Antimutagenicity against Urethane and *In vivo* Nitrosated Methylurea of Three Bakeries Fortified with Fruit and Herbal Wines

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ABSTRACT

Butter cake, pancake and doughnut fortified with each wine, namely, grape wine, Krachaidum wine, Mamao wine and mangosteen wine were determined for their antimutagenicity against urethane and the *in vivo* nitrosated methylurea. The three-day old trans-heterozygous (*mwh flr*$^{+}$*/mwh TM3*) larvae were transferred from regular medium to an experimental medium (substituted each wine fortified bakery for carbohydrate) for mutagenicity test. The antimutagenicity of each bakery was revealed by transferring three-day old trans-heterozygous larvae to an experimental medium containing urethane (20 mM) or the combination of sodium nitrite (36 mM) and methylurea (10 mM). The round wings from the surviving adult flies were analyzed for the occurrence of mutant spots. The results showed that neither wines nor bakeries fortified with wines were mutagenic. Interestingly, fortification of butter cake, pancake and doughnut with most wines improved the antimutagenicity against urethane of bakeries. The antimutagenicity against *in vivo* nitrosated methylurea of some fortified butter cakes, pancakes and doughnuts was also observed. We proposed that these fruit and herbal wines fortified bakeries might either inhibit activating enzymes (phase I), induce detoxifying enzymes (phase II) or trap urethane. Surprisingly, the ordinary pancake strongly enhanced the mutagenicity of *in vivo* nitrosated methylurea, but pancake fortified with grape, mamao and mangosteen wines decreased the enhancing activity of the ordinary bakery. Interestingly, pancake fortified with krachaidum expressed significant antimutagenicity against this mutagen. Thus, some components of pancake could increase the formation of mutagen in the *in vivo* nitrosation of methylurea and those in fortified pancake might inhibit the formation of mutagen. The result in the present study revealed that doughnut fortified with wine possessed strong antimutagenicity and therefore might be the most suitable bakery for wine fortification in order to obtain a functional food that could counteract the action of mutagens.

Keywords: Antimutagenicity, urethane, *in vivo* nitrosation, SMART, fruit, herbal wines, bakeries

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Introduction

Evidence suggests that just one meal high in saturated fat can affect the body's ability to protect itself against some of the underlying causes of heart disease and stroke. For instance, one piece of carrot cake modified to be high in saturated fat can reduce the body's ability to protect itself against heart disease.[1] In addition, diet high in fat has been shown to increase the risk of cancers. The evidence appeared strongest for an association of dietary fat with breast, large bowel, and prostate cancers, and was suggestive for other cancers.[2] Therefore, the development of functional foods that can counteract such disease is of great interest worldwide.

Wine is one of the worldwide popular beverages. Some evidences suggest that it contains high phenolic compounds.[3] Among these phenolics, the major difference is in the flavonoids.[4] It has been suggested that the French benefit from the consumption of wine which has high flavonoid content leading to the postulation that the high consumption of red wine is responsible for "French paradox".[5] Flavonoids in fruits, vegetables and juices appear to play a significant role in cancer and heart disease health benefits.[6] Therefore, the present investigation was proposed to determine the effect of fruit and herbal wine fortified bakeries, namely, buttercake, pancake and doughnut on urethane and on *in vivo* nitrosated methylurea induced somatic mutation and recombination in *Drosophila melanogaster* (SMART).

Materials and Methods

Chemicals and Samples preparation: Urethane (URE) was purchased from Sigma Chemical (St. Louis, Mo, USA). We obtained grape wine and Krachaidum wine from B.J Garden Winery Co., Ltd (Rayong, Thailand). Highland Agricultural Sanghlaburi (Kanchanaburi, Thailand) supplied Mangosteen and Mamao wines. We used commercially available premixes (Table 1) to prepare butter cake, pancake and doughnut as suggested on the labels. Each wine fortified bakery was prepared by substitution the wine for water. Each finished product was homogenized in an electrical blender and stored frozen. Other chemicals were of laboratory grade.

