Effect of Soymilk Residue Substitution for Flour of Some Dishes on Antioxidant Activity and Urethane Induced Mutation in *Drosophila melanogaster*

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Abstract

Four dishes, namely glutinous rice balls in coconut milk (Bua-loi), crisp crackers (Khao-kriep), fried radish cake (Khanom-puk-kad) and cookies that have soymilk residue (SR) substituted for flour as well as their traditional corresponding ones were determined for antioxidant activity and total phenolic content. The antioxidant activity of each SR- substituted samples seemed to be the same as of its corresponding traditional one. Only the phenolic content of SR- fried radish cake increased. The samples were evaluated for their modulating effect on urethane induced wing spots of *Drosophila melanogaster* in somatic mutation and recombinant test (SMART). Firstly, three day-old trans-heterozygous larvae (*mwh flr*$^{+}$*/ mwh TM3*) were transferred to the *Drosophila* medium containing each sample substituted for corn flour; it was found that none was mutagenic. In the antimutagenicity study, each sample was subjected to the same procedure as that of the mutagenicity study except the medium used in bringing up the larvae had each sample substituted for corn flour and 20 mM urethane substituted for deionized water. The number of urethane induced wing spots on flies from both SR substituted samples and their corresponding traditional ones were reduced. Overall data suggested that the substitution of SR in each dish should be benefit to the consumers because it had increased antimutagenicity. The antimutagenicity might be due to some components of each sample modulated the expression of urethane mutagenicity.

Keywords: Soymilk residue, SMART, Antimutagenicity, Antioxidant activity

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ผลของการใช้กากจากผลิตภัณฑ์นมถั่วเหลืองแทนแป้งบางส่วนในอาหารบางสำรับต่อฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านก่อกลายพันธุ์โดยยูรีเทนในแมลงหวี่

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บทคัดย่อ

ในงานวิจัยได้ทำการวัดฤทธิ์ต้านอนุมูลอิสระและปริมาณของสารประกอบฟีนอลิคในอาหารไทย 4 ตำหรับคือ ขนมบัวลอย ข้าวเกรียบ ขนมหัวผักกาด และคุกกี้ ที่เสริมและไม่เสริมกากจากการผลิตนมถั่วเหลือง พบว่าตัวอย่างทั้งหมดมีฤทธิ์ต้านอนุมูลอิสระและมีสารประกอบฟีนอลิคในปริมาณไม่เปลี่ยนมากนักหลังการทดแทนแป้งด้วยกากถั่ว ยกเว้นปริมาณสารฟีนอลิคในขนมหัวผักกาดเพิ่มขึ้นชัดเจน ตัวอย่างทั้งหมดถูกนำมาศึกษาฤทธิ์ก่อกลายพันธุ์และฤทธิ์ต้านการก่อกลายพันธุ์ในแมลงหวี่ (*Drosophila melanogaster*) ด้วยวิธี somatic mutation and recombination test (SMART) โดยการเลี้ยงหนอนแมลงหวี่ trans-heterozygous larvae (*mwh flr*$^{+}$*/ mwh TM3*) อายุ 3 วัน ในอาหารทดสอบ (อาหารเลี้ยงแมลงหวี่ปกติที่แทนที่แป้งข้าวโพดด้วยตัวอย่างอาหารเพื่อทดสอบฤทธิ์ก่อกลายพันธุ์) และอาหารทดสอบที่มีสารละลาย 20 mM ยูรีเทนแทนที่น้ำ (เพื่อทดสอบฤทธิ์ต้านการก่อกลายพันธุ์ของตัวอย่างอาหาร) โดยใช้อาหารเลี้ยงแมลงหวี่ปกติและอาหารเลี้ยงแมลงหวี่ปกติที่มี 20 mM ยูรีเทน เลี้ยงแมลงหวี่กลุ่มควบคุมผลลบและกลุ่มควบคุมผลบวก ตามลำดับ ผลการศึกษาพบว่า ตัวอย่างทั้งหมดไม่มีฤทธิ์ก่อกลายพันธุ์ แต่มีฤทธิ์ต้านการก่อกลายพันธุ์ของยูรีเทน และการเสริมกากถั่วเหลืองใน บัวลอย ข้าวเกรียบ ขนมหัวผักกาด และคุ้กกี้นั้น มีประโยชน์ต่อผู้บริโภค เนื่องจากทำให้ฤทธิ์ต้านการก่อกลายพันธุ์เพิ่มขึ้นจากเดิม ซึ่งอาจเกิดจากการที่องค์ประกอบของตัวอย่างอาหารมีสารที่ไปยับยั้งกระบวนการออกฤทธิ์ก่อกลายพันธุ์ของยูรีเทนได้

