

Effect of miso (A soybean fermented food) on some human cell lines; HEPG2, MCF7 and HCT116Abeer Abu Zaid ^{1,2} and Nahla S. El-Shenawy^{2,3*}¹Food Technology Research Institute, Agriculture Research Center, Giza, Soy product Processing Center, Egypt.²Biology department, Faculty of Science, Taif University (Qurwa), Taif, Saudi Arabia.³Zoology Department, Faculty of Science, Suez Canal University, Ismailia 41522, Egyptelshenawy_nahla@hotmail.com

Abstract: The study was conducted to investigate the antitumor activity of miso, storage at different period or prepared with different starters, on human cell lines {HEPG2 (liver carcinoma), MCF7 (breast carcinoma), and HCT116 (colon carcinoma)}. The highest inhibitory effect on liver and breast carcinoma was seen when miso used after fermentation/aging zero time without storage period. Miso with different storage period (zero, 6 months and 5 years) has the same effect on colon carcinoma. Preparation of miso with different mixture of starters was also investigated on the same human tumor cell lines in culture. Miso prepared with *A. oryzae* and *Bacillus subtilis* starters inhibited the proliferation of human tumor cell lines culture with a wide variation in LC₅₀ values (2.97, 3.37 and 3.37 µg/ml for MCF7, MCT116 and HEPG2, respectively). Miso prepared with *Aspergillus oryzae* and *Pleurotus ostreatus* starters inhibited human tumor cell line cultures with different LC₅₀ values (10.9, 17.5 and 24.3 µg/ml for MCF7, MCT116 and HEPG2, respectively). The miso prepared with *A. oryzae* and *Rhizopus oryzae* effect only on MCF7 and HEPG2 with high LC₅₀ values (25.5 and 35.8 µg/ml, respectively). We can conclude that the mixture of *A. oryzae* and *Bacillus subtilis* has the best effect among the other mixture of starters. The results indicated that all of fermented soybeans products with different mixture of starters contained higher isoflavones compounds than unfermented cooked soybeans. Moreover, soybean fermented with *B. subtilis* showed highest amount of isoflavones. Therefore, miso can be used as anticancer.

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1. Introduction

Soybeans (*Glycine max* (L.) Merr.) are highly regarded as a healthy food in several Asian countries and are widely consumed as soymilk, tofu and fermented products. Fermented soy foods, such as *paste*, are important components of the Korean diet in much of the world. *Soy paste (Doenjang)* has been traditionally manufactured from *Koji*, which is a fermented rectangular block of crushed cooked soybeans. The primary microorganisms involved in *Koji* fermentation are *Bacillus subtilis* and molds such as *Rizopus*, *Mucor*, and *Aspergillus* species (Park and KO 2005; Dajanta et al., 2009). Soy paste fermented with various filamentous fungi including *Aspergillus awamori*, *Aspergillus oryzae*, *Aspergillus sojae*, *Rhizopus azygosporus* and *Rhizopus sp.* lead to

the increased antioxidant activities, total extractable phenolic compounds and anthocyanins content after fermentation (Lee et al., 2007; 2008). Fermentation of soybean and other ingredients by microbial starters is the most important step during manufacturing of soybean fermented products. During fermentation and due to excretion of hydrolytic exoenzymes by microbial starters, precursors such as phenolic compounds (genistin and diadzein) are converted by β- glucosidase into more biologically active forms of isoflavones such as genistein and daidzein (Hsiang et al., 2005). Protein and fats are also converted into amino acids and bioactive peptides, and fatty acids by proteases and lipases respectively (Dziuba et al., 2003).