Experimental Design Each wine was diluted with deionized water (1:1 v/v) and tested for its mutagenicity. The diluted wine or urethane solution (20 mM) was substituted for water (2 ml) of the regular medium and was named *wine medium* or *positive control medium*, respectively. Each ordinary bakery or wine fortified bakery was substituted for the carbohydrate source (corn flour and sugar) of the fly medium and named *bakery control medium* or *experimental medium*, respectively. In addition, the incorporation of final 18 mM sodium nitrite as suggested by Rincon *et al*.[7] to the experimental medium was conducted to prove weather there was an *in vivo* nitrosation of wine fortified bakery to produce mutagenic species in the larvae. The mutagenicity and/or antimutagenicity of each sample in this experiment were evaluated as suggested by Graf *et al*.[8] Virgin females of Oregon wing flare strain (*ORR/ORR*; *flr*^{3}*/TM3, Ser)* were mated with males of multiple wing hair strain *(mwh/mwh)* to produce *trans*-heterozygous larvae of improved high bioactivation cross (IHB). Both strains were obtained from the Institute of Toxicology (Swiss Federal Institute of Technology, and the University of Zurich). They were maintained on the regular medium[9] that had propionic acid as a preservative.

Two models of antimutagenicity studies were used to evaluate all samples. In the first model, urethane solution (20 mM) was substituted for deionized water in the experimental medium and the regular medium which was the *positive control medium* of the urethane model. In the second model, the mixture of sodium nitrite (final 18 mM) and methylurea (final 5 mM) was substituted for deionized water in the experimental medium. The regular medium that had the mixture of sodium nitrite and methylurea substituted for deionized water was used as the *positive control medium* of the latter model.

Table 1 Ingredients of each bakery

Bakery	Ingredients
Butter cake	two and half cups of UFM butter cake mix (wheat flour, sugar, non fat dry milk, baking powder, salt, emulsifier, artificial color and artificial flavor), five tablespoons and one teaspoon of butter, one and half eggs, one tablespoon and two teaspoons of dry milk and seven tablespoons of drinking water (or wine)
Pancake	one cup of IMPERIAL buttermilk pancake mix (wheat flour, starch, shortening, baking powder, sugar), one and quarter cups of dry milk, one egg and one and half cups of drinking water (or wine).
Doughnut	one gram of UNCLE BARNS' doughnut cake mix (wheat flour, sugar, palm oil, baking powder, salt and vanilla powder), one and quarter eggs, one tablespoon of dry milk, one tablespoon of butter, four tablespoons and two teaspoons of drinking water (or wine).

Induction frequency of wing spots of each sample was compared with that of the negative control group. The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed with significant level of $\alpha = \beta = 0.05$. A multiple-decision procedure was used to decide whether a sample was positive, weakly positive, inconclusive or negative mutagenicity as described by Frei and Wurgler.[10] Antimutagenicity was estimated using percent inhibition of total spots per wing calculated as follows: percentage of inhibition = 100(*a* - *b*)/*a*, where *a* is the frequency of spots induced by urethane or nitrosated methylurea alone and *b* the frequency of spots induced by urethane or nitrosated methylurea in the presence of test sample. It was proposed that percentage of inhibition between 0-20 represents negligible effect while expression of percent inhibition between 20-40, 40-60 and more than 60 are the evidences of weak, moderate and strong antimutagenicity, respectively

Results

The mutagenicity of each fruit and herbal wine was firstly determined. The results showed that neither the wines nor wine fortified bakeries (with and without sodium nitrite) was mutagenic since they did not significantly induce the frequencies of mutant spots at any testing concentrations to be higher than that of the negative control medium (data not shown).

Table 2 shows that most wine fortified bakeries (butter cake and doughnut) presented their antimutagenicity against 20 mM urethane in both trials. Surprisingly, the ordinary pancake had no antimutagenic activity but slightly enhanced the mutagenic effect of urethane. However, the fortification of each wine to pancake could abolish such bad effect and rendered pancake to counteract the mutagenicity of urethane.

