Milk Thistle Seeds Powder: A Potential Source of High Value-Added for Functional Pan Bread

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Received: 10 November 2020   Accepted: 11 January 2021   Published: 25 January 2021

ABSTRACT

*Silybum marianum* is a member of the Asteraceae family, also known as milk thistle. The milk thistle seeds contain silymarin, silymarin is the main active compound in seeds and is both an antioxidant and anti-inflammatory. The objective of the work was to determine the influence of milk thistle seed powder on the physical, antioxidant, and organoleptic properties of pan bread enrichment with different ratios (0%, 2.5%, 5%, 7.5% and 10%). Antioxidant properties, chemical composition, color properties, baking quality, texture profile analysis, freshness and organoleptic properties of pan breads enrichment with Milk Thistle Seeds (MTS) powder were analyzed and compared with control sample. Cytotoxicity effect of milk thistle seeds extract against hepatocellular carcinoma cell line (HepG2) using viability assay was investigated in this study. Total flavonoid contents, radical-scavenging activity, specific volume, significantly increased with the addition of MTS powder. Pan breads containing MTS powder also showed better organoleptic evaluation scores. Milk thistle seeds extract at various concentration showed an exhibited a potent anticancer effect against human liver cancer cell (HepG2). Pan breads containing MTS powder can thus be developed as a health-promoting functional food include protecting liver health.

**Key words:** Milk thistle, *Silybum marianum*, silymarin; flavonolignans, pan bread, Antioxidant properties, Texture profile, viability assay

1. Introduction

*Silybum marianum* (L.) Gaertn. (Asteraceae), otherwise called milk thistle, is an important herbal medicine. The edible seeds contain a mixture of flavonoid taxifolin and several flavonolignans compounds commonly named silymarin (Doehmer et al., 2011; Pereira et al., 2013). Silymarin is a mixture of seven main compounds: taxifolin, silydianin, silychristin, isosilybin A, isosilybin B, silybin A and silybin B (Sy-Cordero et al., 2010). Silymarin has been used to cure liver disease, chronic and acute viral hepatitis and toxin-induced liver diseases (Abenavoli et al., 2010). Flavonolignans compounds inhibit hepatitis C virus (HCV) contagion and also shows antioxidant, anti-inflammatory, and immunomodulatory activities that contribute to its hepatoprotective effects (Polyak et al., 2010). Silymarin is a promising agent for adjuvant cancer therapy and cancer prevention (Sagar, 2007).

Milk thistle is commonly cultivated to get silymarin (Alemardan et al., 2013). High variability in silymarin content or composition between wild milk thistle has recently been presented in different studies conducted in Italy and Iran (Shokrpour et al., 2008; Sulas et al., 2016). Silymarin components variation of milk thistle growing in the north, middle, and south of Egypt was studied (AbouZid et al., 2016), up to 40 milk thistle plant samples were collected from seven governorates in Egypt (three replicates were analyzed for each sample).

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Milk thistle dietary enrichments that contain silymarin are widely used and marketed in the USA and other countries for liver enhancement and recovery (Anthony et al., 2013). Milk thistle is recognized as a safe plant as it has not been included in the list of botanicals that contain toxic, psychotropic, addictive or other substances of concern (European Food Safety Authority, 2009). In Spain, *Silybum marianum* has traditionally been used as a vegetable for salads or eaten boiled and fried (Morales et al., 2014). Milk thistle seeds can also be added to bakery products as a healthy ingredient at 9.3% replacement ratios without affecting on sensorial parameters of biscuits (Bortliková et al., 2019).

The goal of this study was to find novel sources of nutraceutical substances such as flavonolignans compounds by studying the possibility enrichment wheat flour with milk thistle seed powder at different ratios to produced functional pan bread.

2. Materials and Methods

2.1. Materials

Milk thistle seeds were purchased from local market in Cairo, Egypt. The sample were identified by Botany Department, Agriculture and Biology Research division, National Research Canter, Egypt. This study was carried out from March – November, 2019 at the Food Technology Department, National Research Canter, Egypt. All-purpose wheat flour, salt, sugar, instant yeast and shortening were purchased from local market in Cairo.

2.2. Methods

2.2.1. Preparation of milk thistle seed

Milk thistle seeds were cleaned seeds were ground to an average particle size of 0.4 mm. The ground samples were then defatted in a Soxhlet apparatus with 200 mL hexane for 4 hr. The residue was air-dried and stored at -18°C freezing until analyzed.

