ฤทธิ์ของแก่นตะวันในการปกป้องจีโนมของแมลงหวี่จากสารไนโทรโซเมทิลยูเรีย

พิสมัย ติง, ปิยะ เต็มวิริยะนุกูล*, แก้ว กังสดาลอาไพ, ครรชิต จุดประสงค์

สถาบันวิจัยโภชนาการ มหาวิทยาลัยมหิดล

บทคัดย่อ

แก่นตะวัน (*Helianthus tuberosus* L.) เป็นพืชหัวที่มีคุณค่าหลายด้าน โดยเป็นทั้งอาหารของมนุษย์ ยาพื้นบ้าน อาหารสัตว์ และในประเทศไทยแก่นตะวันกำลังถูกพัฒนาเป็นอาหารเสริมสุขภาพ ในขณะที่ข้อมูลความปลอดภัยของการบริโภคต่อระบบพันธุกรรมยังไม่ชัดเจน จึงเป็นที่มาของการทดสอบการก่อกลายพันธุ์ด้วยวิธี somatic mutation and recombination test (SMART) เพื่อประเมินความปลอดภัยของแก่นตะวันสด แก่นตะวันต้ม และอินนูลิน (ใยอาหารหลักในแก่นตะวัน) นอกจากนี้ทุกตัวอย่างยังถูกทดสอบความสามารถในการยับยั้งการก่อตัวของสารก่อกลายพันธุ์จากการทำปฏิกิริยาระหว่างโซเดียมไนไทรต์และเมทิลยูเรีย ผลการวิจัยพบว่าแก่นตะวันมีความปลอดภัยต่อระบบพันธุกรรม ไม่เหนี่ยวนำให้เกิดการกลายพันธุ์ อีกทั้งพบว่าทุกตัวอย่างที่ใช้ทดสอบสามารถยับยั้งการก่อตัวของไนโทรโซเมทิลยูเรียได้ในระดับสูง (มากกว่า 80 เปอร์เซ็นต์) ซึ่งอินนูลินแสดงการยับยั้งการเกิดสารก่อกลายพันธุ์สูงสุด (มากกว่า 94 เปอร์เซ็นต์) ดังนั้น อินนูลินอาจเป็นองค์ประกอบหลักในการลดการก่อตัวของสารก่อกลายพันธุ์ที่ได้มาจากปฏิกิริยาระหว่างโซเดียมไนไทรต์และเมทิลยูเรีย ดังนั้น การบริโภคหัวแก่นตะวันอาจเป็นทางเลือกที่ดีต่อผู้บริโภคที่ห่วงใยสุขภาพ เนื่องจากผลการศึกษาครั้งนี้พบว่าแก่นตะวันไม่เหนี่ยวนำให้เกิดการกลายพันธุ์ และสามารถลดความเป็นพิษของไนไทรต์ต่อระบบพันธุกรรมได้

คำสำคัญ: แมลงหวี่ ความเสียหายต่อรหัสพันธุกรรม แก่นตะวัน ไนโทรโซเมทิลยูเรีย

***ผู้รับผิดชอบบทความ**

อ.ดร. ปิยะ เต็มวิริยะนุกูล

สถาบันวิจัยโภชนาการ มหาวิทยาลัยมหิดล นครปฐม 73170

E-mail: piya.tem@mahidol.ac.th

Kaentawan Protects Genome Integrity of *Drosophila melanogaster* from Nitrosomethylurea

Pisamai Ting, Piya Temviriyanukul*, Kaew Kangsadalampai, Kunchit Judprasong
Institute of Nutrition, Mahidol University

Abstract

Kaentawan (*Helianthus tuberosus* L.) tuber has been a valuable crop as human food, traditional medicine and animal feed. In Thailand, it is also being developed as functional food, whereas studies on its genotoxic effect are still scarce. To this concern, the standard genotoxicity test; the somatic mutation and recombination test (SMART), was employed for the sake of safety confirmation. Lyophilized raw Kaentawan, lyophilized boiled Kaentawan and inulin were evaluated for their mutagenicity. Moreover, Kaentawan and inulin were also evaluated whether they could modulate the formation of nitrosomethylurea, which is the mutagen derived from the mixture of sodium nitrite and methylurea. The results indicated that all tested samples were safe in terms of genotoxicity. The strong inhibitory effect towards nitrosomethylurea formation (> 80 percentage of inhibition) was detected in all tested samples. Intriguingly, inulin gave the highest inhibition on mutagen formation (> 94 percentage of inhibition), thus inulin might be the main factor in reducing the formation of mutagen derived from sodium nitrite and methylurea. Thus, the consumption of Kaentawan tuber seems to be a good choice for health concerning consumer since our study demonstrated that Kaentawan was non-genotoxic and could reduce nitrite toxicity.