In the past several years, soy and its constituents have garnered considerable attention, from both

researchers and health practitioners. Epidemiological data which indicated people from Asian cultures have lower rates of certain cancers, including cancer of the breast, prostate and colon, sparked an interest in soy as a contributing factor. Numerous epidemiological, human, animal, and *in vitro* studies have demonstrated that soy isoflavones are effective chemopreventive agents for certain types of cancer (Head, 1997). *In vitro*, high concentrations of the isoflavone genistein inhibit most types of cancer cells and some animal studies suggest that genistein inhibits metastases (Menon et al., 1998). Further controlled human trials are needed to confirm the preliminary findings reported in these studies. Jung et al. (2006) reported that miso could prevent cancer by decreasing tumor formation and increasing natural killer cell activity in spleens. Miso goes a long way towards providing people with their daily needs for the trace minerals zinc, manganese and copper. Miso has antioxidant properties and it has the ability to decrease the lipid peroxidation (El-Shenawy and Abu Zaid, 2011).

In the present study, we have tested the effect of miso that prepared with single starter (*Aspergillus oryzae*) and stored for different period (Zero, 6 months and 5 years) on three human tumor cell lines. In addition, the effects of three different mixture of starters were examined on cell proliferation of the same cell lines [HEPG2 (liver carcinoma), MCF7 (breast carcinoma) and HCT116 (colon carcinoma)].

2. Materials and Methods

2.1 Microorganisms and Media

The microorganisms included in this investigation are commonly used as starters in the fermentation of many traditional, oriental food products. The mold *Aspergillus oryzae*, is used to make traditional doenjang, a Korean fermented soy paste (Kim et al., 2009), *Rhizopus oryzae*, used in preparation of temper, Indonesian fermented food (Haron et al., 2009) and the bacterium *Bacillus subtilis* was found among the microbial community in doenjang (Yoo et al., 1999). Potato dextrose agar was used for growth and maintenance of all mold strains, and nutrient agar medium was used for the bacterium *Bacillus subtilis*.

2.2 Preparation of miso

Miso was prepared in two major steps. In the first step, microbial starters were prepared as follows: wheat bran seeded with *Aspergillus oryzae* fungi as singly at approximately $1-2 \times 10^6$ cfu/g. Then, it was spread at the surface of a layer of 20 Kg of boiled and steamed wheat at a ratio of 1% and incubated at 25 °C for 3 days in order to encourage growth and multiplication of microbial starters. Secondly, the growing starter was added to 40 Kg of boiled and steamed soybean mixed thoroughly in presence of 16% salt and moisture content was controlled at approximately 12.5%. The resulting paste represents the miso at zero time. If the paste was stored in jars for fermentation and aging, the resulting miso represents 6 months or more (ISO, 2006). During all the experiment, fermentation/aging for 6 months has been used except for antitumor activity experiment; three different times of fermentation/aging [zero (1), 6 months (2) and 5 years (3)] have been investigated. Miso was also prepared with different mixture of starters; *Aspergillus oryzae* and *Pleurotus ostreatus* (4) or *A. oryzae* and *R. oryzae* (5) or *A. oryzae* and *B. subtilis* (6) (Abu Zaid et al. 2010). The bacteria were routinely cultured on nutrient agar and their stock cultures were maintained at -80°C in 20% glycerol. For inoculums preparation, the bacteria were grown in nutrient broth at 37°C for 24 h. The cells were then harvested, re-suspended in sterile distilled water and properly adjusted to obtain a concentration of 10^4 CFU/mL. The suspension was served as the inoculums for soybean fermentation.

2.3 Determination of miso total fatty acid and amino acids

Gas liquid chromatography was applied to identify the fatty acids present in different types of miso. Lipids were extracted by a modified method described by Xu and Beardall (1997). Amino acid determination was performed according to the method of the Official Journal of the European Communities 19-9-98 (AOAC, 1995).

2.4 Determination of miso Isoflavones

The method described by Coward et al. (1993) was used after modification. After freeze drying, samples were extracted using 80% aqueous methanol (10 ml/g) and stirred for 1.0 h at 60 °C. The mixture was centrifuged at 2500 g for 10 min and the supernatant was decanted into a round bottom flask. The pellet was re-suspended in 10.0 ml of 80% aqueous methanol and centrifuged. Then, both