2.2.2. Extraction of flavonolignans from milk thistle seeds powder

Defatted seeds powder was extracted with 200 mL methanol for 4 hr. using a Soxhlet apparatus. The extract was evaporated to dryness on a rotary evaporator under reduced pressure at 40°C, and reconstituted in 25 mL methanol. A 1 mL aliquot of the extract was further diluted to 25 mL with methanol and subsequently used for antioxidant analysis.

2.2.3. Antioxidant activity assays of seeds extract

DPPH radical-scavenging activity the free radical-scavenging activity was measured by the DPPH method according to Zhao and Li (2015). The 0.4 mmol/L solution of DPPH in 95% ethanol was freshly prepared daily, and 2 mL of this solution, together with 2 mL of 95% ethanol, were added to 1 mL samples. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank.

2.2.4. Total Flavonoid Content (TFC)

Total flavonoid content was determined by using aluminum chloride (AlCl₃) colorimetric method described by Drouet et al., (2018). Briefly, 10 µL of the extracts samples were mixed with 10 µL of potassium acetate (1 M) and 10 µL of AlCl₃ (10%, w/v) and 170 µL distilled water. This mixture was incubated for 30 min at room temperature (25 ± 2 °C), and then the absorbance was measured at 415 nm by using UV–visible spectrophotometer (UNICO 2100 UV/Vis United products and Instruments INC.). Rutin was used as the standard for the calibration curve, and the total flavonoid contents were expressed as milligrams rutin equivalents per 100 g.

2.2.5. Pan bread preparation

Pan Breads were prepared using different levels of milk thistle seed powder to enrichment wheat flour as ratios: 0, 2.5, 5, 7.5, or 10%. The blends were homogenized and stored in airtight containers and kept at 5–7 °C until further using. Pan bread was prepared according to the method given by AACC (2010) follows as: 100g flour, 60g water, 1.5g salt, 1.5g sugar, 1.5g instant yeast (was dissolved in water at 30 °C for 5 min) and 2 g shortening. Different blends were mixed in a laboratory mixer for
approximately 6 min until forming consistent dough. The dough was shaped and proofed in a bread incubator at 30 °C at a relative humidity of 85%. After 55 min incubation, the dough was removed and punched down, then put back in the incubator, then punched down a second time after 25 min. Then, the dough was equally divided into the same weight portions after fermentation and each section was shaped, put in metal pans and incubated for another 45 min. All dough was then baked conventionally in an oven preheated to 220 °C for 20 min. until loaf is medium golden brown. After baking bread, samples were separated from the metal pan and allowed to cool at room temperature for 1 hr. before organoleptic evaluation and different experiments.

2.2.6. Antioxidant properties of pan bread
Each sample was weighed (50 mg) into a centrifuge tube. Methanol (80%) (1 mL) was then added, and then shaken at 1,000 rpm for 1 h at 25°C. The sample was then centrifuged. Antioxidant activity of the extracted bread samples and TFC was estimated according to the above methods.

2.2.7. Proximate chemical composition of pan bread
The chemical composition (moisture, fat, protein, ash, crude fiber) of samples was determined using standard procedures (AOAC, 2012), and total carbohydrate content was calculated by difference method.

2.2.8. Baking quality (physical evaluation) of pan bread
The weight of pan bread samples was determined after cooling. Bread samples volume was measured by rapeseed displacement method. Specific volumes (cm³)/g of bread were calculated by dividing the volume (cm³) by their weight (g). Density g/(cm³) of bread were calculated by dividing weight (g) by their volume (cm³). The height (cm) pan bread samples were measured with a caliper after cooling AACC (2010).

2.2.9. Color properties of crust and crumb pan bread
The pan bread crust and crumb color was measured. Hunter (a*, b* and L*) parameters were measured with a color difference meter using a spectro-colorimeter (Tristimulus Color Machine) with the CIE lab color scale (Hunter, Lab Scan XE - Reston VA, USA) in the reflection mode. The instrument was standardized (at each time) with a white tile of Hunter Lab Color Standard (LX No.16379): X= 72.26, Y= 81.94 and Z= 88.14 (L* = 92.46; a* = -0.86; b* = -0.16) (Ahmed and Abozed, 2015).

