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ORIGINAL ARTICLE

Influence of natural coagulants on isoflavones and antioxidant activity of tofu

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Abstract Tofu (instead of preparing using synthetic coagulant) was prepared using coagulants of plant origin (*Citrus limonum, Garcinia indica, Tamarindus indica, Phyllanthus acidus* and *Passiflora edulis*). Total crude protein and fat contents were highest in tofu prepared using *G. indica* and *T. indica* (72.5% dbw) compared to synthetic coagulant. Tofu prepared with natural coagulants had significantly higher antioxidant activity compared to synthetic coagulant. Bioconversion of isoflavone glucosides (daidzin and genistin) into their corresponding bioactive aglycones (daidzein and genistein) was observed in tofu. The difference between glucosides and aglycones contents in soy milk was significant but there was not much difference in tofu coagulated with synthetic and natural coagulants.

Keywords Tofu · Isoflavones · Antioxidant activity · Coagulants · Soy milk

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Introduction

Soybean is a phenolic rich legume consumed worldwide, most commonly in Asian countries, such as Japan, Korea and Indonesia (Patrick and Kalidas 2004), and tofu is a widely consumed soybean product (Wu et al. 2004). Tofu is also gaining wide acceptance in the United States and other Western countries. East Asian populations that readily consume soybean and its products seem to have lower incidences of cancers and oxidation-linked old age diseases that are prevalent in Western population who do not traditionally consume soybean foods (Sarkar and Li 2003). Numerous epidemiological studies have demonstrated an association between the consumption of soybean and improved health, particularly as a reduced risk for prostate cancer, breast cancer, cardiovascular disease, and atherosclerosis (Jenkins et al. 2002, Yamakoshi et al. 2000, Yamamoto et al. 2003).

Researchers have postulated that the health benefit may be due to a specific group of phenolic compounds found uniquely within soybean known as isoflavonoids. It may be due to its estrogenic effect or antioxidant activity (Lee et al. 2004). Isoflavones are phytochemicals that exist in two basic categories, the aglycones and the glucosidic conjugates. The main glucosidic isoflavones are daidzin and genistin and the main aglycones are daidzein, and genistein (Batt et al. 2003). However, it is the aglycone (glucoside-free) form of isoflavonoids that is metabolically active (Yuan et al. 2003), which also possesses higher antioxidant acitivity and get absorbed faster in the intestines than their glucoside bound form (Murota et al. 2002, Rao and Muralikrishna 2002, Setchell et al. 2002). In addition, aglycones have been reported to be more stable than isoflavone glycosides during storage at different temperatures (Otieno et al. 2006). Consequently, providing food products with aglycones would be considered as a novel trend for the food industry (Pham and Shah 2007). According to Wang and Murphy (1994), each gram of tofu contains 0.532 mg of isoflavones. In another study, the total isoflavone content in raw tofu and cooked tofu was found to be 0.297 and 0.258 mg/g, respectively (Franke et al. 1999). Variation in isoflavone contents in tofu products was governed by the original content in soybeans and extent of loss in whey during recovery of soy curd.

Antioxidant property, especially radical scavenging activity is important in foods and in biological systems due to its deleterious role on free radicals. Excessive formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance (Min 1998). Superoxide anion, which is a reduced form of molecular oxygen, has been implicated in initiating oxidation reactions associated with aging (Wickens 2001). In this study antioxidant activity of tofu was determined by 1, 1-diphenyl-2-picrylhydrazyl free radical scavenging assay, inhibition of ascorbate autoxidation and measurement of reducing activity.

The objectives of this investigation were to study the isoflavone content and antioxidant activity of tofu coagulated with synthetic and natural coagulants thereby to identify the tofu with high isoflavone content and high antioxidant activity for future application in soy-based functional foods.

Materials and methods

Natural coagulants: Soybean and fruits like *Citrus limonum* (lemon), *Garcinia indica* (garcinia), *Tamarindus indica* (tamarind), *Phyllanthus acidus* (gooseberry) and *Passiflora edulis* (passion fruit) were procured from local market. $CaSO_4$ and $MgCl_2$ were from Rankem Fine Chemicals, USA.