supernatants were combined and taken to dryness using rotary evaporator. Then, dried extract was re-dissolved in 5.0 ml of 50% aqueous methanol, and the lipid fraction was removed by partitioning in hexane (4 X 20 ml). The aqueous methanol phase was evaporated to dryness using rotary evaporator and the dried residue dispersed in 10 ml of 80% aqueous methanol. The mixture was centrifuged using an eppendorf by microfuge just prior to analysis by HPLC. Separation of isoflavones was achieved by high-performance liquid chromatography (HPLC) on a 30 cm x 0.45 cm, Aquapore C8 reversed – phase column with mobile phase consisting of a gradient of 0- 46.4% acetonitril in 0.1 % (v/v) aqueous trifluoroacetic acid at flow rate of 1.5 ml/min. The eluting components were detected from their absorbance at 262 nm. Concentrations of the isoflavones were calculated from standard curves of area responses for authentic isoflavones standards normalized to the constant amount of fluorescein as internal standard was added to each sample.

2.5 Antitumor activities of miso on human cell lines

Antitumor activity of a colorimetric cytotoxicity assay (Skehan et al., 1990). Cell lines: HEPG2 (liver carcinoma), MCF7 (breast carcinoma) and HCT116 (colon carcinoma) were harvested from exponential phase cultures by trypsinization, counted and plated as cell monolayer in 96-multiwell plate (10^4 cells/well) for 24 h. Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS) and 1 mM L-glutamine. Then different concentrations (0, 1, 2.5, 5 and 10 $\mu\text{g/ml}$ in DMSO for HEPG2 and MCF7) or (0, 5, 12.5, 25, and 50 $\mu\text{g/ml}$ in DMSO for HCT116) of miso after fermentation/aging for zero, 6 months and 5 years were added to the wells in triplicate. After 48 h of incubation, cells were fixed, washed and stained with Sulfo-Rhodamine-B (SRB) stain. Excess stain was washed with acetic acid and the attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between survival fraction and extract concentration was plotted to give the survival curve of each tumor cell line after the specified dilution of the extract, and the LC_{50} was calculated.

2.6 Statistical analysis

Results are expressed as mean \pm SE, and were obtained from at least 5 samples. Statistical analysis

was performed using one-way analysis of variance (ANOVA) followed by the Bonferroni's *post-hoc* test (level of significance; $P \leq 0.05$) using SPSS version 15, statistical program (Hill, 1971).

3. Results

3.1 Total fatty acid, total amino acid and total isoflavones

The highest level of fatty acid and total amino acid (Table 1) were detected in the miso that prepared with *A. oryzae* and *R. oryzae* (5) (102.55 ± 7.29 and 30.2 ± 0.27 , respectively). However, the highest level of total isoflavones was determined in miso that prepared with *A. oryzae* and *B. subtilis* (6). The concentration of isoflavones in unfermented soybean was too low as compared with all other types of miso.

3.2 Effect the storage time of miso on human tumor cell lines

The survival fraction of HEPG2 and MCF7 cell line was plotted against the different concentrations (1-10 $\mu\text{g/ml}$) of miso with different storage period (0, 6 months, 5 years). The concentrations of miso that reduced survival of carcinoma cell line of liver (HEPG2) to 50% were 1.96, 2.88 and 3.5 $\mu\text{g/ml}$ for zero, 6 months and 5 years of fermentation, respectively (Fig. 1). The concentrations of miso that reduced survival of breast (MCF7) carcinoma to 50% were 0.982, 1.3 and 1.14 $\mu\text{g/ml}$ for zero, 6 months and 5 years, respectively (Fig. 2). The highest inhibitory effect on liver and breast carcinoma was seen when miso used immediately after fermentation (zero time). However, the survival fraction of carcinoma cell line of colon (HCT116) was plotted against the different concentrations (5-50 $\mu\text{g/ml}$) of miso (Fig. 3). Effect of different storage periods of miso on colon carcinoma was approximately the same; 14.4, 13.0 and 14.3 $\mu\text{g/ml}$ for zero, 6 months and 5 years of fermentation, respectively.