Keywords: *Drosophila melanogaster*, Genotoxicity, Kaentawan, Nitrosomethylurea

***Corresponding author**
Dr. Piya Temviriyanukul
Institute of Nutrition, Mahidol University 73170
E-mail: piya.tem@mahidol.ac.th

Introduction

Kaentawan (*Helianthus tuberosus* L.) is grown around a temperate zone with a high production of its tuber. Unlike most crops that store carbon as starch, the tuber of Kaentawan stores inulin which is a fructose polymer (soluble fiber). This has a pronounced influence on the value and utility of Kaentawan as soluble dietary fiber[1-3]. Inulin provides various health benefits, *e.g.* maintaining the normal blood sugar and lipid concentration in blood circulation, and improving gastrointestinal and immune system[1,3]. Since Kaentawan is being promoted as functional food in Thailand. Various recipes of the Kaentawan can be developed as raw and cooked food components, such as raw Kaentawan with Namphrik, Miang kham Kaentawan, Kaentawan soup, and Spicy Kaentawan. Recently, Kaentawan sausage has been developed using Kaentawan as a fat substitute for pork fat in order to reduce nitrite and increase nutrition value[4]. In addition, Kaentawan has been reported as a medicinal plant in China. It has been applied to treat many symptoms, such as aperient, cholagogue, diuretic, spermatogenic, stomachic, diabetes and rheumatism[1,2,5]. However, the information regarding the safeness of Kaentawan is still scarce. Thus, it is of great interest to investigate the genotocixity of Kaentawan.

Previous studies suggested that direct mutagens formed from nitrite and mutagen precursors in the acid condition similar to the stomach digestion could be one of the etiologies of the human gastric cancer[6-8]. Kaentawan is a source of dietary fibers[9], which are extensively studied for their properties as antimutagens and chemopreventive agent. The hypothesis regarding the chemopreventive properties of dietary fibers in cancer prevention could be due to that dietary fibers are effective in binding of carcinogens[10]. In support, rats, which were fed with inulin, exhibited low pre-neoplastic lesion induced by a mutagen, 1,2 dimethylhydrazine dihydro-chloride[11]. Thus, the present study was also designed to investigate the modulating effect of Kaentawan on the formation of nitrosomethylurea.

The somatic mutation and recombination test (SMART) or wing spot formation test is an *in vivo* standard genotoxicity test. The principle of the test is based on induced loss of the heterozygosity, which may occur through various mechanisms, such as chromosomal rearrangement, chromosome breakage, or chromosome loss as well as mitotic recombination and gene conversion[12]. On the treatment of larvae during the embryogenesis, the imaginal disc cells proliferate mitotically and many genetic

events, such as point mutation, deletion, somatic recombination and non-disjunction can be determined on the wing of adult flies[13]. If a genetic alteration occurs in one cell of the imaginal disc during mitotic proliferation, it will form a clone of mutant cells expressing the phenotype regulated by the specific genetic markers of wing surface *i.e.* the recessive markers multiple wing hair (*mwh*) and flare (flr^3) phenotype[12,14].

Materials and methods

Chemicals and Reagents

N-methylurea was ordered from Fluka AG (Buch, Switzerland). Sodium nitrite was purchased from BDH Chemicals Ltd. (Poole, England). Inulin (98%) was purchased from Beneo (Mannheim, Germany). Other experimental ingredients were of food grade.

Sample preparation

The tuber of Kaentawan (6 kg) was obtained from the largest producer of Thailand namely, Mae Klong Yai Irrigation Water Management Research Station, Nakhon Pathom Province. Then, Kaentawan was cleaned twice with tap water. Subsequently, it was dried with an electric fan. Its outer skin was peeled off before it was divided into two portions. The former was used raw and the latter was boiled for 8 minutes until the texture became soft. Each portion of the sample was blended in a homogenizer and lyophilized.