Preparation of soy milk: Soybean (200 g) was cleaned and soaked in excess water at room temperature (28 ± 2°C) for 12 h. Hydrated soybean was washed once again to dehull the outer layer and ground with water (1:8) in a blender. The soybean slurry was indirectly heated in a water bath to avoid charring for 45 min at 85°C with constant stirring. The hot slurry was filtered through double-layered cheese cloth to separate soy milk from residue (okara) and the creamy layer formed was removed after cooling soy milk. The solid content of soy milk was determined using Abbe refractometer (American Optical Mode, 10450) and was adjusted to 9° Brix using water, which resulted in less foam production.

Preparation of synthetic and natural coagulants: $CaSO_4$ and $MgCl_2$ solutions (0.2% w/v) were used as coagulants in the preparation of tofu samples. Each fruit viz., garcinia, tamarind, lemon, gooseberry and passion fruit (25 g fresh wt) was soaked separately in 50 ml distilled water for 30 min and ground in a pestle and mortar. The slurries obtained were filtered using cheese cloth and the final volume of the individual extract was made up to 100 ml using distilled water. The extract from each fruit was used as a natural coagulant.

Acidity of coagulants: An aliquot of individual fruit extract was titrated against 0.1 N NaOH with phenolphthalein indicator. The acidity of the coagulants was determined (AOAC 1984) and expressed as percent anhydrous citric acid. The final acidity of all filtrates was adjusted to 2% with distilled water and used as natural coagulants.

Preparation of tofu: Soy milk (200 ml) was heated indirectly to 95°C for 5 min and cooled to 80°C. For the preparation of tofu, 10 ml of 0.2% synthetic 20 ml of 2% acidic solution of natural coagulants (extract from each fruit obtained as mentioned earlier), were added separately to soy milk. After the addition of coagulant, soy milk was stirred for 5 min and allowed to coagulate for 15 min without disturbing. Coagulated milk was filtered through cheesecloth lined tofu mold. The curd was pressed with 1 kg weight for the first 20 min and later with 0.5 kg for the next 20 min. The yield of tofu was calculated as the weight of fresh tofu obtained from a specified amount of soy milk used for its preparation. The tofu cake was cooled in water and stored in a refrigerator (4°C).

Proximate composition of tofu: Moisture, crude protein, fat and ash contents were determined as per AOAC (1990) methods.

DPPH free radical-scavenging assay: The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was assessed according to Moon and Terao (1998). To 1.0 ml DPPH (500 µM in ethanol) 200 µg of lyophilized tofu were added and the reaction mixture was made to 2 ml with Tris-HCl buffer (100 mM, pH 7.4). The mixture was shaken vigorously and incubated at room temperature (28 ± 2°C) for 30 min. The absorbance of the resulting solution was measured at 517 nm using spectrophotometer (Schimadzu UV-160A, Japan). Reaction mixture without DPPH was used as control.

Measurement of inhibition of ascorbate autooxidation: The method described by Mishra and Kovachich (1984) was used to determine the inhibition of ascorbate auto oxidation. Lyophilized extract from tofu of 0.25 µg was mixed with 0.1 ml of ascorbate solution (5.0 mM, Sigma, USA) and 9.8 ml of 0.2 M phosphate buffer and placed at 37°C for 10 min. The absorbance of this mixture was measured at 265 nm using spectrophotometer. Similar reaction mixture wherein distilled water in place of tofu was used as control. The ascorbate autooxidation inhibition rate of the sample was calculated as:

$$\%\ \text{Inhibition effect} = \left[\frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} - 1\right] \times 100\ \%$$

Measurement of reducing activity: Reducing activity was determined according to the method of Oyaizu (1986). Lyophilized extract from tofu (0.25 µg) was mixed with 0.5 ml of potassium ferricyanide (1%) and 0.5 ml of sodium phosphate buffer (0.02M, pH 7). This was incubated at 50°C for 20 min and then 0.5 ml of trichloro acetic acid (10%) was added. The mixture was centrifuged at 780 g for 5 min and the supernatant was collected. The supernatant (1.5 ml) was mixed with 0.1% ferrichloride (0.2 ml) and the absorbance was measured at 700 nm using spectrophotometer. Similar reaction mixture wherein tofu was replaced by distilled water was used as control.