3.3 Effect of different methods of miso preparation on human tumor cell lines

The survival fraction of MCF7, MCT116 and HEPG2 cell line was plotted against the different concentrations (5-50 $\mu\text{g/ml}$) of miso that prepared with different mixture of starters. The concentrations

of miso that reduced survival of breast (MCF7) carcinoma to 50% were 2.97, 10.9 and 25.6 $\mu\text{g/ml}$ for 6, 4 and 5, respectively (Fig. 4). The concentrations of miso that reduced survival of carcinoma cell line of liver (HEPG2) to 50% were 3.73, 24.3 and 35.8 $\mu\text{g/ml}$ for 6, 4 and 5, respectively, (Fig. 5). The

highest inhibitory effect on liver and breast carcinoma was seen when miso prepared using mixture of *A. oryzae* and *B. subtilis* (6). However, effect of different preparations of miso on colon carcinoma was 3.73 and 17.5 for 6 and 4, respectively. The miso prepared by *A. oryzae* and *R. oryzae* (5) did not effect on the HCT116 (Fig. 6).

Table 1: Effect of different starters on total fatty acid, total protein and total isoflavones of miso

Miso starters	Total fatty acids (mg/100g)	Total amino acids (%)	Total isoflavones (mg/100g)
Raw materials (unfermented soybean)	---	---	71.46 \pm 6.5
<i>Aspergillus oryzae</i> and <i>Pleurotus ostreatus</i> (4)	93.88 \pm 6.66 ^{**}	28.6 \pm 0.23	571.02 \pm 5.12 ^{**}
<i>A. oryzae</i> and <i>R. oryzae</i> (5)	102.55 \pm 7.29 ^{***}	30.2 \pm 0.27 ^{***}	562.73 \pm 4.95 ^{***}
<i>A. oryzae</i> and <i>B. subtilis</i> (6)	77.24 \pm 4.3	26.52 \pm 0.24	1090.95 \pm 9.69

The data presented as mean \pm SE (n=5). Statistical analyses were performed with SPSS version 11.5 (SPSS Institute, Chicago, IL). One-way analysis of variance (ANOVA) was used to assess the statistical significance of the continuous variables. --- Not detected. * $p < 0.01$, (4) compared with (5). ** $p < 0.05$, (4) compared with (6). *** $p < 0.05$, (5) compared with (6).

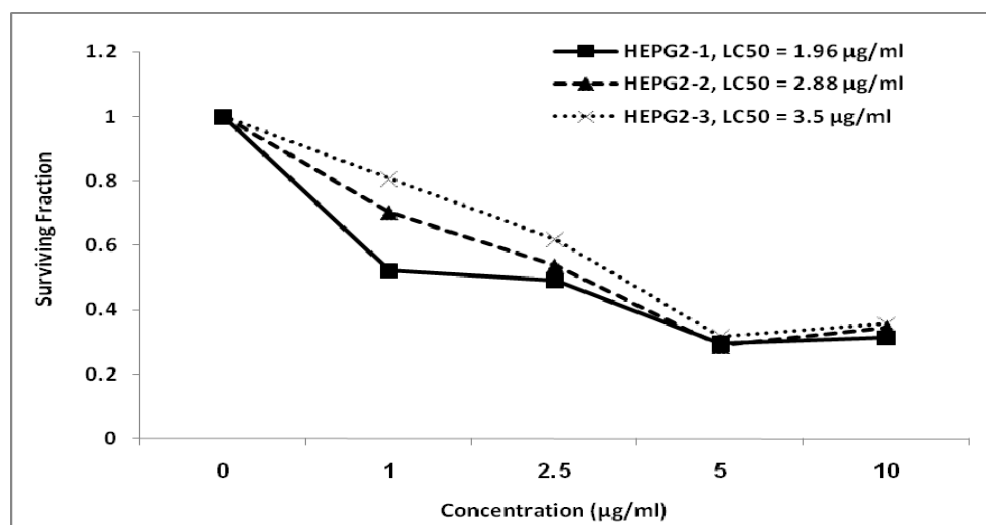


Figure 1. Potential antitumor activities of miso on human cell lines HEPG2 (liver carcinoma), with LC_{50} value denoted on curve. HEPG2-1, HEPG2-2 and HEPG2-3 represent fermentation/aging of miso for zero, 6 months and 5 years, respectively.