2.2.10. Texture profile of pan bread (Crumb firmness)
The bread crumb texture was evaluated according to Sangmark and Noomhorm (2004). The analyses were carried out using a texture analyzer, model TA-XT2 and the XTRA Dimension program (Stable Micro Systems, Surrey, England) with the p/25 cylindrical aluminium sensor probe. The parameters established were: test option and mode = measurement of the compression force, hold until time, pretest speed = 10 mm/s, test speed = 1.7 mm/s, posttest speed = 10 mm/s, distance = 40%, time = 60 s and auto trigger = 10 g.

2.2.11. Freshness of pan bread
Freshness of pan bread samples was tested at room temperature during storage for 1 and 3 days by alkaline water retention capacity (AWRC) test according to the method of Licciardello et al., (2014).

2.2.12. Organoleptic evaluation of pan bread
Organoleptic evaluation of pan bread samples was evaluated after cooling by 15 members of the Food Science and Technology Department, National Research Centre, according to the method given by Hijazi and El-Gazar (2018) with minor modifications. The panelists scored appearance, flavor, taste, exterior color, interior color, cell uniformity and overall acceptability of the bread using a using a 9-point hedonic scale (1 = “disliked extremely”, 9 = “liked extremely”).

2.2.13. Cytotoxicity activity of milk thistle seeds
Cytotoxicity activity of milk thistle seed against cancer cells (Hep-G2) was evaluated by percentages of cell viability using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide] assay as described by (Sladowski et al., 1993). Hepatocellular carcinoma (Hep-G2) was supplied by the Naval American Research Unit-Egypt (NAmRU). Briefly, cells were seeded in 96-well microplates (3 × 103 cells/well) in 100 µL RPMI-1640 culture medium and incubated at 37 and 5 % CO2 overnight. The cells were treated with different concentration from milk thistle extract and staurosporine as a positive standard drug (0.39, 1.56, 6.25, 25 and 100µg/ml), and re-incubated for 24 h. MTT (0.5 mg/mL) solution was added to each well (100 µL), and the cells were incubated over night until the purple formazan crystals appeared. The medium was discarded; 100 µL of DMSO was added to dissolve the crystals. The optical density (OD) of solubilized formazan was measured at 570 nm using an automatic microplate reader and percentages of cell viability of cells were calculated: % cell viability = Absorbance sample/Absorbance control × 100.

2.2.14. Statistical Analysis
The experimental results were expressed as mean ± Standard Deviation (SD) of three replicates. Data obtained were statistically analyzed using one way Analysis Variance (ANOVA), a tool in Statistical Packages for SPSS 18.0 software. The level of significance was set at p < .05. Means were separated with Duncan Multiple Range Test.

3. Results and Discussion

3.1. Antioxidant properties (Free radical scavenging and total flavonoid content) of seeds extract
Free radical scavenging antioxidant activity of the milk thistle seed powder extract was reached (78.401%). Zhao and Li (2015) who noticed that antioxidant activity of 5-10 mg/L milk thistle seeds extract was increased in radical scavenging activity from 10 % to 74 %, thus it can use milk thistle seeds extract as a functional food material against oxidative stress.

The mean value of the total flavonoid content of the milk thistle seeds powder samples under study was 997.539 (mg/100 g dry weight basis), these total flavonoid mean was higher than those detected in milk thistle seeds by Lucini et al., (2016) who found the total flavonoid contents was ranged from 30 to 84 (mg/100 g) in different milk thistle cultivars. On the other hand, the results of this study are in line with Stancheva et al., (2010) where were reported the total flavonoid contents ranged from 390 to 800 (mg/100 g dry weight basis). The variation in flavonolignans may be caused by different genotype, growing conditions and the environmental conditions (Martin et al., 2006).

3.2. Antioxidant properties (Free radical scavenging and total flavonoid content) of pan bread
The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging ability of the pan bread samples at different enrichment ratios of milk thistle seeds compared to control were presented in Table 1. All the level of milk thistle seeds enriched in pan breads showed a good ability in radical scavenging activity (11.189% - 22.100%), while it was only 7.377% in the case of the control. The high antioxidant activity of enriched pan bread samples is due to phytochemical compounds such as silymarin, the antioxidant activity of silymarin extract from milk thistle seeds in order to synergic effect of flavonoids (Lucini et al., 2016).