Investigation of the effect of wine fortified bakeries on the mutagenicity of *in vivo* formed nitrosomethylurea revealed that the ordinary butter cake had no antimutagenicity while all wines except mamao wine fortified ones expressed their antimutagenicity in both trials. On the other hand, ordinary doughnut surprisingly expressed its antimutagenicity and the fortification with each wine improved its antimutagenicity. Contradictory, most wine fortified pancake and the ordinary one enhanced the mutagenicity of the *in vivo* formed nitrosomethylurea. Interestingly, Krachaidum wine fortified pancake was the only one that could counteract the mutagenicity of this mutagen in both trials.

Table 2 Antimutagenicity of each wine fortified bakery against 20 mM urethane (URE) and in vivo formed nitrosomethylurea (NMU)[a] in *Drosophila melanogaster*

Bakery	Trial	Fortified wine	Chemical	No. of wing	Spots per wing (Number of Spots), statistical diagnoses[c]				% Inhibition (rate[c])
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m= 2)	
Butter cake	1	-	-	40	0.30(12)	0	0.08(3)	0.38(15)	-
		-	urethane	40	2.90(116)+	3.95(158)+	0.25(10)+	7.10(284)+	-
		none[d]	urethane	40	3.60(144)+	2.75(110)+	0.50(20)+	6.85(274)+	3.52(n)
		Grape	urethane	40	2.60(104)+	1.88(75)+	0.43(17)+	4.90(196)+	30.99(w)
		Mamao	urethane	40	2.98(119)+	2.43(97)+	0.40(16)+	5.80(232)+	18.31(n)
		Mangosteen	urethane	40	1.50(60)+	2.05(82)+	0.30(12)+	3.85(154)+	45.77(m)
		Krachaidum	urethane	40	1.58(63)+	1.85(74)+	0.25(10)+	3.68(147)+	48.24(m)
		-	-	40	0.30(12)	0	0.08(3)	0.38(15)	-
		-	NMU	40	5.50(220)+	2.30(92)+	0.68(27)+	8.48(339)+	-
		none[d]	NMU	40	4.55(182)+	5.00(200)+	1.20(48)+	10.75(430)+	-26.84(n)
		Grape	NMU	40	2.18(87)+	1.65(66)+	0.45(18)+	4.28(171)+	49.56(m)
		Mamao	NMU	40	2.70(108)+	4.28(171)+	1.13(45)+	8.10(324)+	4.42(n)
		Mangosteen	NMU	40	2.30(92)+	2.55(102)+	0.48(19)+	5.33(213)+	37.17(w)
		Krachaidum	NMU	40	2.48(99)+	2.75(110)+	0.55(22)+	5.78(231)+	31.86(w)
	2	-	-	40	0.40(16)	0	0	0.40(16)	-
		-	urethane	40	4.00(160)+	2.75(110)+	0.45(18)+	7.20(288)+	-
		none[d]	urethane	40	4.20(168)+	2.68(107)+	0.40(16)+	7.28(291)+	-1.04(n)
		Grape	urethane	40	3.30(132)+	2.10(84)+	0.45(18)+	5.85(234)+	18.75(n)
		Mamao	urethane	40	3.85(154)+	2.00(80)+	0.63(25)+	6.48(259)+	10.07(n)
		Mangosteen	urethane	40	1.98(79)+	2.43(97)+	0.50(20)+	4.90(196)+	31.94(w)
		Krachaidum	urethane	40	2.73(109)+	1.83(73)+	0.55(22)+	5.10(204)+	29.17(w)
		-	-	40	0.40(16)	0	0	0.40(16)	-
		-	NMU	40	5.43(217)+	2.15(86)+	0.68(27)+	8.25(330)+	-
		none[d]	NMU	40	4.53(181)+	3.43(137)+	0.90(36)+	8.85(354)+	-7.27(n)
		Grape	NMU	40	2.58(103)+	2.85(114)+	0.35(14)+	5.78(231)+	30.00(w)
		Mamao	NMU	40	3.20(128)+	4.08(163)+	0.73(29)+	8.00(320)+	3.03(n)
		Mangosteen	NMU	40	2.25(90)+	2.45(98)+	0.55(22)+	5.25(210)+	36.36(w)
		Krachaidum	NMU	40	2.08(83)+	2.85(114)+	0.73(29)+	5.65(226)+	31.52(w)

