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Effect of natural sources rich in calcium on treated rats induced osteoporosis

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Received: 12 June 2022 / Accepted: 26 July 2022
Published online: 31 August 2022

Abstract
The present study aimed to treat female rats' osteoporosis using natural sources rich in calcium (permeate with kiwi or fig). Thirty-two female rats weighting 150 ± 10 g were used in this experiment. After adaptation period (7 days), rats were divided to 8 groups (four in each group); first group worked as a control negative and other groups were injected with 1 mg Dex. /kg/bw glucocorticoid (dexamethasone) for 7 days to induce osteoporosis. Second group was considered as a positive group and other groups were treated with beverage (kiwi with permeate or fig with permeate) at different concentrations, i.e., 20, 30 and 40%. The DPPH test was recorded 69.19, 87.01, 68.95, and 44.88% for fresh kiwi, refrigerator kiwi beverage, fresh fig, and refrigerator fig beverage respectively. Meanwhile, using ABTS recorded of 32.6, 39.07, 36.66 and 41.99 Trolox/100 g for above mentioned treatments. In general, total phenol and total flavonoid values of fig treatments were high compared with kiwi treatments. The antimicrobial examination showed an increase in lactic acid bacteria due to refrigeration in both beverages and less total count. Mold, yeast, and coliform not detected. The biological assay resulted in decrease weight gain and feed intake while feed efficiency ratio slightly affected due to beverages. Ca and P content of osteoporotic femur have shown an increase more than the potassium content. Also, serum Ca, P, PTH, Vit. D and protein increased significantly as the result of beverage treatments. The X- ray showed an improved of bone in all treatments compared with (+) control. It could be concluded that permeate mixed with kiwi or fig can be used as a therapeutic diet for subjects, who suffered from osteoporosis.

Keywords Milk permeate · Kiwi · Fig. functional beverages · Bacteria · Osteoporosis · Glucocorticoid

Abbreviations

DPPH	2,2 Diphenyl-1-Picrylhydrazyl
ABTS	Azino bis 3-Ethylbenzothiazoline-6- sulfonic acid
IDF	Inhibin disc fungi
APHA	American pub1.Health Assoc
T.C	Total count
PTH	Parathyroid hormone
TTA	Total titratable acidity
ELISA	Enzyme linked immunosorbent assay

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1 Introduction

Osteoporosis is the most common health condition among metabolic bone disease, which is related to a decrease in bone minerals density. And microstructural deterioration of bone tissue. A good relation between osteoporosis and increasing constituents a major public health problem, i. e., hyper thyrodism, hyperparathyroidism, hypogonadism, bone destruction which led to tumor metastasis, rheumatoid arthritis and chronic obstructive pulmonary disease [1]. The diagnosis of osteoporosis should be established by the measurements of the bone minerals density [2]. Which recommended by WHO [3]. Due to the demographic changes, more than of 40 million American citizens over 50 years of age are risk of osteoporosis fracture, this number will at least double until year 2040 [4]. Also mentioned that the increase in body mass density (BMD) is leading to an increase in bone fragility and susceptibility fractures of some confectioned antiosteoporotics are effective in the treatments and prophylaxis of osteoporosis [5]. Calcium is vital for life muscle function, nerve activity and bone mineralization depend on an accurate balance between the extracellular and intracellular calcium [6]. Vitamin D directly raise the serum calcium by protein synthesis in the intestinal wall which promotes intestinal calcium transport and absorptions. Vitamin D increases the reabsorptions of calcium and phosphorus from renal tubular [7]. Parathyroid hormone is very sensitive and response to any change in extracellular calcium which acts in conjunction with active vitamin D to regulate calcium. Many studies were carried out to investigate the different mechanisms of parathyroid hormones regulation and restricted that in three distinct mechanisms: (1) it increase reabsorptions of calcium from the renal tubular, (2) increase bone turnover to release calcium and phosphorus from the bone, (3) enhance the conversion of hydroxyl vitamin D to an active form [6]. As far as food health goods are concerned, milk permeate is one that hasn't been widely used. More than 85% of the milk used to make cheese is wasted in this way, although it is still considered a waste product of the ultrafiltration (UF) processes [8]. When milk is ultra filtered, a substantial amount of permeate is produced as a byproduct. Getting rid of the rest amount of liquid was produced by the dairy sector needs a considerable outlay of resources [9]. Adding milk permeate to meal increase its nutritional value since it includes electrolytes (sodium, potassium, magnesium, zinc and calcium) that are present in alternative products [10]. The use of permeate in the manufacturing of several beneficial products has received great attention [8]. On the other hand, employing milk permeate was able to generate an excellent grade produce fruit drinks [11]. However, employing the permeate has economic relevance since it is regarded useful alternative to skim milk as partial or entire replacement [12]. Fig and kiwi are rich sources of sugar, organic acid, mineral, vitamins, polyphenols, flavonoids and fiber [13, 14]. They found that fruity milk residue is rich source of important electrolytes.