Table 1: DPPH and Total Flavonoids Content of MTS and pan bread produced by different enriched ratios

| Antioxidant properties | Milk Thistle Seeds | Enriched ratios of pan bread |
|------------------------|--------------------|-----------------------------|
|                        | Control            | 2.5% MTS | 5% MTS | 7.5% MTS | 10% MTS |
| DPPH/RSA (%)           | 78.401± 0.86       | 7.377± 0.25d | 11.189± 0.16c | 11.562± 0.04c | 14.303± 0.57b | 22.100± 1.66a |
| TFC (mg Rutin/100g)    | 997.539± 1.86      | 25.451± 0.35c | 68.842± 0.39d | 79.607± 0.61c | 199.340± 1.19b | 223.760± 0.44c |

Values with different superscripts within the same row are statistically significant from each other (p<0.05)
Presented data are mean value of three replications± standard deviation
From the data declared in the same table, it could be noticed that MTS are a good source of total flavonoids. The results indicated that the total flavonoids contents of pan bread samples were significant increased by rising levels of MTS addition, their contents ranged from 68.842 mg/100g to 223.760 mg/100g compared to control bread which given 25.451 mg/100g.

3.3. Proximate chemical composition of pan bread

Chemical composition of pan bread samples enriched with 2.5%, 5%, 7.5% and 10% MTS compared to control samples were evaluated and the results are tabulated in Table 2. The results indicated that adding MTS to prepared pan breads caused significant differences in all chemical composition between control and other bread samples. The moisture content ranged from 27.927% for the 5% MTS to 29.904% for control pan bread sample. Pan bread enriched by 10% MTS caused relatively high mean value of fat content 3.204% when compared to other enriched ratios and control breads sample.

Bread samples with 7.5% and 10% milk thistle seeds recorded significant (P ≤ 0.05) increase in ash contents being 2.193% and 2.159%, respectively, compared to control bread, this result could be attributed to the high ash content of used MTS. The same trend was found in the case of crude fiber, where the highest level 2.140% was given by breads sample prepared by adding 10% of milk thistle with significant (P ≤ 0.05) increase compared to other breads samples and control one. These findings are in agreement with previous study conducted by Faiza et al. (2018) whose reported that milk thistle seeds powder has high content of crude fiber (24±0.36 %).

Table 2: Proximate chemical composition of the pan bread produced by different enriched ratios of MTS

| Treatments | Moisture (%) | Fat (%) | Protein (%) | Ash (%) | Crude fiber (%) | Total Carbohydrate* (%) |
|------------|-------------|---------|-------------|---------|----------------|------------------------|
| Control    | 29.904±     | 2.402±  | 12.415±     | 1.578±  | 0.843±         | 82.760±                |
|            | 0.32abc     | 0.12ab  | 0.33bc      | 0.06c   | 0.076d         | 0.29a                  |
| 2.5% MTS   | 29.300±     | 2.051±  | 13.282±     | 1.911±  | 0.915±         | 81.839±                |
|            | 0.67ab      | 0.05d   | 0.33a       | 0.03b   | 0.03d          | 0.34c                  |
| 5% MTS     | 27.927±     | 3.028±  | 12.850±     | 1.952±  | 1.178±         | 80.989±                |
|            | 0.77c       | 0.03b   | 0.33b       | 0.22b   | 0.02c          | 0.50b                  |
| 7.5% MTS   | 28.997±     | 3.012±  | 11.314±     | 2.193±  | 1.376±         | 82.103±                |
|            | 0.53abc     | 0.02b   | 0.23c       | 0.01a   | 0.06b          | 0.20bc                 |
| 10% MTS    | 28.106±     | 3.204±  | 11.702±     | 2.159±  | 2.140±         | 80.794±                |
|            | 0.76abc     | 0.15a   | 0.34c       | 0.09a   | 0.11b          | 0.38c                  |

Values with different superscripts within the same column are statistically significant from each other (P<0.05)
Presented data are mean value of three replication± standard deviation
*Total Carbohydrate calculated by difference