Table 2 Antimutagenicity of each wine fortified bakery against 20 mM urethane (URE) and in vivo formed nitrosomethylurea (NMU)[a] in *Drosophila melanogaster* (continued)

Bakery	Trial	Fortified wine	Chemical	No. of wing	Spots per wing (Number of Spots), statistical diagnoses[b]				% Inhibition (rate[c])
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m= 2)	
Pancake	1	-	-	40	0.18(7)	0	0	0.18(7)	-
		-	urethane	40	3.00(120)+	2.38(95)+	0.35(14)+	5.73(229)+	-
		none[d]	urethane	40	3.30(132)+	2.55(102)+	0.35(14)+	6.20(248)+	-8.30(n)
		Grape	urethane	40	2.08(83)+	2.28(91)+	0.25(10)+	4.60(184)+	19.65(n)
		Mamao	urethane	40	1.95(78)+	2.55(102)+	0.43(16)+	4.90(196)+	14.41(n)
		Mangosteen	urethane	40	1.83(73)+	2.10(84)+	0.33(13)+	4.25(170)+	25.76(w)
		Krachaidum	urethane	40	2.25(90)+	2.58(103)+	0.35(14)+	5.18(207)+	9.61(n)
		-	-	40	0.18(7)	0	0	0.18(7)	-
		-	NMU	40	5.00(200)+	2.83(113)+	0.75(30)+	8.58(343)+	-
		none[d]	NMU	40	9.40(376)+	7.65(306)+	2.68(107)+	19.73(789)+	-130.03(n)
		Grape	NMU	40	4.13(165)+	4.50(180)+	1.13(45)+	9.75(390)+	-13.70(n)
		Mamao	NMU	40	4.28(171)+	5.33(213)+	1.30(52)+	10.90(436)+	-27.11(n)
		Mangosteen	NMU	40	5.13(205)+	5.08(203)+	1.05(42)+	11.25(450)+	-31.20(n)
		Krachaidum	NMU	40	3.18(127)+	2.68(107)+	1.00(40)+	6.85(274)+	20.12(w)
	2	-	-	40	0.28(11)	0.03(1)	0	0.30(12)	-
		-	urethane	40	3.43(137)+	3.30(132)+	0.60(24)+	7.33(293)+	-
		none[d]	urethane	36	5.81(209)+	3.36(121)+	0.58(21)+	9.75(351)+	-19.80(n)
		Grape	urethane	40	2.60(104)+	2.75(110)+	0.83(33)+	6.18(247)+	15.70(n)
		Mamao	urethane	40	2.73(94)+	2.90(116)+	0.53(21)+	6.15(231)+	16.04(n)
		Mangosteen	urethane	40	2.35(109)+	2.90(116)+	0.53(21)+	5.78(246)+	21.16(w)
		Krachaidum	urethane	40	3.35(134)+	2.83(113)+	0.83(33)+	7.00(280)+	4.44(n)
		-	-	40	0.28(11)	0.03(1)	0	0.30(12)	-
		-	NMU	40	5.60(224)+	2.88(115)+	0.65(26)+	9.13(365)+	-
		none[d]	NMU	40	7.60(304)+	5.63(225)+	2.58(103)+	15.80(632)+	-73.15(n)
		Grape	NMU	40	4.20(168)+	4.25(170)+	1.05(42)+	9.50(380)+	-4.11(n)
		Mamao	NMU	40	4.55(182)+	2.70(108)+	0.95(38)+	8.20(328)+	10.14(n)
		Mangosteen	NMU	40	5.60(224)+	5.28(211)+	1.35(54)+	12.23(489)+	-33.97(n)
		Krachaidum	NMU	40	2.10(84)+	1.53(61)+	0.43(17)+	4.05(162)+	55.62(m)

Table 2 Antimutagenicity of each wine fortified bakery against 20 mM urethane (URE) and in vivo formed nitrosomethylurea (NMU)[a] in *Drosophila melanogaster* (continued)