The present investigation aimed to produce fruity milk residue and study its effects as natural source rich in calcium on osteoporosis female rats.

2 Material and methods

Milk permeate was obtained from the Animal Production Research Institute's, Agriculture Research Center. The starter culture namely (*Lactobacillus delbrueckii subsp bulgaricus, Bifidobacteria bifidus, Streptococcus thermophilus, and Lactobacillus acidophilus*) were obtained from Mersin, faculty of agriculture. Ain shams university. kiwi, fig, and sugar were obtained from the local market, Giza, Egypt. All chemicals were used in the present investigation were purchased from Sigma Aldrich. Dexamethasone (glucocorticoid) kits and basal food components were purchased from Al- Gomhorya Pharmaceutical Industries Company, A.R.E. Thirty female albinos Sprague Dawley strain weighing 150 ± 10 gm were obtained from Food Tech. Res. Ins. A.R.C.

2.1 Preparation of the functional beverages

Both the kiwi and fig were carefully cleaned before being peeled and chopped into "small pieces". The fruit flesh was blanched at 80 °C for 10 min after then the flesh of both fruits (kiwi and fig) were kept frozen (−20 °C). The milk permeate was heated to 40 °C, 5 g/100 ml sucrose was added, and the mixture was heated to 85 °C for 15 min and quickly cooled. 2% of a starter culture *of Lb. delbrueckii subsp bulgaricus, Bifidobacteria bifidus, Lb. acidophilus, and Str. thermophilus* was added to the sweetened milk permeate, incubated at 42 °C until the pH was reached to 5, and then rapidly cooled to 4 °C [15]. Treated permeate was added to the blanched fruits in the ratio of 3:1, 2:1 and 1:1 (v/w), respectively. The beverages

were subjected to sensory evaluation by test panelists of FTRI and reported that the ratio of sweetened permeate to the fruits of 1:1 (w/v of fruit and permeate) was the best ratio to produce the beverage which was in parallel with the finding of Atallah [16].

2.2 Chemical composition analysis

As stated by the American Association of Analytical Chemistry (AOAC), titratable acidity and pH values were obtained using the AOAC [17].

Mineral composition of the raw material i.e. kiwi, fig and permeate and the produced beverage were determined according to procedures archived in A.O.A.C [17].

2.3 Microbiological examinations

IDF [18] and APHA [19] were used to count lactic acid bacteria (LAB), yeasts and Moulds, and coliform bacteria, respectively. *Lb. delbrueckii subsp. bulgaricus, Str. thermophilus*, and *Bifidobacteria sp.* were counted in the produced beverage according to Ryan et al. [20].

2.4 Determination of DPPH and ABTS radical savaging activity

DPPH was determined according to the method described by Liu et al. [21] while ABTS was determined according to the method of Re et al. [22].

2.5 Determination of total phenols

Total phenols were determined of the produces beverages according to the method described by Chang et al. [23].

2.6 Determination of total flavonoids

The method of Liu et al. [21] was used to determined total flavonoids.