3.4. Physical evaluation of pan bread

Volume is an important measure of pan bread quality that has traditionally been determined by seed displacement methods and it is critical to customer acceptance of these products. The volume, weight, height, density, specific volume for prepared pan bread samples containing MTS were analyzed and the data are shown in Table 3. The results showed that all selected enriched ratios resulted in significant (P ≤ 0.05) increase in pan breads volume and specific volume when compared to control which recorded 347.666 cm³ and 2.447 cm³/g, respectively. The higher specific volume (2.750 cm³/g) was given by using 10% of MTS without significant difference with breads sample containing 5% and 7.5% of MTS which recorded 2.725 cm³/g and 2.731 cm³/g, respectively. However, the data could be observed that no significant (P ≥ 0.05) difference in weight between control and breads enriched with 2.5%, 5%, 7.5% and 10% of MTS. Data also indicated that enrichment of 10% MTS caused significantly (P ≤ 0.05) increase in height (9.533 cm) compared to other enrichment ratios and control which given 7.466 cm. Otherwise, the control sample was the highest in density than the other pan bread samples. From the previous results, it can be summarized that adding the milk thistle seeds powder improved the baking quality of pan bread.
defines the proportion of yellow (positive value) or blue color (negative value). Where presented data are mean value of three replication ± standard deviation. Values with different superscripts within the same column are statistically significant from each other (p<0.05).

3.5. Crust and crumb color properties

Color is a key quality parameter that often affects consumer acceptability and it depends on raw materials. Crust and crumb color of pan bread were evaluated using a Hunter lab. The L*, a*, b* and ΔE values for crust and crumb of pan bread samples with MTS are shown in Table 4. It could be observed that control pan bread had the significant (P ≤ 0.05) increase in lightness (L*) for crust and crumb which given 67.68 and 73.08 respectively, while the crust lightness was gradually decreased from 57.83 to 51.05 and crust lightness was decrease from 66.41 to 58.72 with increased the adding level of MTS in bread from 2.5% to 10%, respectively. The same trend was found in b* (yellowness) values, the yellowness was decreased with increased the adding levels of MTS in pan bread. However, the highest value 31.48 for the crust and 22.70 for crumb were given by control pan bread with significant (P ≤ 0.05) increase compared to other pan breads enriched. Regarding to redness (a*), data revealed that using MTS at 10% in crust and crumb layers for pan bread resulted significant (P ≤ 0.05) high in crust 15.88 and in crumb 3.94 compared to other treatments and control bread which recorded the lowest value in crust 9.34 and in crumb 1.66. Total color difference (ΔE) was increased with increasing the adding levels of MTS in pan bread. The crust and crumb pan bread with 7.5% and 10% MTS recorded significant (P ≤ 0.05) increase in total color difference compared to other enriched ratios for pan bread. These results may be occurring because of light brown color of milk thistle seeds.

Table 4: Crust and crumb color of the pan bread produced by different enriched ratios of MTS

| Treatments | Crust | Crumb |
|------------|-------|-------|
|            | L*    | a*    | b*    | ΔE   | L*    | a*    | b*    | ΔE   |
| Control    | 67.68 ± 0.31<sup>a</sup> | 9.34 ± 0.14<sup>a</sup> | 31.48 ± 0.25<sup>b</sup> | ---   | 73.08 ± 0.354<sup>a</sup> | 1.66 ± 0.7<sup>c</sup> | 22.70 ± 0.41<sup>c</sup> | ---   |
| 2.5% MTS   | 57.83 ± 0.88<sup>b</sup> | 10.85 ± 0.24<sup>d</sup> | 32.43 ± 0.18<sup>b</sup> | 9.83 ± 0.88<sup>c</sup> | 66.41 ± 1.19<sup>c</sup> | 2.96 ± 0.18<sup>c</sup> | 19.72 ± 0.13<sup>c</sup> | 7.44 ± 1.04<sup>c</sup> |
| 5% MTS     | 56.00 ± 1.49<sup>c</sup> | 11.71 ± 0.12<sup>c</sup> | 32.33 ± 0.44<sup>b</sup> | 11.78 ± 1.44<sup>bc</sup> | 65.27 ± 0.07<sup>e</sup> | 2.93 ± 0.05<sup>d</sup> | 18.93 ± 0.18<sup>c</sup> | 8.77 ± 1.11<sup>b</sup> |
| 7.5% MTS   | 55.61 ± 0.94<sup>c</sup> | 13.31 ± 0.10<sup>d</sup> | 28.01 ± 0.89<sup>c</sup> | 13.02 ± 1.05<sup>c</sup> | 59.27 ± 1.05<sup>c</sup> | 3.86 ± 0.04<sup>d</sup> | 18.38 ± 0.08<sup>d</sup> | 14.63 ± 0.19<sup>a</sup> |
| 10% MTS    | 51.05 ± 0.49<sup>d</sup> | 15.88 ± 0.29<sup>d</sup> | 27.15 ± 0.30<sup>a</sup> | 18.21 ± 0.62<sup>d</sup> | 58.72 ± 0.09<sup>a</sup> | 3.94 ± 0.04<sup>d</sup> | 17.86 ± 0.35<sup>a</sup> | 15.32 ± 0.06<sup>a</sup> |