คำสำคัญ: กากถั่วเหลือง SMART ฤทธิ์ต้านการก่อกลายพันธุ์ ฤทธิ์ต้านอนุมูลอิสระ

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Introduction

Soybean is used to make soymilk, tofu, tempeh, miso, natto and yogurt. They provide additional health benefits for the consumer besides their nutritional properties. The principal isoflavones found in soy proteins and soy foods are daidzein, genistein, and glycitein[1] which are antioxidants, therefore, they might play a role in the prevention of a number of chronic diseases such as cancer and cardiovascular disease[2].

The production of soymilk and tofu produce soymilk residue (SR) or okara[3]. SR contains mostly crude fiber composed of cellulose, hemicellulose and lignin, about 25% protein, 10-15% oil, but little starch or simple carbohydrates[4]. It can be widely utilized as an ingredient of food or as animal feed. It has been used as culture media of some microorganism[5], e.g. SR of tofu manufacturing was used to *Ganoderma lucidum* culture in solid-state fermentation[6]. SR is also used as an ingredient of bakery product. For example, it was added into biscuits and snacks to reduce calorie intake and to increase dietary fiber[7]. Trongpanich *et al*.[8] increased dietary fiber and protein content of extruded cereal snacks with the addition of SR. Although several researchers tried to increase the amount of SR added to bakery products or other snacks without having a bad impact on sensory characteristics by the substitution of SR in food has never been investigated on other health benefit. Thus, the present study was proposed to determine the modulating effect of SR substitution on urethane mutagenicity in *Drosophila melanogaster* as well as the antioxidant and total phenolic content of the SR-substituted products.

Materials and Methods

Chemicals and reagents

Urethane, 2, 4, 6-tripyridly-s-triazine (TPTZ), ferric chloride hexahydrate and ferrous sulfate heptahydrate were purchased from Sigma Chemical (St. Louis, MO, USA). Diethylether and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Propionic acid, 2, 2-diphenyl-1-picrylhydrazl (DPPH), gallic acid and Folin-Ciocalteu reagent were supplied from Fluka Chemika (Buchs, Switzerland). Trolox was brought from Aldrich Chemical (Milwaudee, WI, Germany). All other chemicals and reagents were of analytical grade.

Sample preparation

Soy milk was prepared by soaking soybean brought from a local supermarket (Salaya, Nakhon Pathom Province) in water at room temperature overnight, then it was added

with deionized water at the ratio of 1:1 (w/v) and homogenized in an electric blender for 1 min. Soy (milk) residue (SR) was separated using cheesecloth for further study.

Glutinous rice ball in coconut milk (Bua-loi), crisp cracker (Khao-kriep), fried radish cake (Khanom-puk-kad) and cookie were prepared with partial substitution of SR for flour as described elsewhere[9,10]. Corresponding traditional dish of each sample was used as a control. Each sample was chopped into small pieces and ground, and then it was lyophilized. Each dried sample was kept refrigerated before revealing its antioxidant activity, total phenolic content and antimutagenicity against urethane.

Antioxidant activity and total phenolic content

A portion of sample (5 g) was mixed with 80% methanol (50 ml) in a 250-ml Erlenmeyer flask. It was stirred for 2 h at room temperature, and then the extract was filtered through Whatman filter paper No.1 and collected into a glass bottle. The antioxidant capacity was measured by the DPPH radical method according to Fukumoto & Mazza[11] and modified the procedures of measurement by using a microplate reader[12]. The reducing power of methanolic extract was also determined according to the method of Benzie & Strain[13]; Li *et al.*[14] with slight modification[12]. The phenolic content of methanolic extract was determined with Folin-Ciocalteu reagent method[15] and modified the procedures of measurement by using a microplate reader[12].