HPLC analysis of isoflavones: The stock solutions of each of the standard compounds of daidzein, genistein, daidzin and genistin were prepared by dissolving 1 mg of each in 10 ml of 80% aqueous methanol and were stored in refrigerator (4°C). Each isoflavone standard solution was injected into HPLC and the peak areas were determined.

The procedure of Chiou and Cheng (2001) was used for HPLC analysis of isoflavones. One mg of lyophilized tofu was taken in 10 ml centrifuge tube, 4 ml methanol was added and the tube was screw capped. After vortexing, the tube was heated at 70°C for 30 min. During heating, the tubes were inverted by hand for agitation at 5 min interval. The tubes were centrifuged at 20°C at 15000 rpm for 30 min. One ml of the sample was withdrawn from the middle layer, filtered through 0.45 µm and 20 µl of the solution was injected into HPLC system (Model LC 10A, Shimadzu, Japan) and the compounds were detected with UV detector (265 nm). A reversed-phase water C18 column (Spherisorb ODS 2, 4.6 × 250 mm) with a gradient solvent system starting with methanol 20% and Milli Q water 80% and progressed to 80% methanol and 20% Milli Q water within 16 min followed by holding for an additional 2 min. The flow rate was 1.0 ml/min.

Statistical analysis: Data were analyzed by Duncan's multiple range tests using statistical package Statistica V 5.5 software. A significant level was defined as a probability of 0.05 or less. Determinations were carried out in triplicate.

Results and discussion

Preparation and proximate composition of tofu: Tofu prepared using lemon had highest moisture content (80.4%) followed by garcinia (75.6%) and gooseberry (75.3%), which is reflected in lower yield of whey (Table 1). Tofu prepared using the coagulants gave clear whey, indicating that the level of coagulants added was sufficient for complete coagulation of soy proteins. The solids in whey are most probably soluble sugars and low molecular weight protein (Lee et al. 2000). The variation in the whey volume was most likely due to a change in water holding capacity of tofu, which may be affected by coagulants (Lim et al. 1990). Highest yield of tofu was with garcinia extract (22.5%) followed by synthetic coagulant (21.3%) and extract of passion fruit (21.3%) while the yield was less in tofu prepared with the extract of gooseberry (15.6%). Protein content was highest in tofu coagulated with the extracts of garcinia and tamarind (72.5%) and lowest in tofu prepared with synthetic coagulant (56.3%).

The highest protein recovery in tofu prepared with the extracts of garcinia and tamarind was probably due to its higher protein content and more whey proteins retained in it during pressing. The coagulation and pressing process removes some carbohydrates, which results in an increase in protein content. The cause of difference in protein recovery may be related to the effect of coagulants on cross linking of glysin and β-conglycinin (Trinde and Kow-ching 1999). Fat content was maximum in tofu prepared using passion fruit (27.8%, db) and minimum in synthetic coagulant tofu (22.0%, db).

DPPH scavenging activity: The free radical scavenging activities (FRSA) of tofu coagulated with natural coagulants were comparatively higher than the tofu with synthetic coagulant (Table 2). Tofu prepared with garcinia extract showed the highest FRSA (82.1%) and the least antioxidant activity was with synthetic coagulant (63.1%). The increase in antioxidant activity may be due to the polyphenolic compounds present in fruit extracts (Lee et al. 2004). This is true in garcinia as reported by Fumio et al. (2000), wherein the garcinol present in garcinia exhibited potent radical scavenging activity almost 3 times higher than DL-α-tocopherol and comparable to 85% of the activity of ascorbic acid. Eriocitrin from lemon fruit is also an antioxidant compound (Yoshiaki et al. 1997), which may contribute to increase in antioxidant activity of tofu. It is reported by Martinello et al. (2006) that the crude tamarind extract is rich in polyphenols (34.0 nmol/ml) and flavonoids (35.5 µg/ml), which are well known antioxidant agents. The carotenoids which give characteristic colour to the yellow passion fruit rind, flesh and juice are important source of