The somatic mutation and recombination test (SMART)

Briefly, virgin *ORR; flr^3* females and *mwh* males were mated on the standard medium (corn flour [0.25 g], sugar [0.20 g], agar [0.03 g], yeast [0.10 g] and distilled water [2 ml]). Six days after mating, 100 of 3-day old larvae were collected, washed with water and transferred to the experimental medium.

To determine the mutagenicity of Kaentawan, the standard medium was substituted for corn flour by lyophilized raw Kaentawan, lyophilized boiled Kaentawan or inulin for 50% and 100%, respectively [0.125 or 0.250 g] and mix well with other ingredients, such as sugar [0.2 g], agar [0.03 g], yeast [0.10 g] and distilled water [2 ml] to make experimental medium. The standard medium was used as a negative control, while standard medium containing nitrosomethylurea was used as a positive control. All experiments were performed at 25±1°C. The surviving adult flies were collected, at least 40 wings, were analyzed under a compound microscope for exhibiting the multiple wing hairs (*mwh*) or the flare (flr^3) phenotype[12, 15].

To determine the antimutagenicity of Kaentawan, the experimental medium was added with the combination of sodium nitrite (36 mM, 1 ml) and methylurea solution (10 mM, 1 ml). The combination of sodium nitrite (36 mM, 1 ml) and methylurea (10 mM, 1 ml) was used as positive control medium. In each experimental medium, the final concentration of nitrite is 18 mM[16]. All experiments were performed as mentioned above. Overall steps are also shown in Figure 1.

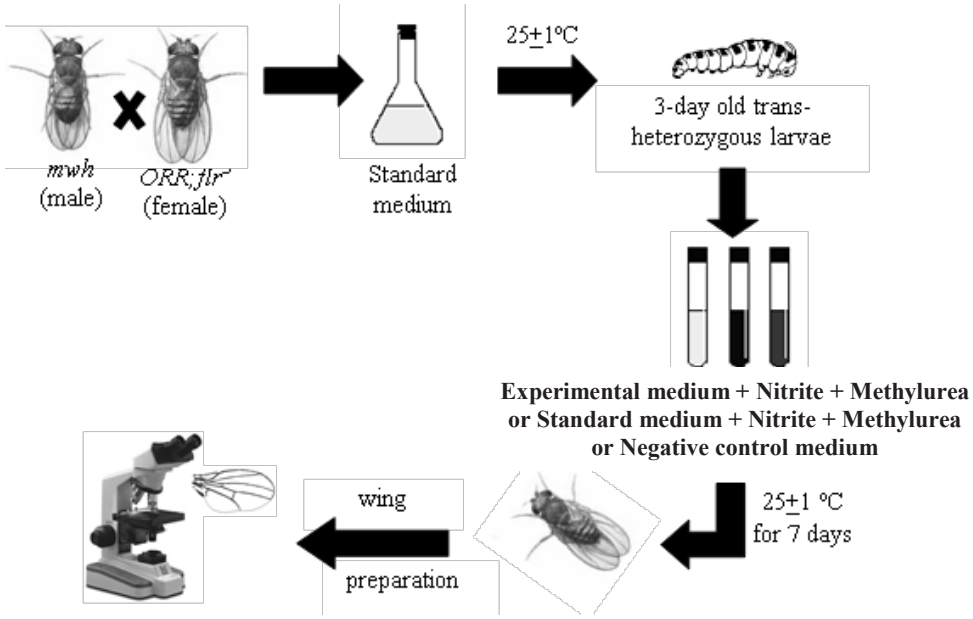

Figure 1 Antimutagenicity study of samples on mutagenicity of *in vivo* formation of nitrosomethylurea induced wing spots of *Drosophila melanogaster* (courtesy of Assoc. Prof. Kaew Kangsadalampai)

The wing spots data were scored as frequency of spot per wing and evaluated for mutagenicity using the statistical procedure as described by Frei and Würgler[17]. Test responses were classified into three categories; (I) positive (+), a strong response was found; (II) inconclusive (i), no acceptance at the same time of two mutually exclusive hypotheses; and (III) negative (-), no effects under the conditions of the test. Furthermore, the modulating activity of each sample was evaluated and the result was expressed as follows:

Percentage of inhibition = (a-b)/a × 100

Where "a" is the number of total spots/wing induced by nitrosomethylurea, "b" is the number of total spots/wing induced by nitrosomethylurea simultaneously administered with each

sample. It is proposed that percent of inhibition between 0-20%, 20-40%, 40-60% and higher than 60% indicate negligible, weak, moderate and strong inhibition of sample on *in vivo* formed nitrosomethylurea, respectively[15].