Bakery	Trial	Fortified wine	Chemical	No. of wing	Spots per wing (Number of Spots), statistical diagnoses[b]				% Inhibition (rate[c])
					Small single (m=2)	Large single (m=5)	Twin (m=5)	Total (m= 2)	
Doughnut	1	-	-	40	0.18(7)	0.08(3)	0	0.25(10)	-
		-	urethane	40	4.50(180)+	3.75(150)+	0.58(23)+	8.83(353)+	-
		none[d]	urethane	40	2.93(117)+	3.00(120)+	0.30(12)+	6.23(249)+	29.46(w)
		Grape	urethane	40	1.98(79)+	1.70(68)+	0.23(9)+	3.90(156)+	55.81(m)
		Mamao	urethane	30	2.27(68)+	1.57(47)+	0.30(9)+	4.13(124)+	64.87(s)
		Mangosteen	urethane	40	2.63(105)+	2.35(94)+	0.15(6)+	5.13(205)+	41.93(m)
		Krachaidum	urethane	40	2.75(110)+	2.10(84)+	0.25(10)+	5.10(204)+	42.21(m)
		-	-	40	0.18(7)	0.08(3)	0	0.25(10)	-
		-	NMU	40	3.88(155)+	1.60(64)+	0.45(18)+	5.93(237)+	-
		none[d]	NMU	40	1.20(48)+	1.53(61)+	0.38(15)+	3.10(124)+	47.68(m)
		Grape	NMU	40	0.78(31)+	1.43(57)+	0.33(13)+	2.53(101)+	57.38(m)
		Mamao	NMU	40	1.28(51)+	1.15(46)+	0.25(10)+	2.68(107)+	54.85(m)
		Mangosteen	NMU	40	1.45(58)+	1.13(45)+	0.28(11)+	2.85(114)+	51.90(m)
		Krachaidum	NMU	30	1.43(43)+	1.40(42)+	0.33(10)+	3.17(95)+	59.92(m)
	2	-	-	40	0.30(12)	0.03(1)	0.03(1)	0.35(14)	-
		-	urethane	40	4.15(166)+	3.68(147)+	0.45(18)+	8.28(331)+	-
		none[d]	urethane	30	3.53(106)+	2.70(81)+	0.67(20)+	6.90(207)+	37.46(w)
		Grape	urethane	40	1.93(77)+	1.98(79)+	0.55(22)+	4.45(178)+	46.22(m)
		Mamao	urethane	40	2.30(92)+	1.98(79)+	0.60(24)+	4.88(195)+	41.09(m)
		Mangosteen	urethane	40	2.28(91)+	2.48(99)+	0.33(13)+	5.08(203)+	38.67(w)
		Krachaidum	urethane	30	2.23(67)+	2.20(66)+	0.43(13)+	4.87(146)+	55.89(m)
		-	-	40	0.30(12)	0.03(1)	0.03(1)	0.35(14)	-
		-	NMU	40	2.90(116)+	1.83(73)+	0.65(26)+	5.38(215)+	-
		none[d]	NMU	40	2.10(84)+	1.45(58)+	0.65(26)+	4.20(168)+	21.86(w)
		Grape	NMU	40	0.63(25)+	1.25(50)+	0.38(15)+	2.25(90)+	58.14(m)
		Mamao	NMU	40	1.95(78)+	1.60(64)+	0.50(20)+	4.05(162)+	24.65(w)
		Mangosteen	NMU	40	1.45(58)+	1.48(59)+	0.60(24)+	3.53(141)+	34.42(w)
		Krachaidum	NMU	40	1.33(53)+	1.58(63)+	0.63(25)+	3.53(141)+	34.42(w)

[a]Produced by administration 36 mM sodium nitrite and 10 mM methylurea to3-day-old larvae (Rincon *et al.*, 1988)

[b]Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Wurgler (1988) for comparison with distilled water: + = positive; - = negative; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical tests. [c]w = weak antimutagenicity, m = moderate antimutagenicity, s = strong antimutagenicity, n = negligible antimutagenicity, [d]ordinary bakery

Discussion

The survivals of adult flies fed on both ordinary and all wine fortified bakeries were more than fifty percents and the flies had big size. It indicated that none of the samples was toxic, and they were then suitable for further studies on their antimutagenicity. These bakeries are also good sources of nutrients for adult flies. Results of this study have revealed that both ordinary and wine fortified bakeries are safe to the consumers.