Table 1 Yield and proximate composition of tofu prepared using synthetic and natural coagulants

Coagulants	Yield, g/100 ml soy milk	Moisture, %	Volume of whey, ml/ 100 ml of soy milk	Fat, % db	Protein, % db	Ash, % db
*Synthetic coagulant	21.3 ± 0.16^{b}	74.9 ± 0.52^{ab}	83 ± 0.42^{a}	22.0 ± 0.21 ^{a}	56.3 ± 0.86^{a}	2.3 ± 0.03^{ab}
Lemon	17.6 ± 0.18^{ab}	80.4 ± 0.48^{b}	82 ± 0.68^{a}	26.2 ± 0.24^{ab}	69.0 ± 0.90^{b}	2.6 ± 0.02^{bc}
Tamarind	21.0 ± 0.14^{b}	71.2 ± 0.46^{a}	85 ± 0.50^{a}	24.0 ± 0.21^{ab}	72.5 ± 0.84^{b}	2.0 ± 0.08^{a}
Garcinia	22.5 ± 0.16^{b}	75.6 ± 0.34^{ab}	82 ± 0.64^{a}	23.7 ± 0.20^{ab}	72.5 ± 0.98^{b}	2.8 ± 0.02^{c}
Gooseberry	15.6 ± 0.16^{a}	75.3 ± 0.48^{ab}	81 ± 0.50^{a}	26.0 ± 0.18^{ab}	58.2 ± 0.40^{a}	2.8 ± 0.03^{c}
Passion fruit	21.3 ± 0.18^{b}	72.1 ± 0.56^{a}	86 ± 0.60^{a}	27.8 ± 0.20^{b}	70.0 ± 0.80^{b}	2.6 ± 0.04^{bc}

Means followed by different superscripts in a column are significantly different ($p \leq 0.05$) (n=3). *($CaSO_4$ + $MgCl_2$)

provitamin A and antioxidant activity (Stephen et al. 2003). Gooseberry contains flavonoids like Kaempferol and 2, 3-dihydroxy benzoic acid and other bioactive compounds (Li and Wang 2004) which possess antioxidative activities. These results suggested that plant origin coagulant extract might react as free radical scavengers and contribute hydrogen from phenolic hydroxyl groups themselves, thereby forming stable free radicals that do not initiate or propagate further oxidation of lipids.

Inhibition of ascorbate autoxidation: Tofu prepared with synthetic coagulant showed lowest ability to inhibit ascorbate autooxidation (Table 2). On the other hand, tofu prepared with natural coagulants significantly increased the inhibition rate of ascorbate autooxidation which may be due to polyphenols present in the fruits. Inhibition of ascorbate autooxidation ranged from 36.8 to 70.3% depending on the coagulants used. Tofu prepared with the extracts of passion fruit exhibited the highest inhibition of ascorbate autooxidation (70.3%).

Reducing activity of tofu: The reducing activity of tofu is expressed as an equivalent amount of cysteine (µM). Tofu coagulated with the extract of passion fruit exhibited the highest reducing activity (0.565 µM) among the natural coagulants and lowest was in synthetic coagulant tofu (0.485 µM) (Table 2). In addition to the fruit extract with antioxidative activity, soybean isoflavones may also add to the antioxidative activity (Drumm et al. 1990).

Isoflavones: The isoflavones genistin, daidzin, genistein and daidzein were successfully separated and identified (Fig. 1). The HPLC chromatogram of isoflavones of tofu coagulated with lemon is shown in Fig. 2. The glycosidic isoflavone genistin and daidzin (glucosides) were abundant in soy milk but genistein and daidzein (aglycones) were in less concentration. In soy milk, the daidzin and genistin contents were 0.282 and 0.030 mg/g (db), respectively. In contrast, genistein and daidzein were quantitatively lesser in soy milk accounting of 0.003 and 0.007 mg/g, respectively.