Values with different superscripts within the same column are statistically significant from each other (p<0.05). Presented data are mean value of three replications ± standard deviation. Where L* represents lightness of color, a* represents red (positive value), and green color (negative value), while b* defines the proportion of yellow (positive value) or blue color (negative value).

3.6. Crumb firmness of pan bread

Texture parameters such as hardness, cohesiveness, gumminess, chewiness adhesiveness and springiness of pan bread samples prepared by adding 2.5%, 5%, 7.5 and 10% MTS compared to control were measured and the data are shown in Fig (1). From the results, it could be observed that the all pan bread samples produced by different enrichment ratios was given lower hardness values than the control.
bread (Fig. 1a). The decrease in the crumb hardness can be explained by the increase in the bread volume (Table 2). These results are consistent with those obtained by Yamsaengsung et al., (2010) who reported that the increased bread volume is clearly related to the decreased hardness values. Moreover, Wang et al., (2002) reported that texture profile analysis revealed softer crumbs when fiber is added to wheat flour when the manufacture of bread. The cohesiveness values decreased with increasing the enriched ratios (Fig. 1b). Again, the results showed that all enriched ratios of pan bread resulted decrease in gumminess and chewiness values when compared to control which recorded 22.97 N and 614.80 mj, respectively. (Fig. 1c and d). The results are in accordance with Barak et al., (2014) who reported that both gumminess and chewiness are parameters dependent on hardness. Data also indicated that the 5% and 10% MTS pan bread became more adhesive when compared to control pan bread samples and other enriched ratios (Fig. 1e). The springiness values of pan bread tested samples were ranged from 23.66 to 38.63 (mm) for 2.5% and 7.5% MTS respectively, While the springiness values of enriched bread at 5% is close to the values of the control bread samples, which recorded 26.54 and 26.77(mm), respectively. (Fig. 1f).

Fig. 1: Texture profile analysis of pan bread produced by different enriched ratios of MTS: (Fig.1a) Hardness, (Fig.1b) Cohesiveness, (Fig.1c) Gumminess, (Fig.1d) Chewiness, (Fig.1e) Adhesiveness and (Fig. 1f) Springiness.
3.7. Alkaline water retention capacity of pan bread

Alkaline water retention capacity is a quick and simple test to follow freshness of pan bread. Higher values this test means higher freshness of the bread. The change in freshness of pan bread enriched with different MTS ratios stored at room temperature for 0, 1 and 3 days are shown in Table 5. From the results, it could be observed that the control pan bread had the lowest values of freshness after 3 days storage. Results indicate that the pan bread prepared by 7.5 MTS had the highest values of freshness compared to control and the other three MTS ratios during storage. In addition, the data showed that the loss of freshness for pan bread prepared by different MTS ratios ranged from 25.682 to 27.078% with no significant differences after 3 days storage. All enriched samples showed positive effects on retarding staling of the pan bread during storage periods at room temperature. Such effect might be related to the physicochemical properties of milk thistle flour.

Table 5: Alkaline water retention capacity as indicator for freshness properties of pan bread produced by different enriched ratios of MTS

| Treatments | Storage periods | Alkaline water retention capacity (%) | Loss of freshness (%) |
|------------|----------------|----------------------------------------|------------------------|
|            | Zero           | 1 days                                 | 3 days                 | 1 days | 3 days |
| Control    | 331.243±      | 271.050±                               | 232.565±               | 18.171± | 29.790± |
|            | 0.91d         | 1.33b                                  | 1.78c                  | 0.53a   | 0.48a   |
| 2.5% MTS   | 338.663±      | 291.596±                               | 251.690±               | 13.897± | 25.682± |
|            | 1.94c         | 1.54a                                  | 2.29ab                 | 0.14c   | 0.26b   |
| 5% MTS     | 341.356±      | 292.310±                               | 250.946±               | 14.367± | 26.487± |
|            | 1.13bc        | 0.96a                                  | 4.96bc                 | 0.40bc  | 1.31b   |
| 7.5% MTS   | 343.803±      | 292.730±                               | 255.360±               | 14.854± | 25.724± |
|            | 1.53a         | 0.65a                                  | 1.11a                  | 0.18b   | 0.08b   |
| 10% MTS    | 339.513±      | 290.790±                               | 247.583±               | 14.350± | 27.078± |
|            | 1.12bc        | 1.14a                                  | 3.28b                  | 0.43bc  | 0.74b   |