Results and discussion

To determine the mutagenic properties of Kaentawan, the standard medium was substituted for corn flour by lyophilized raw Kaentawan, lyophilized boiled Kaentawan or inulin for 50% and 100%, respectively. The data is shown in Table 1. The results showed that Kaentawan induced the spot/wing between 0.30-0.65 spot/wing, while the negative control was 0.08 spot/wing. The statistical analysis according to Frei and Würgler[17] revealed that Kaentawan at any tested concentration was not mutagenic. In consistent with the literature, nitrosomethylurea is potent mutagen[18] as shown in Table 1. Inulin, a soluble fiber mainly found in Kaentawan, was also investigated for its mutagenic properties. As expected, inulin did not statistically induce wing/spot in tested *Drosophila* compared to control (Table 1). This is in agreement with a study that inulin extracted from chicory was not mutagenic when inulin was tested using several strains of bacteria including *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100, and *Escherichia coli* strain WP2uvrA[21]. Thus, the daily intake of inulin has been accepted at up to 10 g in U.S. and Europe[19]. The NOEL (no observed effect level) of inulin for the laxative effect is at 30 g per day[20].

Beside the mutagenic properties of Kaentawan, we also investigated the antimutagenic properties of Kaentawan in order to promote its healthy value. To this aim, the solution of sodium nitrite and methylurea solution were added to experimental medium. The number of total spot per wing of adult files obtained from each experimental medium containing nitrosated methylurea was compared with that of adult files obtained from positive control group in order to derive the percentage of inhibition on the formation of nitrosomethylurea; the result is shown in Table 2. As compared to the positive control, lyophilized raw Kaentawan, lyophilized boiled Kaentawan and inulin exhibited potential inhibitory effect to reduce wing spots induced by *in vivo* formed *N*-nitrosomethylurea (88-94 percentage of inhibition) (Table 2). The result that inulin inhibited the formation of mutagen derived from the mixture of sodium nitrite and methylurea implied that soluble fibers, especially inulin might be the major components of Kaentawan in

inhibiting the mutagen formation. Since the concentration of inulin used here was higher than that of inulin found in tested lyophilized Kaentawan (both 50% and 100%), hence we conclude that inulin from 50% lyophilized Kaentawan or even lower was effectively enough to prevent the formation of nitrosomethylurea. Guzmán-Rincón and his co-workers[22] suggested that the *in vivo* nitrosation between nitrite ion and its nitrosable compound occurred in the digestive tract of *Drosophila melanogaster* larvae in a similar manner to that of mammalian stomach. Therefore, it is tempting to speculate that Kaentawan might act as carcinopreventive agent in order to prevent the formation of potent mutagen from nitrosated methylurea.

Sausage is a well-known source of nitrite compound that is subsequently interacted with suitable amine precursors in an acid condition similar to stomach digestion resulting in a potent mutagen, *N*-nitroso compounds formation, such as nitrosomethylurea[18,23]. Thus, a special sausage was formerly developed using Kaentawan as a fat substitute for pork fat, which could reduce the amount of nitrite added and increase its nutritive value while the texture was acceptable[4]. In addition, Beriain *et al.* [24] also substituted inulin and olive oil for fat in the sausage and achieved good acceptability rating. Our data revealed that raw and boiled Kaentawan possessed the same inhibitory effect against the formation of *N*-nitroso compounds (Table 2), so replacement of Kaentawan in food, for example sausage or bologna, might be worth developing as healthy food.

However, the safety evaluation of such modified meat products in terms of the occurrence of botulinum toxin has to be seriously considered since the presence of nitrite residue in fermented meat product generally guarantees the absence of such toxin.

Table 1 The mutagenicity of Kaentawan as well as inulin reported as wing spot induction on *Drosophila melanogaster* derived from trans-heterozygous *mwh+/+flr*^{3} larvae of the improved high bioactivation cross fed medium containing each sample.