Table 2 Antioxidant activities of tofu coagulated with synthetic and natural coagulants

Coagulants	DPPH scavenging, %	Inhibition of ascorbate auto oxidation, %	Reducing activity equivalent cysteine, µM
Synthetic tofu	63.1 ± 0.64[a]	36.9 ± 0.42[a]	0.485 ± 0.006[a]
Lemon	69.1 ± 0.82[a]	37.3 ± 0.34[a]	0.487 ± 0.004[a]
Tamarind	80.4 ± 0.57[b]	57.2 ± 0.67[c]	0.540 ± 0.003[c]
Garcinia	82.1 ± 0.92[b]	45.3 ± 0.56[b]	0.540 ± 0.006[c]
Gooseberry	82.0 ± 0.80[b]	57.8 ± 0.45[c]	0.503 ± 0.004[b]
Passion fruit	81.3 ± 0.68[b]	70.3 ± 0.80 [d]	0.565 ± 0.008[d]

Means followed by different superscripts in a column are significantly different ($p \leq 0.05$) (n=3). DPPH: 1, 1-diphenyl-2-picrylhydrazyl.

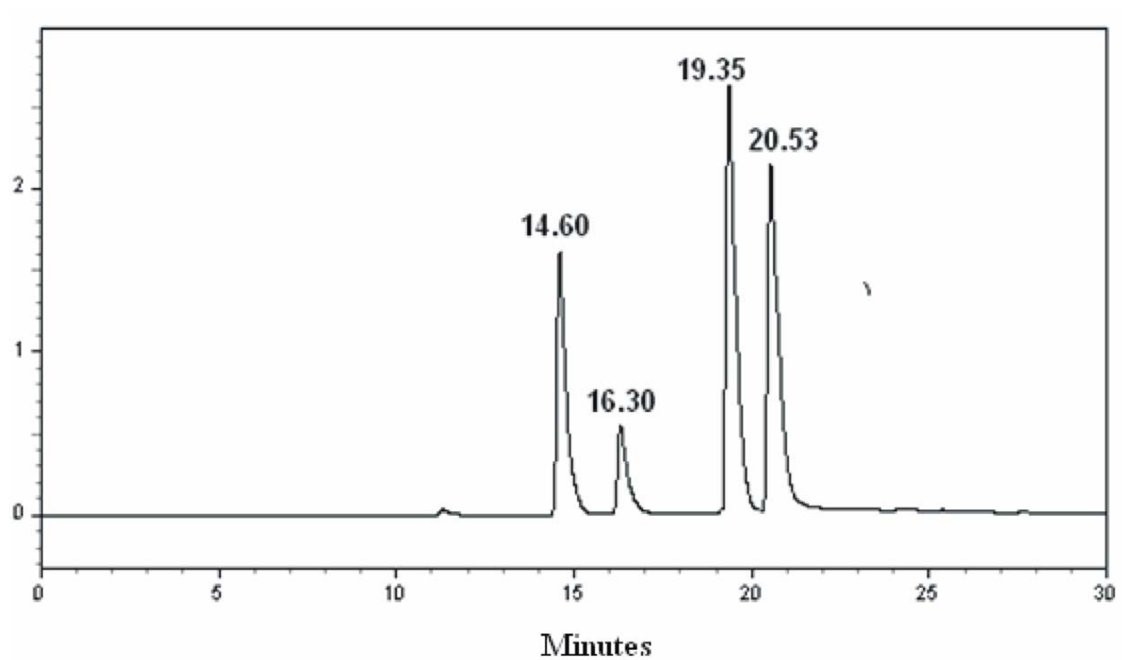

Fig. 1 HPLC chromatogram showing the retention time of standard isoflavones Daidzin (14.60 min), Genistin (16.30 min), Daidzein (19.35 min) and Genistein (20.53 min)