2.7 Fractionation of sugar components by HPLC

The chromatographic system Agilent (series 1200) coupled to the refractive index detector was equipped with a quaternary pump, degasser and auto injector. The chromatographic data were acquired using the Agilent software. The samples obtained as described above were analyzed using an Aminex-carbohydrate HPX-87 column under isocratic condition with deionizes water. The flow rate was 0.5 ml/min. The column temperature was maintained at 85 °C and the detector at 50 °C. Sample detection was performed by comparing retention time's standards [24].

2.8 Biological assay

Thirty-two female albino rats of Sprague Dawley strain, weight 150 ± 10 gm (3 months as age) were raised in the animal house of Food Technology Research, Agriculture research center. Giza, Egypt.). The animals were allowed to free access to water and basal diet for 7 days as an adaptation period. The basal diet was formulated according to AIN [25]. All animal experiments were conducted according to guidelines of the ethical principles established by national institutes of health guide for the care and use of laboratory of animal (NIH Publication 2011). The rats were divided into 8 groups (G1–G8 n = 4 for each group) according to the following scheme (Fig. 1), functional beverage were added to Basel diet at different concentration (20, 30, 40 ml/ 100 ml). (G1: Control, G2: Control +, G3: Kiwi functional beverage groups 20%, G4: Kiwi functional beverage groups 30%, G5: Kiwi functional beverage groups 40%, G6: Fig functional beverage groups 20%, G7; Fig functional beverage groups 30%, G8: Fig functional beverage groups 40%).

Biological experimental design32 female rats fed basal diet for 7 days as an adaptation period.

At the end of the experimental period, (45 days), the rats were fasted overnight. The blood samples were obtained from orbital plexus Venus by means of fine capillary glass tubes [26]. The blood samples were placed in dray and clean