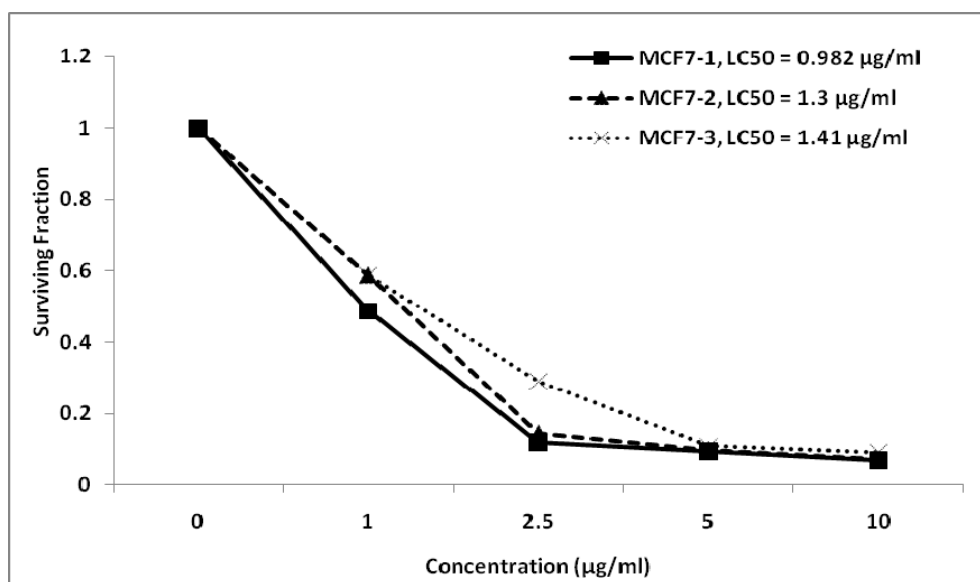


Figure 2. Potential antitumor activities of miso on human cell lines MCF7 (breast carcinoma), with LC_{50} value denoted on curve. MCF7-1, MCF7-2 and MCF7-3 represent fermentation/aging of miso for zero, 6 months and 5 years, respectively.

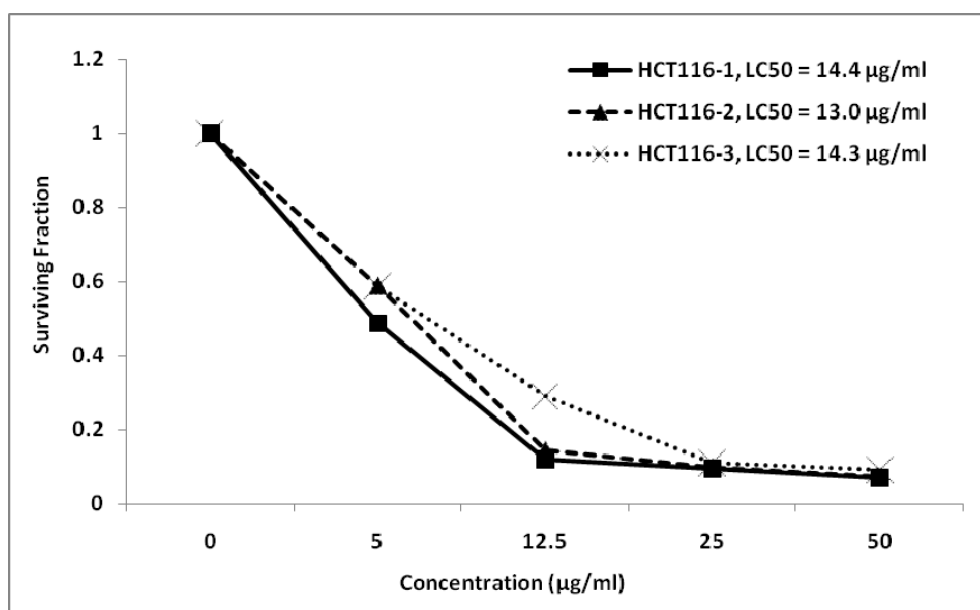


Figure 3. Potential antitumor activities of miso on human cell lines HCT116 (colon carcinoma), with LC_{50} value denoted on curve. HCT116-1, HCT116-2 and HCT116-3 represent fermentation/aging of miso for zero, 6 months and 5 years, respectively.