Somatic Mutation and Recombination Test (SMART)

The test was performed as described by Graf *et al*[16]. Three-day-old, trans-heterozygous larvae (*mwh flr*$^{+}$*/mwh TM3*) were transferred to the *Drosophila* medium[17] that each dessert was substituted for corn flour (used for the mutagenicity evaluation of each sample) and the *Drosophila* medium that each dessert was substituted for corn flour and 20 mM urethane was substituted for water (used for antimutagenicity evaluation of each sample). The standard medium[17] was used as the negative control and that containing 20 mM urethane was used as the positive control. The wings of the surviving flies were analyzed for the occurrence of mutant spots. The wing spots data were evaluated using the statistical procedure and a multiple decision procedure as described by Frei and Wurgler[18]. The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed with significance level of $\alpha = \beta = 0.05$. Each experiment was done twice.

The antimutagenicity of each sample was determined from the percentage of inhibition calculated as the following equation:

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

When *a* is the frequency of spots induced by urethane alone and *b* is the frequency of spots induced by urethane in the presence of sample. It is proposed that percentages of inhibition between 0-20 represent a negligible effect while expression of percent inhibitions between 20-40, 40-60 and more than 60 are the evidences of weak, moderate and strong antimutagenicity, respectively as suggested by Abraham[19].

Results

The antioxidant activity of each food substituted with SR and its corresponding traditional one is shown in Figure 1. The reducing of DPPH by antioxidant of each sample expressed as Trolox equivalent antioxidant capacity (mg TEAC/g dry weight) is shown in Figure 1a. Free radical scavenging activity of each food substituted with SR is between 0.134 to 0.727 TEAC/g dry weight. The FRAP value (mg Fe(II)/g dry weight) of each food substituted with SR is between 232.32 -1006.81 mg Fe(II)/g dry weight (Figure 1b). The total phenolic content of each sample (mg GAE/g dry weight of each food substituted with SR) is in the range between 48.50 and 436.75 of mg gallic acid/g dry weight (Figure 1c).

The number of induced wing spots of adult flies derived from larvae of brought up on *Drosophila* medium containing each sample substituted for corn flour was not different from that of the flies brought up on the negative control medium (data not shown). This indicated that most samples contain no mutagen. The antimutagenicity of each food substituted with SR has been compared with that of its corresponding traditional one. SR substituted glutinous rice ball in coconut milk, crisp cracker, fried radish cake and cookie presented greater antimutagenicity than that of their corresponding traditional ones (Figure 2). Partial substitution of SR for rice flour in cookie, glutinous rice ball in coconut milk and fried radish cake increased their antimutagenicity to be higher than that of their corresponding traditional ones. However the substitution of SR did not change the levels of inhibition. It, therefore, indicated that SR influenced on the mutagenicity of urethane in this study.

Discussion

The study on traditional food samples revealed that they were good sources of antioxidants including phenolic compounds.

This may be due to the fact that almost traditional samples contained many herbs and spices that are the sources of antioxidants[20,21] . Crisp cracker has garlic, pepper and coriander as its flavor. Bozin *et al.*[22] reported that the extracts of garlic reduced the DPPH radical (IC_{50} ranging from 1.03 to 6.01 mg/ml) and neutralized H_2O_2 (IC_{50} ranging from 0.55 to 2.01 mg/ml) in a dose-dependent manner. Strong inhibition of lipid peroxidation in both systems of induction was observed for all tested garlic extracts. Agbor *et al.*[23] suggested that peppercorns especially black pepper, which constitutes an important component in the diet of many sub-Saharan and oriental countries, can be promoted for their nutritional importance as antioxidants and radical scavengers. Moreover, Wangensteen[24] suggested that the addition of coriander to food increased the antioxidant content and might have potential as a natural antioxidant and thus inhibit unwanted oxidation processes.

The water extract from fragrant screw pine (*Pandanus amaryllifolius*) was the main flavor of glutinous rice ball in coconut milk. Nor *et al.*[25] reported that fragrant screw pine leaf extract, which had a polyphenol content of 102 mg/g wet weight, exhibited an excellent heat-stable antioxidant property and may be a good natural alternative to existing synthetic antioxidants in the food industry.