Values with different superscripts within the same column are statistically significant from each other (p<0.05)

3.8. Organoleptic evaluation of pan bread

Organoleptic evaluations of prepared pan bread with different levels of MTS Fig. (2) were evaluated compared to control pan bread and the results are found in Fig. (3). It was noticed that enrichment of 7.5% and 10% MTS in bread caused relatively low score in appearance (7.125 and 6.875) and exterior color (7.00 and 7.00), respectively when compared to control bread which recorded the highest scores in appearance 8.750 and exterior color 8.750.

Fig. 2: Pan bread produced by different enriched ratios of MTS
The increase in darkness was reflected in L* values (Table 4); which due to the color of added MTS. The flavor of breads containing 7.5% and 10% of MTS was gradually less scores 7.375 and 7.375, respectively, compared to control bread which had 8.75, data also indicated that taste of studying bread samples containing 2.5%, and 5% of MTS were acceptable by panelists and without significant (P ≥ 0.05) difference with control bread. It could be observed that no significance (P ≥ 0.05) difference in overall acceptability between control bread 8.87 and the breads enriched with 2.5% and 5% of MTS which given 9.00 and 8.625, respectively. These data are in agreement with Bortliková (2019) who stated that, recommended limits for good technological behavior and, consequently, good quality of biscuits equal up to the 9.3 % wheat flour replacement with milk thistle seeds flour.

3.9. Cytotoxicity activity of milk thistle seeds

Results of in vitro cytotoxic activity of flavonolignan compounds extract from *Silybum marianum* (L.) seeds against HepG2 cell lines using viability assay was presented in Fig 4. Viability assay is a test to evaluate the ability of cell to recover or maintain a state of survival. In this part, the ability of hepatocellular carcinoma (HepG2) cancer cells to recover was studied when treated with different concentrations of milk thistle seeds extract, and the half-maximal inhibitory concentration (IC₅₀) of the extract was compared to a staurosporin as a positive standard, where used as extensively to in antitumor therapy for their anticancer activity.

Figure 4 showed that when hepatocellular carcinoma (HepG2) cancer cells were treated with 6.25 µg/ml seeds extract, the percentage of viability of these cells reached 61.62 %. Whereas, in case increasing the concentration of seeds extract up to 100 µg/ml, the percentage of viability was decreased to 41.53%. Percentage of viability for 100 µg /ml staurosporineas (positive standard) was reached 33.74%. While, 6.25 µg /ml were reached 49.4 %.

The half-maximal inhibitory concentration (IC₅₀) was recorded 32.1±1.56 and 9.71±0.47 µg/ml (for 24 h exposure) for milk thistle seeds extract and staurosporineas, respectively. The results are clear that the viability of hepatocellular carcinoma (HepG2) cancer cells decreased with increase in the concentration of the seeds extract due to the cytotoxic effect of the phytochemical compounds in the
seeds. The anticancer activity of flavonoids is related to their antioxidant ability and induction of apoptosis and cell cycle arrest (Abou Baker, 2020).

![Graph](image)

**Fig. 4:** Cytotoxicity effect of *Silybum marianum* (L.) seeds extract against HepG-2 cell line at different concentration

**4. Conclusion**

It could be concluded that the antioxidant properties, baking quality and freshness of pan bread enriched with milk thistle seed powder improved with an increase in the additive ratios. Cell viability was reduced when treated with different concentration of *Silybum marianum* (L.) seeds extract. Generally, enrichment of about 5 to 7.5% milk thistle seeds powder did not significant difference in taste and overall acceptability when compared to the control sample and at the same time gave higher nutritive values without drastically affecting the pan bread quality. Furthermore, production of milk thistle pan bread may provide a platform for further development of high-value functional food products.

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