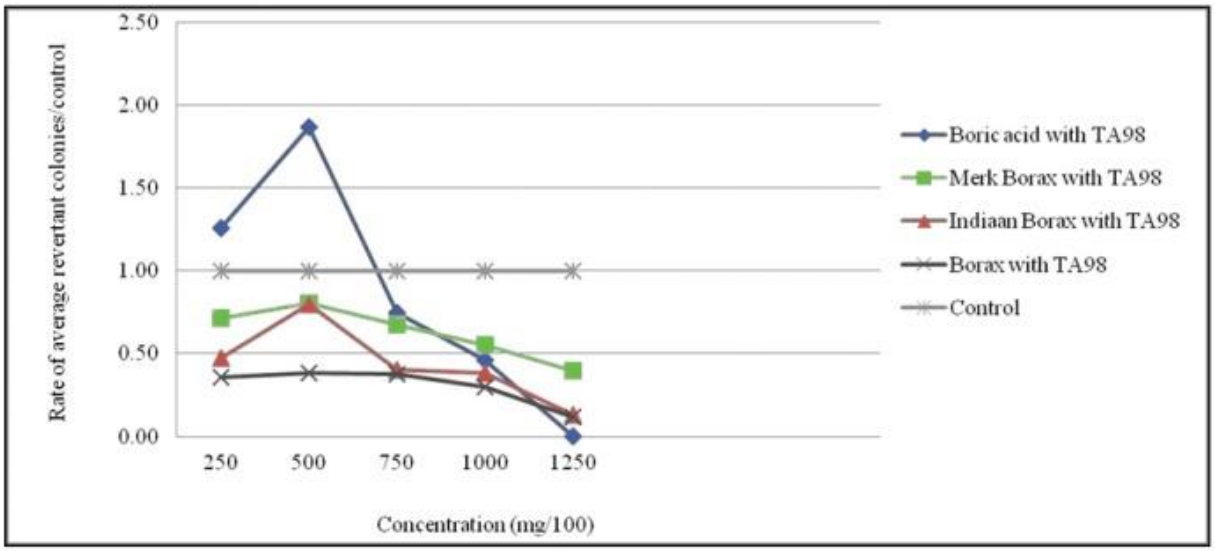

Fig. 2. Effect of boric acid and different borax concentrations on TA98 strain

Table 2. Different concentrations of Boric Acid and Borax types' performance in reversion of TA98 strain colonies

A	The average number of reverse colonies with commercial Borax	A	The average number of reverse colonies with Indian Borax	A	The average number of reverse colonies with Merck Borax	A	The average number of reverse colonies with Boric acid	Sample concentration, mg/100
0.354	9.2±2.21	0.473	12.3±2.31	0.716	18.6±2.58	1.258	4.50±2.60	250
0.385	10±2.55	0.8	20.8±3.14	0.804	20.9±3.10	1.866	48.4±4.78	500
0.377	9.8±1.72	0.4	10.4±1.91	0.677	17.6±3.07	0.747	19.24±4.01	750
0.289	7.5±1.62	0.385	10±1.82	0.554	14.4±2.46	0.462	12±1.75	1000
0.116	3±0.97	0.131	3.4±1.10	0.393	10.2±2.04	0	0	1250