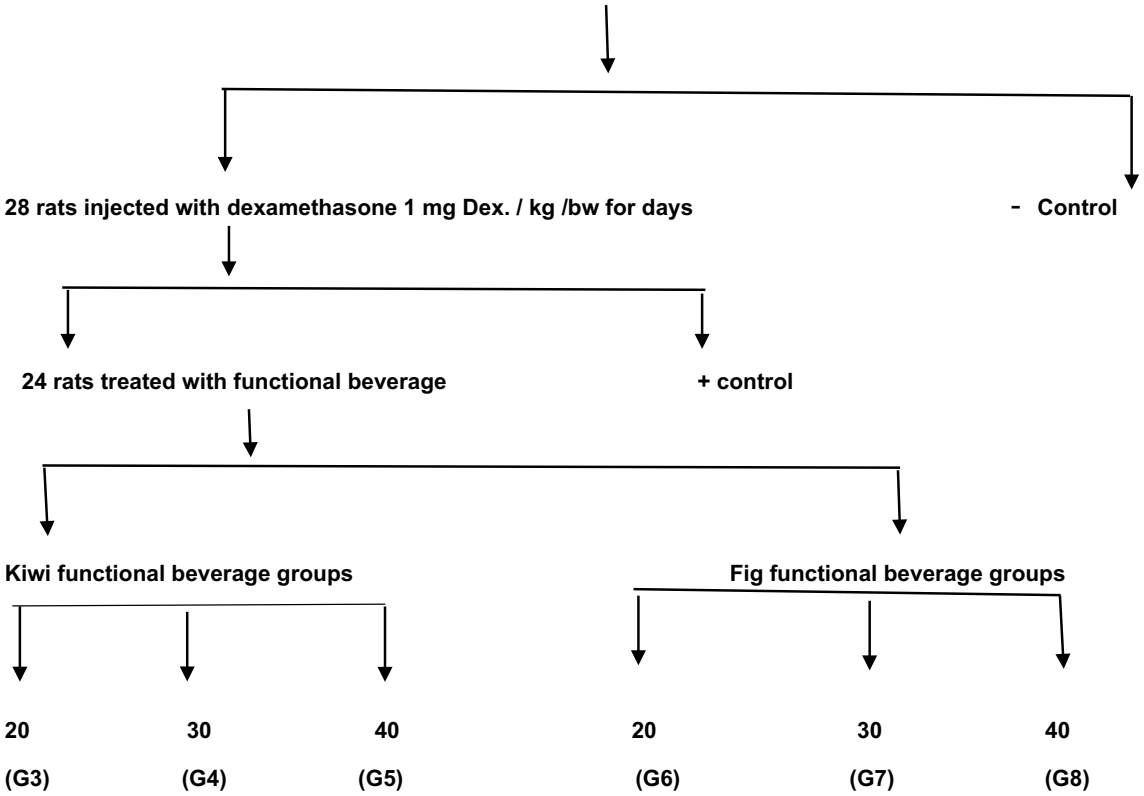


Fig. 1 Scheme diagnosed for osteoporosis female rats treated with kiwi and fig beverage

centrifuge tubes and allowed to clot for 1 h at room temperature. Serum was removed using a Pasteur pipette and centrifuged for 20 min at 3000 rpm. The clean supernatant sera were then kept frozen until analysis.

2.8.1 Collection of the feed intake

The food intake was calculated daily and the body weight gain was recorded weekly.

$$\text{Calculate DM\%} = (\text{feed dry weight} \div \text{feed wet weight}) \times 100\%$$

2.8.1.1 Feed efficiency ratio Feed Efficiency Ratio (FER) for all treatments were given in 1 ml volume from stock solution by stomach tube all over the period of the study was assigned for 3 months. The feed intake was calculated daily and the body weight gain was recorded weekly [27]**.**

2.8.2 Serum analysis

Serum total calcium and PTH parathyroid hormone were estimated using ELISA technique according to the manufacturer's instructions (BioSource host- osteocacin, INC., Camarillo, California, USA. Total Vit. D [28]**.** While total calcium and phosphorus were determined according to the method of Weatherburn et al. [29].

2.8.3 Radiography of bones by X-ray

Both femurs were separated and cleaned from the surrounding soft tissue. Radiography was performed using X-ray (Fischer Imaging) at 40 Kv and 10 Mas [30]. The length of each femur was measured using a Varnier caliper [31].

2.8.4 Determination of bone mineral content

After incinerated femur bone (550 °C until ashing) using muffle furnace, the ash content was calculated and solubilized using HCL 6N and quantitatively transferred into volumetric flask and complete to 100 ml using HCL (6N). Atomic spectrophotometer (parker Elmer) was used for determination of calcium [32]. Meanwhile phosphorus content in bone was determined colormetrically using uv- visible spectrophotometer [29].

2.9 Statically analysis

The obtained data were statistically analyzed using computerized SPSS version 16 (Statistical Package for the Social Sciences). Effects of different treatments were analyzed by one-way ANOVA (Analysis of variance) test using Duncan's multiple range test and $P \leq 0.05$ was used to indicate significance between different groups [33].

3 Results and discussion

3.1 The chemical composition of raw materials

Data in Table 1 displayed the chemical composition of raw materials used in manufacture of the functional beverages. Data presentenced are similar to Nasir et al. [34] for fig fruit, and with Ragab et al., [35] for kiwi fruit, as for permeate the results is in agreement with Rizk [36].

3.2 Chemical composition of functional beverages

The chemical composition of the prepared functional beverages (the total solids, ash, fat and protein contents) increased slightly in all treatments during the storage periods. Generally, there were significant differences ($P \leq 0.05$). The slight increase of total solids, acidity and ash during storage may be attributed to the loss of some moisture content and fermentation during the cold storage. And these findings are in agreement with other authors [16, 37, 38] that the acidity values of fermented beverages prepared with deferent LAB strains for fermentation gradually increased in the course of fermentation, as a result of the LAB metabolism of carbohydrates presented there in milk permeate.