Treatment		**Spots per wing (Number of spots from 40 wings)**[a]			
Compound	**Amount of corn flour substitution (g/tube)**	**Small single spot (m = 2)**	**Large single spot (m = 5)**	**Twin spot (m = 5)**	**Total spot (m = 2)**
Negative control	-	0.03 (1)	0.05 (2)	0 (0)	0.08 (3)
Nitrosomethylurea	-	14.33 (430)+	2.27 (68)+	0.10 (3)+	16.73 (501)+
Raw Kaentawan 50%	0.125	0.53 (21)i	0.08 (3)i	0.05 (2)i	0.65 (26)i
Raw Kaentawan 100%	0.250	0.35 (14)i	0.03 (1)-	0.03 (1)i	0.40 (16)i
Boiled Kaentawan 50%	0.125	0.45 (18)i	0.05 (2)-	0 (0)i	0.50 (20)i
Boiled Kaentawan 100%	0.250	0.43 (17)i	0.03 (1)-	0 (0)i	0.45 (18)i
Inulin 50%	0.125	0.25 (10)i	0.05 (2)-	0 (0)i	0.30 (12)-
Inulin 100%	0.250	0.30 (12)i	0 (0)-	0 (0)i	0.30 (12)-

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparison with deionized water; + = Positive; - = Negative; i = Inconclusive. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical test
"m" is an increased mutation frequency compared with the spontaneous frequency (m times).

Table 2 The modulating effect of Kaentawan as well as inulin on the combination of sodium nitrite and methylurea administered to *Drosophila melanogaster* derived from trans-heterozygous *mwh+/+flr*^{3} larvae mutagenicity expressed as percentage of modification.

Treatment		Spots per wing (Number of spots from 40 wings)[a]				
Compound	**Amount of corn flour substitution (g/tube)**	**Small single spot (m=2)**	**Large single spot (m = 5)**	**Twin spot (m = 5)**	**Total spot (m = 2)**	**Percent inhibition[b]**
Negative control	-	0.03 (1)	0.05 (2)	0 (0)	0.08 (3)	
Nitrosomethylurea	-	14.33 (430)+	2.27 (68)+	0.10 (3)+	16.73 (501)+	
Raw Kaentawan 50%	0.125	0.90 (36)	0.25 (10)	0.03 (1)	1.18 (47)	91
Raw Kaentawan 100%	0.250	1.08 (43)	0.13 (5)	0 (0)	1.2 (48)	90
Boiled Kaentawan 50%	0.125	1.18 (47)	0.15 (6)	0.15 (6)	1.48 (59)	88
Boiled Kaentawan 100%	0.250	1.10 (44)	0.15 (6)	0.03 (1)	1.28 (51)	90
Inulin 50%	0.125	1.00 (40)	0.05 (2)	0 (0)	1.05 (42)	92
Inulin 100%	0.250	0.73 (29)	0.03 (1)	0 (0)	0.75 (30)	94

[a]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Würgler (1988) for comparison with deionized water; + = Positive; - = Negative; i = Inconclusive. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical test.

[b]Percent of inhibition ((a-b)/a) x 100. Where "a" is the number of total spots per wing of nitrosomethylurea group, "b" is the number of total spots per wing of each experimental group.

"m" is an increased mutation frequency compared with the spontaneous frequency (m times).