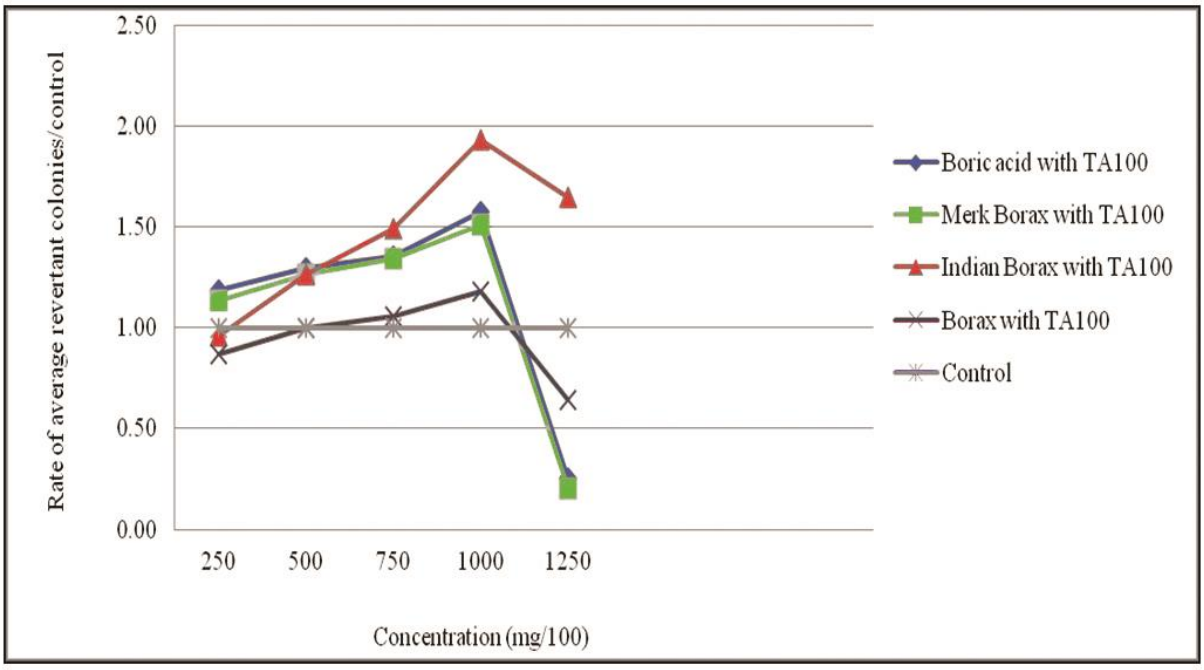

Fig. 3. The effect of boric acid and different borax concentrations on TA100 strain

Table 3. Different concentrations of Boric Acid and Borax types' performance in reversion of TA100 strain colonies

A	The average number of reverse colonies with commercial Borax	A	The average number of reverse colonies with Indian Borax	A	The average number of reverse colonies with Merck Borax	A	The average number of reverse colonies with Boric acid	Sample concentration, mg/100
0.87	68.7±8.23	0.959	75.66±10.15	1.133	89.4±11.41	1.183	93.4±5.72	250
0.995	78.6±10.84	1.265	99.9±10.15	1.261	99.50±11.04	1.293	102.1±9.56	500
1.059	83.6±13.74	1.492	117.8±11.85	1.343	106±12.14	1.355	107±8.03	750
1.179	93.1±13.80	1.93	152.4±17.33	1.509	118.5±13.05	1.576	124.5±10.20	1000
0.64	50.5±7.62	1.646	130±16.05	0.202	16.3±2.14	0.254	20±1.90	1250

- *Sodium Nitrite's Mutagenicity Effects*

As reported in the previous studies, sodium nitrite forms N-nitrosamines and nitrosamides with the existent amines and amides in the foods and medicines (Maron Ames, 1983; Kunisaki, 1979). The mutagenicity effects of the produced chemicals were studied through different methods and revealed their mutagenicity property (Liener, 1974; Tannenbaum, 1983). In this study, the sodium nitrite mutagenicity effect was assayed by Ames test. Table and figure 1 show that the revertant colonies number of the TA98 strain at 50-500 mg/100g concentration that increases by the increase in the concentration of sodium nitrite. Dose-response curve of sodium nitrite to 50-500 mg/100g concentrations depends on the concentration. When a value is about one or less, it might be concluded

that there is no mutagenic effect for that chemicals for that specific concentrations on TA98 strain. Sodium nitrite's effect on TA100 strain indicated that the revertant colonies number in 50-1000 mg/100g concentrations has increased. Dose-response curve of these points indicated the colonies number dependency on sodium nitrite concentration, therefore, sodium nitrite did not show mutagenicity effects on TA100 strain. Although sodium nitrite has not shown any mutagenicity effects on either TA98 or TA100 strains, the results of the test with TA100 indicated that in higher concentrations of this chemical, the revertant colonies number has increased and Dose-Response curve follows the concentration elevation in several points. In addition, the high ratio of the revertant colonies in TA100 in comparison with TA98 has been clear and might be due to the higher sensitivity of TA100 in relation to mutagenic chemicals.