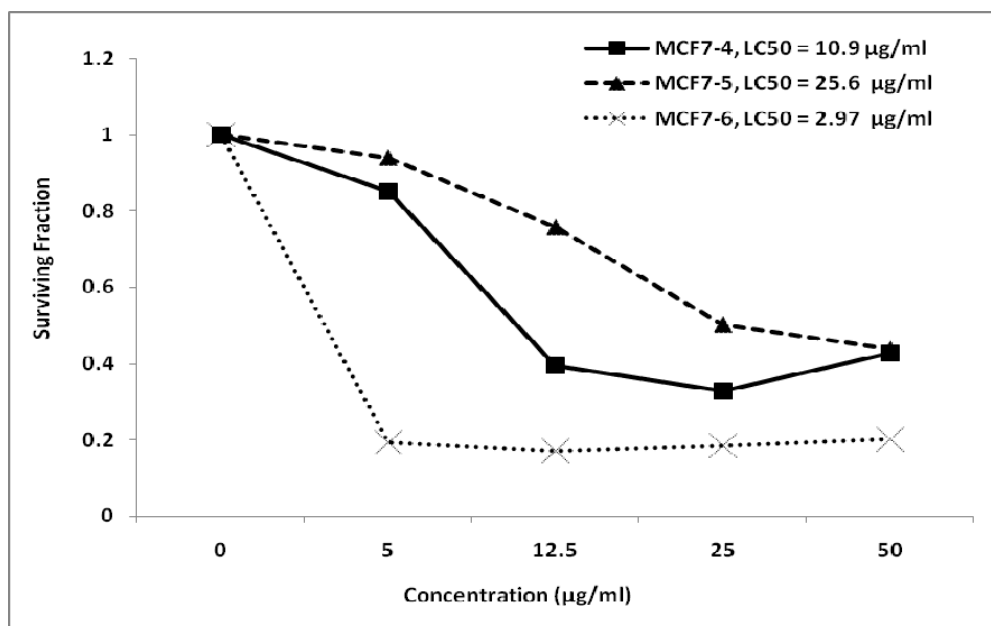


Figure 4. Potential antitumor activities of miso on human cell lines HEPG2 (liver carcinoma), with LC_{50} value denoted on curve. HEPG2-1, HEPG2-2 and HEPG2-3 represent miso for 8, 2 and 4, respectively.