3.3 Mineral content of the raw material and produced beverage

Mineral content of raw materials i.e. kiwi, fig as fruits and permeate were reported in Table 2. The data showed that Ca content was the highest mineral in all raw material being 52 ± 0.32, 58 ± 0.45 and 45 ± 0.12 mg/100 g respectively. For permeate, fig and kiwi. Kiwi fruit was characterized by the highest amount of phosphorus (40 ± 3.45 mg/100 g) which was about 3 times as that of fig (14 ± 2.75 mg/100 g) while permeate was found to be poorest material. On the other hand, permeate showed to superior in Mg content (140 ± 5.7 mg/100 g) which was about 8.2 and 4.7 fold as that of fig and kiwi. The data reveled that potassium found to be the predominate mineral in kiwi (332 ± 8.3 mg/100 g) followed by fig (232 ± 5.7 mg/100 g) and permeate (12.6 ± 2.4 mg/100 g). the other minerals showed a small amounts ranged between 0.01 and 3.0 ± 1.63 mg/100 g. the same minerals were determined after producing the beverage and refrigerated stored for 15 days (10 °C). The results showed that the mineral for kiwi beverage were ranked in

Table 1 Chemical composition of permeate and fig and kiwi

Parameter	Permeate	Fig	Kiwi
T.S	5.68 ± 0.12^{c}	78 ± 0.23^{b}	82 ± 0.54^{a}
Ash	0.26 ± 0.02^{c}	1.0 ± 0^{a}	0.6 ± 0.01^{b}
Fat	0.1 ± 0.03^{c}	0.3 ± 0.02^{b}	0.44 ± 0.03^{a}
Protein	0.22 ± 0.05^{c}	0.70 ± 0.03^{b}	0.9 ± 0.03^{a}
Total sugar	5.02 ± 0.15^{c}	17 ± 0.36^{a}	13 ± 0.27^{b}

a, b, c letter for significant differences between treatments at the same Colum

Standard division (±)

Table 2 Mineral composition of raw material and beverage

Minerals	Fe	Mg	K	Ca	P	Na
Material						
Raw material permeate	0.01 ± 0.02^{c}	140 ± 5.72^{a}	12.60 ± 2.38^{c}	52 ± 0.23^{b}	0.13 ± 1.9^{c}	1.5 ± 0.06^{b}
Kiwi	0.41 ± 0.03^{a}	30 ± 3.93^{b}	332 ± 8.27^{a}	45 ± 0.12^{c}	40 ± 3.45^{a}	5 ± 1.03^{a}
Fig	0.37 ± 0.02^{b}	17 ± 3.85^{c}	58 ± 0.45^{a}	58 ± 0.45^{a}	14 ± 2.75^{b}	1.0 ± 0.02^{c}
Fresh fermented beverages						
Kiwi	0.80 ± 0.12^{b}	6.43 ± 0.04^{c}	71.07 ± 1.73^{a}	66.30 ± 5.51^{a}	49.0 ± 4.78^{b}	76.91 ± 3.76^{a}
Fig	0.45 ± 0.01^{d}	7.02 ± 1.00^{b}	38.60 ± 2.01^{d}	37.74 ± 3.35^{c}	18.0 ± 2.34^{d}	23.60 ± 1.08^{c}
Cold storage beverage (15 days)						
Kiwi	1.47 ± 0.23^{a}	8.48 ± 1.20^{a}	69.40 ± 3.54^{b}	59.31 ± 2.98^{b}	53.5 ± 2.94^{a}	$37,93 \pm 4.55^{b}$
Fig	0.54 ± 0.0^{2c}	5.16 ± 0.23^{d}	46.08 ± 2.55^{c}	32.93 ± 3.42^{d}	22.3 ± 1.56^{c}	22.92 ± 1.56^{c}

a, b, c letter for significant differences between treatments at the same colum

Standard division (±)

the following sequence Na (76.91 ± 3.76) ˃ K (71.07 ± 1.73) ˃ Ca (66.3 ± 5.51) ˃ P (49.0 ± 4.78) ˃ Mg (6.43 ± 0.04) ˃ Fe (0.8 ± 0.12) mg/100 g. After refrigerated a slight fluctuation was found. Concerning the fig beverage, a decrease in Na, P, Ca, K and Fe by about 69.3%, 63.3%, 63.3%, 43.1%, 32.5% and 74.8% for the above mentioned minerals except that of Mg which increased by 8.14 compared with kiwi after production. Meanwhile, the fig beverage resulted in increase in P, K, and Fe and decrease in Na, Ca and Mg by about 19.3%, 16.3% and 53.2% and 28.8%, 12.7% and 25.7%, respectively after refrigeration compare with fresh one due to growth of probiotic bacteria and their ability to produce mineral and vitamin during fermentation. The obtained data were in the line with those of Atallah [16].