The results that fried radish cake and cookie had antioxidants activity and contained phenolic compounds may also be due to their recipe. The components of fried radish cake, namely Chinese radish and Chinese chive leaves were found to have radical scavenging capacity[26]. Chinese chive (*Allium tuberosum*) belongs to the same family as garlic, onion and leek and is an important ingredient in Asian cooking[27]. Allicin and thiosulfinates could be identified in Chinese chive[28,29]. They are both antioxidant[30,31]. This seemed to be the reason why fried radish cake had antioxidant activity.

Brown color that formed through Maillard reaction might take its responsibility as antioxidant of cookie. Gu *et al.*[32] reported that Maillard reaction products of amino acid-sugar exhibited antioxidant activity. Benjakul *et al.*[33] reported that Maillard reaction products derived from fructose or galactose at the level of 2% showed the increase in reducing power and DPPH radical-scavenging activity.

The addition of SR into glutinous rice ball in coconut milk, crisp cracker and cookie did not make much difference on their antioxidant activity and levels of phenolic compounds. Only the phenolic content of fried radish cake significantly increased. Since the substitution slightly changed all parameters

determined in this study, it was suggested that SR still contained antioxidants including phenolic compounds at the amounts that did not change the overall content in the substituted samples compared with those of their corresponding traditional samples. It is well known that soybean and its products are important sources of antioxidant like polyphenols, including isoflavones[34]. Soy isoflavones showed significant antioxidant activities by inhibiting lipid oxidation, scavenging free radicals, and promoting the expression of antioxidative enzymes[35]. It was suggested that isoflavones were responsible for health benefits, including protection against oxidative stress[36]. Patel *et al.*[37] reported that soy isoflavones were capable of inhibition lipoprotein oxidation *in vitro* and suppressing formation of plasma lipid oxidation products *in vivo*.

The investigation on the antimutagenicity of the samples against urethane in *Drosophila melanogaster* in this study was the first attempt to demonstrate that all samples had a health benefit in terms of reducing the mutagenicity of mutagen. The reason might be due some active components in the herbs used in sample preparations that possessed antimutagenic properties. Garlic contains allyl sulfurs[38] that could modulate the activity of several metabolizing enzymes which activate (cytochrome P450s) or detoxify (glutathione S-transferases) carcinogens and inhibit the formation of DNA adducts in several target tissues[39]. Black pepper (*Piper nigrum* L.) that contains flavonoids[40] effectively counteracted the mutagenicity of ethyl carbamate in SMART[41]. Aqueous crude coriander juice that contains flavonoids and polyphenols[42] significantly decreased the mutagenicity of metabolized aromatic amines (AA) in the Ames test using *Samonella typhimurium* TA98.[43] The mutagenicity of urethane might also be reduced by chlorophyll in the fragrant screw pine that Negishi *et al.*[44] demonstrated its inhibitory effect on wing spot formation in *Drosophila* induced by 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2).

The antimutagenicity against urethane of each sample might be due to the fact that urethane is metabolically activated by cytochrome P-450 enzyme system[45] to be vinyl epoxide, the carcinogenic active metabolite[46], that is further detoxified with glutathione-*S*-transferase (GST) conjugation[47]. If number of spots per wing reduces, it is postulated that the sample might act as an inducer of glutathione-*S*-transferase (GST) or an inhibitor of cytochrome P-450 system. The reason why fried radish cake could inhibit the mutagenicity of urethane might be due to

Chinese radish and Chinese chive. Chinese radish has isothiocyanates[48]. Uda *et al*.[49] reported that isothiocyanates induced glutathione *S*-transferase and quinone reductase in animal tissues. Some isothiocyanates were shown to have an antimutagenic effect against heterocyclic amines, namely IQ and Trp-P-1[49] via an inhibition of cytochrome P-450 mediated metabolic activation of the mutagens. Chinese chive has allyl methyl disulfide and allyl methyl trisulfide. They could inhibit benzo[*a*]pyrene induced neoplasia and increase glutathione *S*-tranferases (GST) activity in the stomach of female A/J mice.[50]

It was demonstrated in this investigation that traditional cookie decreased number of urethane induced *Drosophila malanogaster* wing spot. Maillard reaction products (MRPs) occurred during baking of cookie might act as an antimutagen. However, there is scarce information that browning reaction compounds inhibited mutagenicity of any chemicals. Borrelli *et al.*[51] reported that melanoidins extracted from biscuits was able to inhibit the activity of Phase I (NADPH-cytochrome-C reductase) and phase II (Glutathione-*S*-transferase) enzymes. In addition, MRPs exhibited antioxidant activity against fenton reaction-induced hydroxyl free radicals[52].