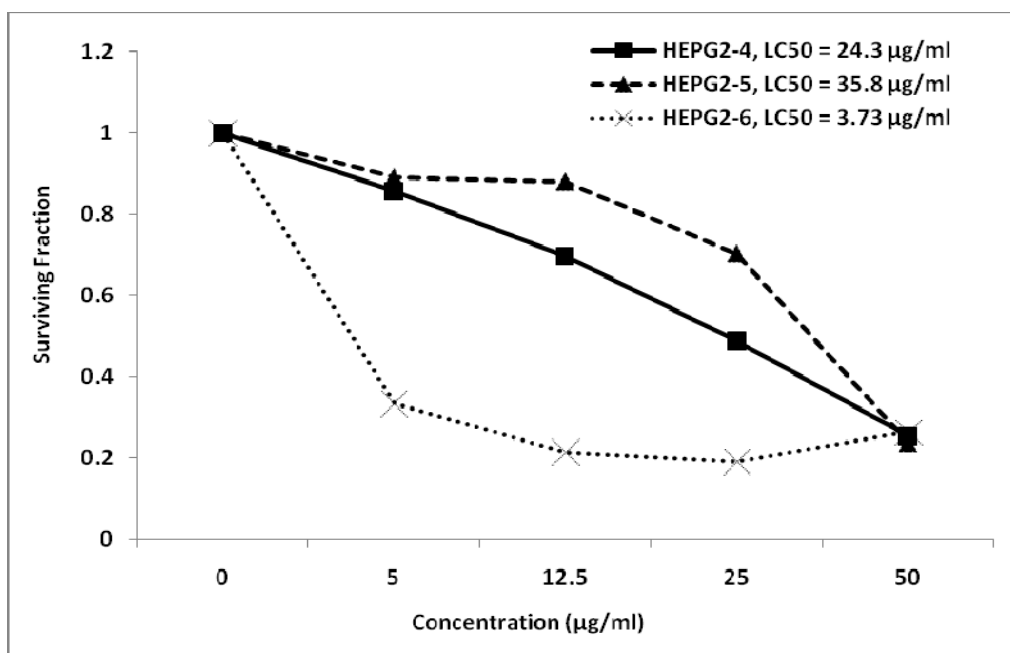


Figure 5. Potential antitumor activities of miso on human cell lines MCF7 (breast carcinoma), with LC_{50} value denoted on curve. MCF7-1, MCF7-2 and MCF7-3 represent fermentation/aging of miso for zero for 8, 2 and 4, respectively.

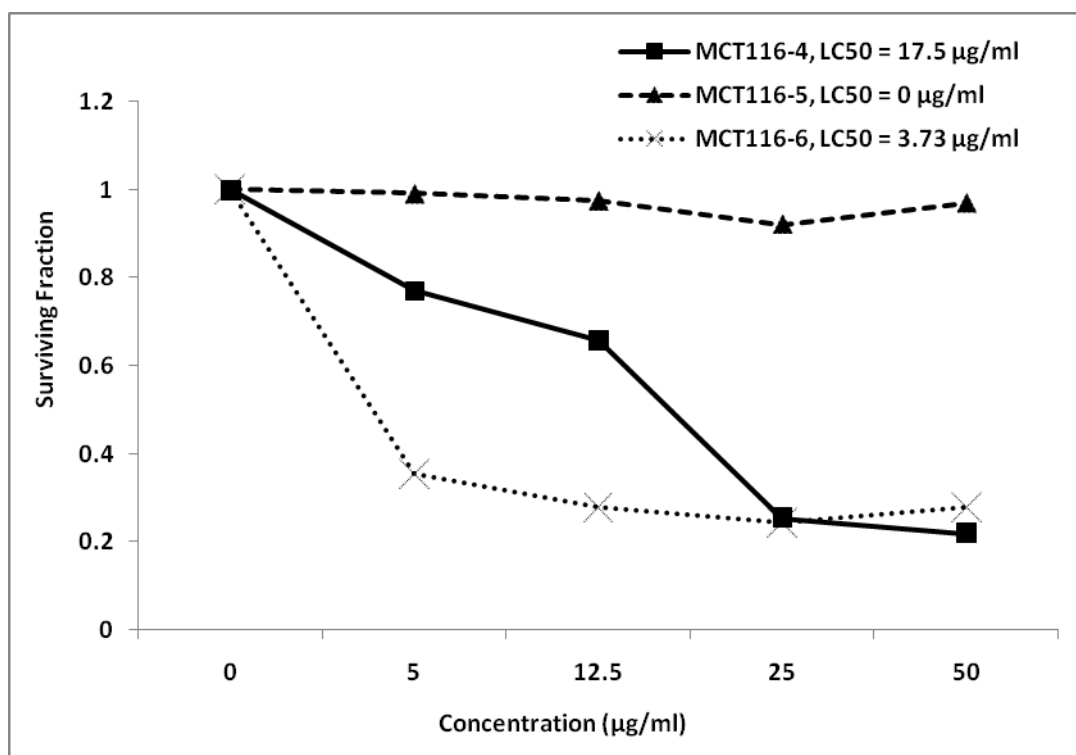


Figure 6. Potential antitumor activities of miso on human cell lines HCT116 (colon carcinoma), with LC_{50} value denoted on curve. HCT116-1, HCT116-2 and HCT116-3 represent fermentation/aging of miso for 8, 2 and 4, respectively.