Widespread studies have been performed through various mutagenicity evaluation methods towards sodium nitrite mutagenicity effects. Balmandua and his colleagues' researches represented the mutagenicity effect of this chemical on TA100 strain and non-mutagenic effect of it on TA98 strain by Ames test. Although, the revertant colonies number in TA100 strain was higher in comparison with TA98 strain (in similar concentrations), but the difference is not significant to be called mutagenic. These studies recommended that sodium nitrite might be more effective in the creation of base substitution mutations (Balimandawa, 1994). Similar results including the present study indicated the non-mutagenic effect of sodium nitrite (Toyoizumi *et al*., 2010). Some other studies, particularly the one performed by Helel and her colleagues indicated that sodium nitrite was recognized as a mutagenic chemical, that effects cellular division and chromosome aberrations; in addition, the limited usage of this food additive is suggested in this research (Helal *et al*., 2008).

- Boric acid and Borax Mutagenicity Effects

The short-term studies towards assessment of carcinogenic effects of boric acid have not indicated the carcinogenicity of this chemical in rats. Further studies, for the same tests have been carried out for short, middle, and long term forms on B6C3F1 rats and the results indicated that boric acid carcinogenicity was not significant and generally, the studies carried out do not approve the chemical's carcinogenicity at this product. (Dieter, 1994). In the present study, boric acid and borax kinds' mutagenic effects were analyzed directly by Ames test. The results of table 2 and figure 2 indicate that the revertant colonies number has increased according to boric acid effect on TA98 strain in 500 mg/100g concentration in comparison with 250 mg/100g concentration, but this increase do not approve the mutagenic activity.

Moreover, by increasing the boric acid concentration, the colonies number decreases because of the toxic effects occurrence. In TA100 strain, the revertant colonies number will increase as the result of boric acid effect on 250-1000 mg/100g concentrations in comparison with the simultaneous revertant colonies number; in addition, Dose-Response curve follows the concentration in these points, but the colonies increase is not that much to call boric acid to have mutagenic effects.

The effect of Borax from the Merck Company on TA98 strain indicated that revertant colonies number increase in 500 mg/100g concentration in comparison with 250 mg/100g concentration. In upper concentrations this amount decreased to reach the cellular toxicity at the end. Therefore, the mentioned chemical has not represented any mutagenic effects. On the other hand, its effect on TA100 strain with

the same previous concentrations was studied and the results indicated that there was no effective mutagenicity in this strain, too. The ratio of the revertant colonies number in the same concentrations of Merck Company's borax is slightly higher than its different types in the market this would be due to high purity of Merck borax as compared to the other types.

The results of tables 2 and 3 and figures 2 and 3 indicate that the borax in the market does not have mutagenic effect on TA98 and TA100 strains in none of the tested concentrations.

The studies show that Indian borax has the same effect on TA98 and TA100 strains as the borax in the market and the Merck borax.

Total results of the study represent the non-mutagenicity of boric acid and borax types on TA98 and TA100 strains by Ames test. These results agree with the previous researches about the carcinogenicity of boric acid on animals. Similar results based on the non-mutagenicity of boric acid and borax was obtained through other studies (Landolph, 1985). In comparison with the above researches, some reports offered several evidences based on mutagenicity of these chemicals. For instance, a research about carcinogenicity effects of borax on human's cultured Lymphocytes indicated that the mentioned chemical was effective on human cells and chromosomes and might lead to chromosome aberrations and genetic deficiencies (Arslan *et al*., 2008; Malinee, 2009).

Conclusion

The results showed non-mutagenic effects of sodium nitrite, boric acid and borax types through Ames test. However, these chemicals should also be evaluated further by using various tests to confirm the above results.

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