The addition of soymilk residue to food samples namely glutinous rice ball with coconut milk, crisp cracker, fried radish cake and cookie could increased their antimutagenicity to be higher than that of their corresponding traditional ones. It is well known that soybean is a source of isoflavone[53] and Kangsadalampai and Sommani[54] revealed the antimutagenicity of soybean products currently consumed in Thailand in *Drosophila melanogaster*. Previous data indicated that isofavones activated phase II detoxification enzymes such as UDP-glucuronyl transferase, glutathione-*S*-transferase and quinone reductase[55]; such activations might take responsibility in reducing the mutagenicity of urethane. The isoflavones isolated from soybean seeds had a suppressive effect against 3 - amino -1,4 - dimethyl - 5H-pyrido [4,3b] indole[56] and benzo [*a*] pyrene induced genotoxicity in Swiss albino mice[57].

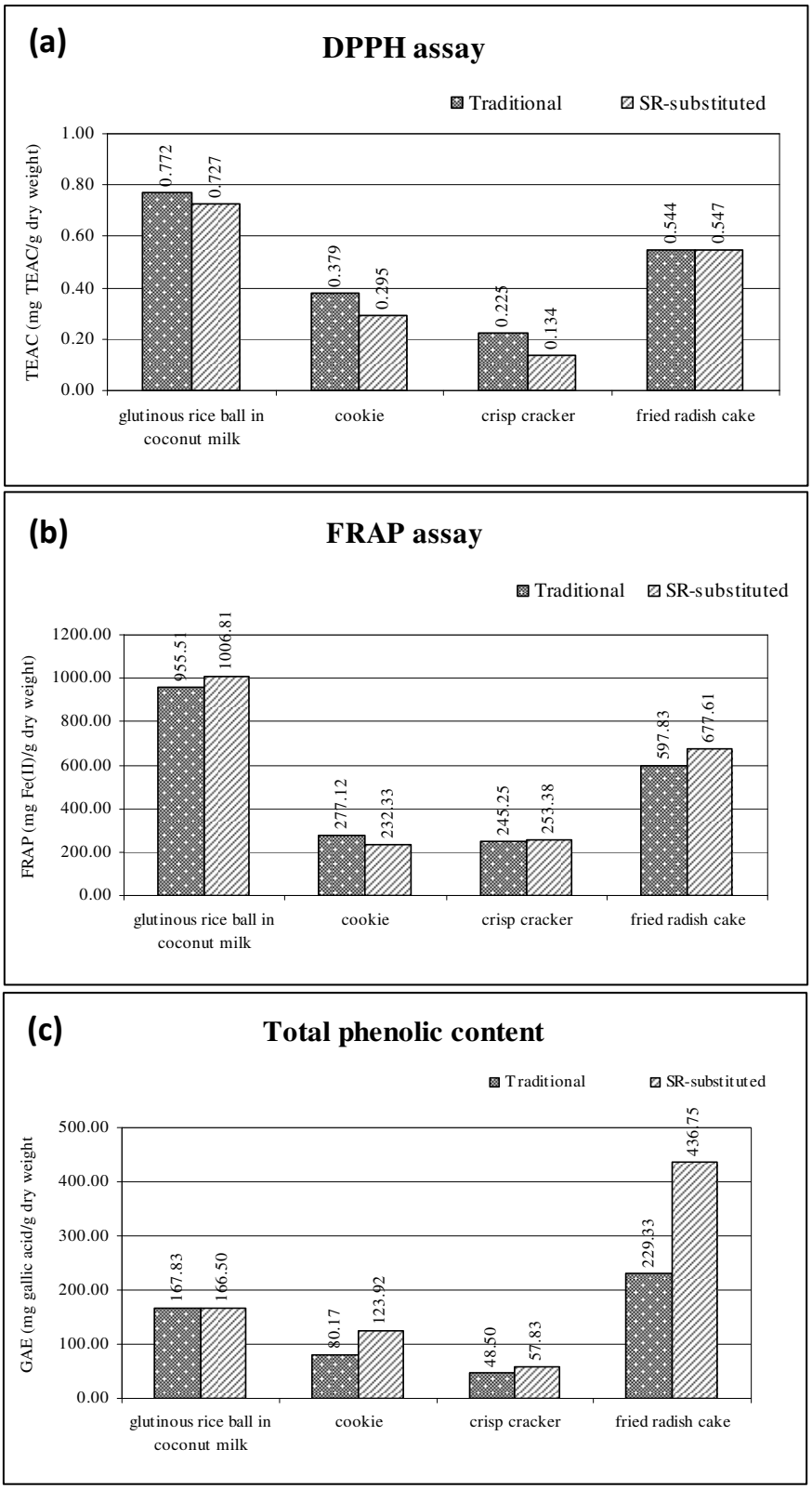


Figure 1 Antioxidant activity in DPPH assay (a), antioxidant activity in FRAP assay (b) and phenolic content (c) of methanolic extracts from each food substituted and unsubstituted with SR (traditional one).

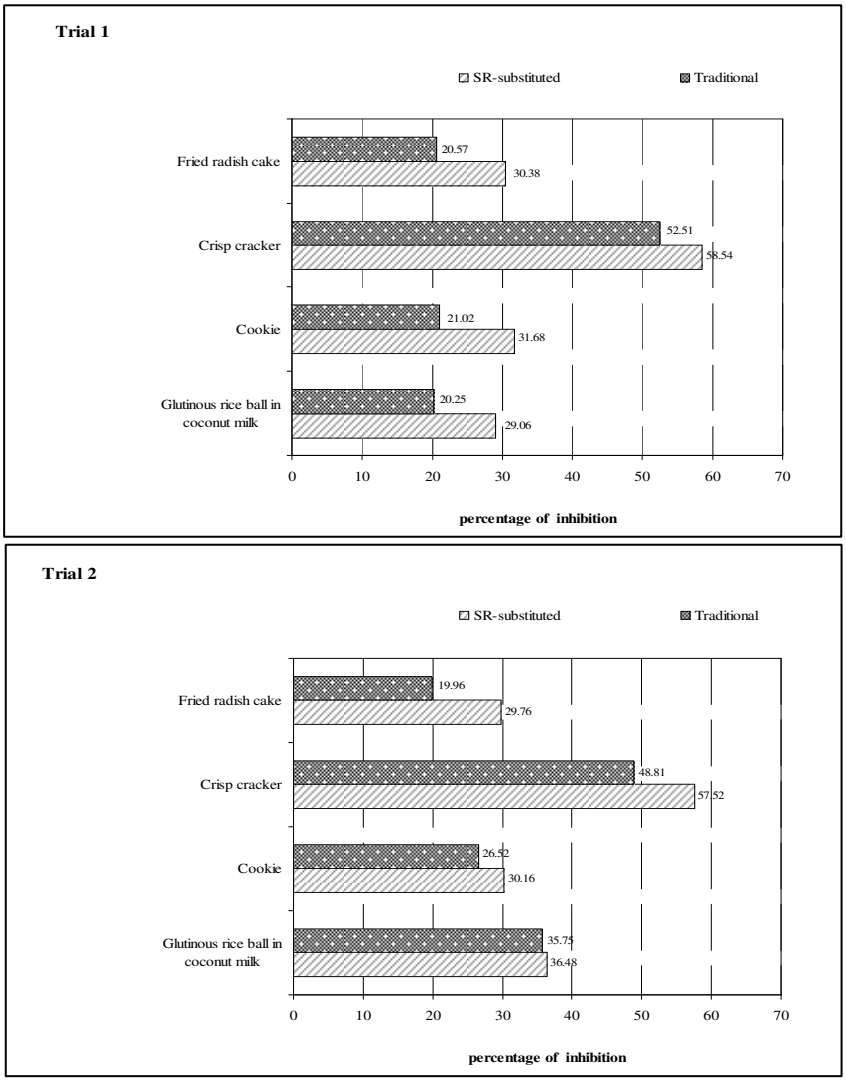


Figure 2 The comparison between percentage of inhibitions on urethane mutagenicity of substituted foods and those of their corresponding traditional ones.

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