References

1. Kay SJ, Nottingham SF. Biology and Chemistry of Jerusalem Atrichoke *(Helianthus tuberosus L*). Northwest, Florida: CRC press, 2007.
2. Pan L, Sinden MR, Kennedy AH, *et al*. Bioactive constituents of *Helianthus tuberosus* (Jerusalem artichoke). *Phytochem Lett* 2009; 2: 15-8.
3. Judprasong K, Tanjor S, Puwastien P, *et al.* Investigation of Thai plants for potential sources of inulin-type fructans. *J Food Compos Anal* 2011; 24: 642-9.
4. Rojanakor T. Use of Kaen-ta-wan (Jerusalem Artichoke) powder and tomato paste for producing reduced fat and nitrite emulsion sausage as a functional Food. Bangkok: National Research Council of Thailand, 2009.
5. Yuan X, Gao M, Xiao H, *et al.* Free radical scavenging activities and bioactive substances of Jerusalem artichoke (*Helianthus tuberosus L.*) leaves. *Food Chem* 2012; 133: 10-4.
6. Wakabayashi K, Nagao M, Chung TH, *et al.* Appearance of direct-acting mutagenicity of various food stuffs produced in Japan and Southeast Asia in nitrite treatment. *Mutat Res* 1984; 158: 119-24.
7. Laires A, Gaspar J, Borba H, *et al.* Genotoxicity of nitrosated red wine and of the nitrosatable phenolic compounds present in wine: Tyramine, quercetin and malvidine -3- glucoside. *Food Chem Toxicol* 1993; 31: 989-94.
8. Higashimoto M, Akada Y, Sato M, *et al.* Inhibitory effects of tea extracts on the mutagenicity of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid on treatment with nitrite in the presence of ethanol. *Food Chem Toxicol* 2000; 38: 7-13.
9. Towviriyakul A. Formulation of foods for the elderly containing Kaentawan (*Helianthus tuberosus* L.). [M.S. Thesis in Food and nutrition for development]. Nakhon Pathom: Institute of Nutrition, Mahidol University, 2013.
10. Møller ME, Dahl R, Bøckman OC. A possible role of the dietary fibre product, wheat bran, as a nitrite scavenger. *Food Chem Toxicol* 1988; 26: 841-5.
11. Verma A, Shukla G. Administration of prebiotic inulin suppresses 1,2 dimethylhydrazine dihydrochloride induced procarcinogenic biomarkers fecal enzymes and preneoplastic lesions in early colon carcinogenesis in Sprague Dawley rats. *J Funct Foods* 2013; 5: 991-6

12. Graf U, Wurgler FE, Katz AJ, *et al.* Somatic mutation and recombination test in *Drosophila melanogaster*. *Environ Mutagen* 1984; 6: 153-88.
13. Würgler FE, Vogel EW. *In vivo* mutagenicity testing using somatic cells of Drosophila melanogaster. In: Serres FJD, editor. Chemical mutagens, principles and methods for their detection (1-72). New York: Plenum Press, 1986.
14. Szabad J, Soós I, Polgár G, *et al.* Testing the mutagenicity of malondialdehyde and formaldehyde by the Drosophila mosaic and the sex-linked recessive lethal tests. *Mutat Res Genet Toxicol Environ Mutagen* 1983; 113: 117-133.
15. Abraham SK, Graf U. Protection by coffee against somatic genotoxicity in *Drosophila*: Role of bioactivation capacity. *Food Chem Toxicol* 1996; 34: 1-14.
16. Rincón JG, Espinosa J, Graf U. Analysis of the in vivo nitrosation capacity of the larvae used in the wing somatic mutation and recombination test of *Drosophila melanogaster*. *Mutat Res Genet Toxicol Environ Mutagen* 1988; 412: 69-81.
17. Frei H, Würgler FE. Statistical methods to decide whether mutagenicity test data from Drosophila assay indicate a positive, negative or inconclusive result. *Mutat Res* 1988; 203: 297-308.
18. ROC (Report on Carcinogens) Thirteenth Edition. N-Nitroso-N-methylurea. U.S. Department of Health and Human Services, 2014. p 15-17.
19. Coussement PAA. Nutritional and health benefits of inulin and oligofructose. *J Nutr* 1999; 129: 1412S-17S.
20. Briet F, Achour L, Flourie´ B, *et al.* Symptomatic response to varying levels of fructooligosaccharides consumed occasionally or regularly. *Eur J Clin Nutr* 1995; 49: 501-7.
21. Johannsen FR. Toxicological profile of carboxymethyl inulin. *Food Chem Toxicol* 2003; 41: 49-59.
22. Guzmán-Rincón J, Espinosa J, Graf U. Analysis of the *in vivo* nitrosation capacity of the larvae used in the wing somatic mutation and recombination test of *Drosophila melanogaster*. *Mutat Res* 1998; 412: 69-81.
23. Mirvish SS. N-Nitroso compounds, nitrite, and nitrate; Possible implications for the causation of human cancer. Proceedings of the Conference on Nitrogen As a Water Pollutant; 2013; 8: 195-207.

24. Beriain MJ, Gómez I, Petri E, *et al.* The effects of olive oil emulsified alginate on the physico-chemical, sensory, microbial, and fatty acid profiles of low-salt, inulin-enriched sausages. *Meat Sci* 2011; 88: 189-97.