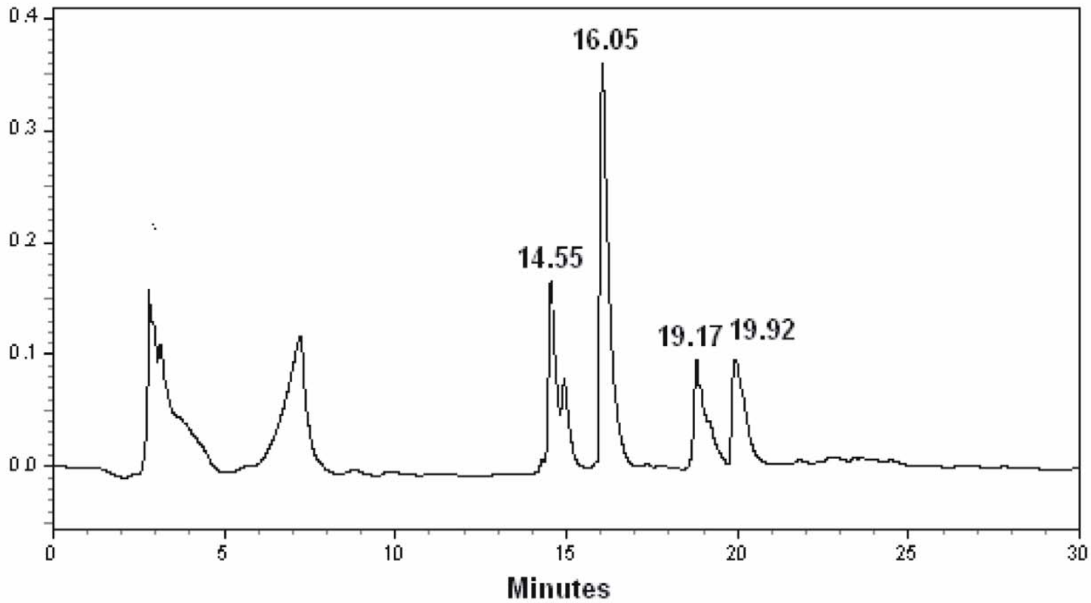

Fig. 2 HPLC chromatogram showing the retention time of isoflavones in lemon tofu: Daidzin (14.55 min), Genistin (16.05 min), Daidzein (19.17 min) and Genistein (19.92 min)

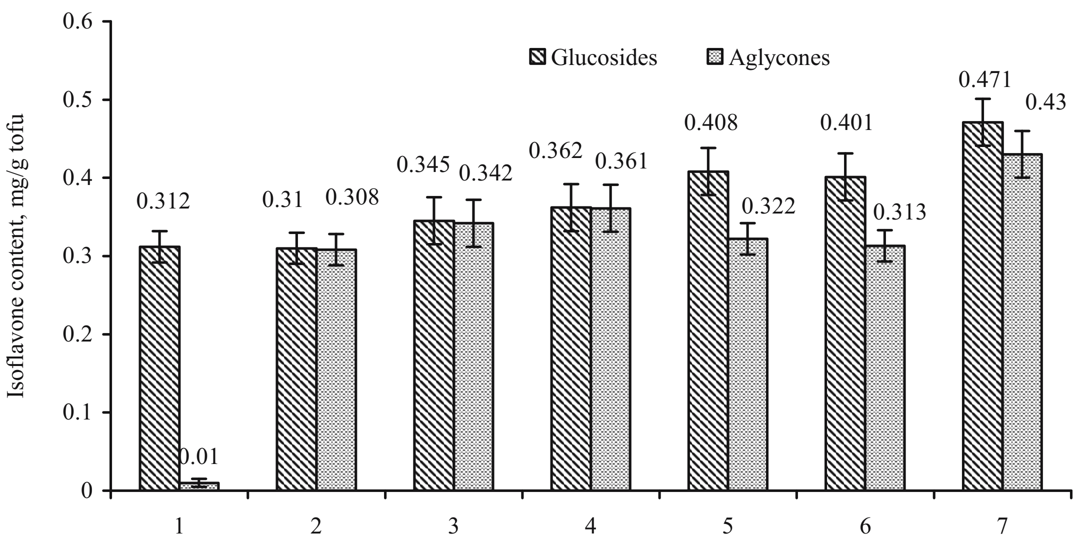

Fig. 3 Isoflavone content in soy milk and tofu coagulated with synthetic and natural coagulants (n=2) 1 Soy milk, 2 Synthetic tofu, 3 Lemon, 4 Tamarind, 5 Garcinia, 6 Gooseberry, 7 Passion fruit

Tofu has an extremely variable isoflavone concentration, ranging from 200 to 3500 μg/g depending on the variety of beans and environmental conditions (Wang and Murphy 1994, Tsukamoto et al. 1995). Processing, enzymatic hydrolysis and fermentation significantly alter the distribution of isoflavones (Wang and Murphy 1994).