The significant antimutagenicity of all except mamao wine fortified butter cake against urethane was observed. Their antimutagenicity might be due to some phytochemicals. Xanthone is an example of chemically active compound found in the rind and pulp of mangosteen. Its molecule possesses a six carbon conjugated ring structure with multiple double carbon bonds which makes the molecule very stable. This structure keeps the xanthone's properties from being destroyed when heated.[11] α-Mangostin, a xanthone derivative increased the expression of glutathione S-transferase (GST) at 0.5-5.0 μM for 12 h treatment in human hepatocellular carcinoma HuH-7 cells.[11] The flavonoids of krachaidum are heat stable and the degradation due to cooking and frying are relatively low.[12] Further study on identification and the health benefit of such flavonoids of krachaidum should be of interest. Resveratrol (in grape wine) has been shown to participate in the prevention of carcinogenesis by the inhibition of some forms of cytochrome P450, an enzyme of phase I[13,14] and also through the induction of phase II xenobiotic metabolising enzymes.[15,16] It is sensitive to light and oxygen exposure[17] but it is heat stable. Therefore the preparation of bakery could not change the resveratrol content of red wine. The reason that mamao wine fortified butter cake exhibited least antimutagenicity may be due to the fact that anthocyanin in mamao is highly sensitive to oxygen, heat and light.[18] Hagiwara *et al*.[19] reported that some anthocyanins inhibited chemical-induced carcinogensis in a rat model. It was proposed that anthocyanins contributed to mark antioxidant activities in preventing DNA damage *in vitro*.

The result that ordinary pancake enhanced the mutagenicity of urethane but the enhancement was abolished with the fortification of each wine to pancake might be explained by the finding of Jägerstad and Skog.[20] They showed that cooking and processing at high temperature generated various kinds of genotoxic substances. Mutagens generated in hamburger during pan-frying are dependent upon both temperature and time.[21] Therefore, it is a need to verify whether there are any mutagens occurring during pan-frying of pancake.

It was found that ordinary doughnut was antimutagenic against urethane in both trials. All wine fortified doughnut presented greater antimutagenicity than the ordinary one. In fact this bakery is the one which is fried in palm oil. Among vegetable oils, palm oil is the richest natural source of tocotrienols.[22] Tocotrienols might act as free radical scavengers.[23] It was documented that *N*-hydroxyurethane, a urethane metabolite,[24,25] was hydrolyzed by esterase to generate hydroxylamine and exerted its mutagenic effect in multiple organs via generating $O_2^{\bullet -}$ and $NO^{\bullet}$ to cause oxidation and depurination of DNA.[26] Thus, it was possible that antioxidant activity from palm oil used in preparation of doughnut might reduce $O_2^{\bullet -}$ and/or $NO^{\bullet}$ in urethane metabolism. Antioxidants have been suggested to scavenge free radicals, and prevent their interactions with cellular DNA.[27] Overall results suggest that fortification of doughnut with each wine that contains phenolic compounds is a good choice for consumers who want to have a functional food that contains a potent antioxidant.

Evaluation of the antimutagenicity against *in vivo* formed nitrosomethylurea demonstrated that fortifying butter cake with each wine could abolish the mutagenicity enhanced by the ordinary one as well as expressed the antimutagenic activity. The fortification of doughnut with each wine slightly increased its antimutagenicity. The beneficial effect of each wine might be possibly due to its components that could reduce the formation of nitrosomethylurea or enhanced the detoxification of this direct acting mutagen. The

result that fortification of wines especially krachaidum wine to pancake could abolish the phenomenon that ordinary pancake enhanced the mutagenicity of *in vivo* formed nitrosomethylurea was quite similar to that of butter cake presented above. Rujjanawate *et al*.[28] revealed that the ethanol extract of krachaidum possessed gastro-protective potential which was related partly to preservation of gastric mucus secretion. Therefore, this wine might be of interest to be analyzed for the compounds that could counteract the notorious effect of pancake on both mutagens.

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