4. Discussion

This study describes the *in vitro* antitumor activity of Miso, a fermented soybean food, on liver, breast and colon cancer human cell lines. Different types of miso, prepared with three starter culture mixtures and of different fermentation periods, were evaluated and their fatty acid, amino acid and isoflavone contents were determined. Finally, the effect of different miso on the inhibition of proliferation of cancer cell lines was studied.

Traditionally fermented soybeans contained higher concentration of Isoflavones than unfermented cooked soybeans. However, *B. subtilis* fermented soybeans appear to be a better source of bioavailable soy isoflavones as it has been reported (Kano et al., 2006; Dajanta et al., 2009). Miso may be of particular interest for using as antitumor agents against liver, breast and colon

carcinoma. The present finding indicates that miso storage for different period were able to inhibit the liver, breast and colon carcinoma. Miso has the ability to inhibit the carcinoma of liver and breast with low concentration at zero time (without storage period) after preparation. However, there is no significant different in the LD_{50} of miso that effect on colon carcinoma by using different storage fermentation period. However, Jung et al. (2006) demonstrated that prolonging the fermentation period (24 months) when making soy paste increases its antitumor effects *in vivo*. The longer storage period of miso over three years led to increase isoflavones concentrations (Yamabe et al., 2007). The time course of the isoflavone composition during the fermentation/aging process of rice-koji miso indicated that glycosides decreased from 86.4% to 44.9% after 6 months but aglycones increased from 9.6% to 53.3% (Yamabe et al., 2007). Soy

isoflavones have also been reported to reduce the risk of prostate cancer by acting as anti-cancer agents blocking the growth of hormone dependents cancers (Heald et al., 2007).

The present study proved that fresh prepared miso is better than miso after storage periods against liver and breast cancer. This result confirmed by the observation of Weed et al., 1985 and Park et al., 2003. They reported that the cooked soybeans showed less inhibition of the mutagenicity than raw soybeans, probably due to the destruction of the trypsin inhibitor by heat treatment (Weed et al., 1985). Park et al. (2003) also noted a marked antimutagenic activity in doenjang, For example, miso, tempeh and natto, fermented soybean products prepared with *A. oryzae*, showed an antimutagenic activity 2.5-fold more than that of unfermented milk. Soybeans inhibit mammary tumors in models of breast cancer. The lower antimutagenic activity of miso is probably due to the smaller portion of soybeans used and short fermentation period (Park et al., 2003).

In this study, the relationship between two types of starts that could be bacteria or fungi was evaluated for preparation of miso. Human cell lines have not been used as an *in vitro* model for studying the potency of miso that prepared with mixture of starters as anticancer. There is no data concerning the influence of miso on the cancer human cell lines MCF7, MCT116 and HEPG2 and for this reason it is very difficult to compare our results with those reported by other authors. Miso was prepared from *A. oryzae* and *B. subtilis* (6) has more potent effect on human tumor cell line MCF7 and reached almost plateau level at all tested concentrations (5-50 µg/ml), causing 50% cell death. There was a dose-dependent relationship of increased number of dead cells (MCF7) with increasing concentration of *A. oryzae* and *R. oryzae* (5). The concentration of miso that prepared with mixture of starters (6) has the most potent effect of the human cell lines HEPG2 and MCT116 as compared to other mixtures (4) and (5). These results could be due to the highest level of isoflavones that detected in miso that prepared with the mixture (6). This present study suggests a promising use of *Bacillus* starter cultures in improving isoflavone compounds especially the isoflavones which would benefit for novel functional food development. This result is in agreement with Dajanta et al. (2009).

We can conclude that miso decreased growth of various human cancer cells and its effect depends on the storage time of miso and the type of the starters. The results indicated that isoflavones suppressed tumor growth of the human cell lines *in vitro*. These results suggest that these different types of miso could induce inhibition of human cell lines in dose-dependent manner.

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