Tofu coagulated with synthetic and coagulants of plant origin had the isoflavone glucosidic content ranging from 0.310 to 0.471 mg/g of tofu and aglyconic content ranging from 0.308 to 0.430 mg/g of tofu (Fig. 3). There was no significant variation in glycosidic and aglyconic form of isoflavones in tofu coagulated with synthetic and coagulants of plant origin. Basically tofu contains both glucosides and aglycones. The aglycones are generated by the action of soybean's native β-glucosidase during soaking of soybean in tofu and soy milk production. Aglycones in tofu were higher than in soy milk (Fig. 3), suggesting the action by native glucosidases during tofu production. This is in accordance with the findings of Matsura and Obata (1993).

There was a decrease in daidzein compared to its conjugate daidzin. This decrease in aglycone is most likely due to its leaching into the water. Temperature played a significant role in the reduction of daidzein. Interesting feature was that genistein contributed to the greatest concentration of agly-

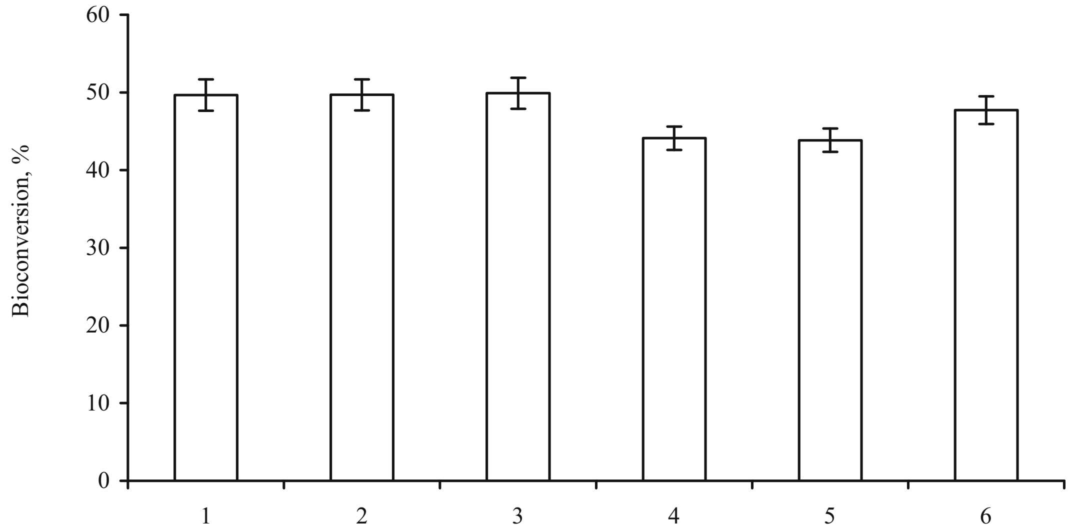

Fig. 4 Bioconversion of glucosides to aglycones in tofu coagulated with synthetic and natural coagulants (n=3). 1 Synthetic tofu, 2 Lemon, 3 Tamarind, 4 Garcinia, 5 Gooseberry, 6 Passion fruit

conic form (0.235–0.334 mg/g of tofu) than daidzin (0.075–0.96 mg/g of tofu) in tofu coagulated with synthetic and coagulants of plant origin. There was an average of 47.4% of the original glucosides bioconverted into aglycones (Fig. 4). Sensory analysis of tofu prepared with different natural coagulants has been reported by Sanjay et al. (2008).

Conclusion

There was an increase in protein and antioxidant activity in tofu coagulated with natural coagulants. Fruit extracts, which are water soluble, rich sources of vitamins, carotenoids and other bioactive molecules, could be an alternative to synthetic coagulants in the preparation of tofu. Tofu prepared with lemon extract was the most preferred tofu which had a smooth, soft, but firm texture with whitish colour. The presence of more water soluble glycosidic isoflavones did not decrease the antioxidant potency of tofu. It is further contributed by the phytochemicals like polyphenols.

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