3.4 Lactose utilization by LAB

Data in Table 3 observed sugar fraction in functional beverage when fresh or during cold storage. In the beginning the results concluded that the lactic acid bacterial used *(Str. thermophiles, Lb. bulgaricus, Lb. acidophilus, bifibacteria bifidus*

Table 3 Sugar fraction of functional beverages when fresh and during cold storage (mg/100 gm)

Sugar fraction	T1	T2	T3	T4
Lactose	1183.95	156.005	2281.997	2013.631
Glucose	136.9917	88.42416	269.4807	1779.326
Galactose	1764.661	709.0599	1047.576	1269.973
Fructose	3165.641	2215.93	0	0
Sucrose	8685.013	3747.111	2998.642	15705.78
Inulin	6364.465	430.2493	221.9837	2085.731
Mannitol	84.29882	2404.244	10.07855	17531.22
Sorbitol	662.1732	5505.933	2513.949	4582.054
Xylose	334.0473	0	0	0
Maltose	551.3501	0	0	0
Ribose	6990.498	1421.971	1405.827	2780.605
Glucuronic	0	0	20668.22	0
Rhamnose	606.484	0	19345.18	0
Mannose	0	0	22496.96	0
Sorbose	895.3562	229.4134		0
Stachyose	0	360.4833	531.5206	1302.947
Arabinose	4014.272	4792.247	0	6418.122
Raffinose	0	0	3831.285	0

T1 (fresh functional beverage with Kiwi Fruit), T2 (functional beverage with kiwi after cold storage), T3 (fresh functional beverage with fig fresh), T4 (functional beverage with Fig after cold storage)

utilized lactose in functional beverage especially lactose contents in permeate to convert him to glucose and lactic and organic acid. In T1(KF) lactose content was 1183.95 mg/100 gm then at the end of storage period (15 days) it become 156.005 mg/100 gm that concluded LAB exhausted most of lactose which excites in permeate. In T2 (FF) lactose content was 2281.997 mg/100 gm after storage period it becomes 2013.631 m/100 gm. This results indicated that the growth of LAC and its activity in permeate kiwi fermented beverage was higher than in permeate fig fermented beverage. The results were in agreement with those authors Hossein et al. [39] stated that during the fermentation of milk permeate the LAB starter cultures showed post-acidification, thus with the concomitant reduction of pH and increase of TTA. Atallah [16] reported that the total carbohydrate of the prepared functional beverages significantly decreased ($P \leq 0.05$) during storage at ~ 4 °C up to 30 days. Also, Zokaityte et al. [38] added that as a matter of fact, the main sugar in milk permeate was lactose, which was fermented by LAB. All LAB grew well in MP and LAB strain exhibited a significant ($P \leq 0.05$) influence on galactobiose and galactotriose synthesis in the fermentable MP substrate.

3.5 Total sugar fraction in functional beverage (mg/100 ml)

Sugar fraction in T1 included: sucrose$^{>}$ ribose$^{>}$ arabinose$^{>}$ fructose $^{>}$ galactose at the end of storage period it becomes sorbitol$^{>}$ arabinose $^{>}$sucrose $^{>}$ribose $^{>}$ galactose. This results indicated that there is degradation in poly saccharide during cold storage and the activity of probiotic bacteria to utilization of polysaccharide for her growth and fermentation.

Sugar fraction in T2 mannose$^{>}$ glucuronic$^{>}$ rhmanose$^{>}$ arabinose at the end of storage period mannitol $^{>}$ arabinose$^{>}$ sorbitol$^{>}$ inulin. The predominant sugars of kiwi polysaccharides reported are Ara, Gal and Glc, although the molar ratios of the simple sugar composition [40].

3.6 Antioxidant activity of the produced beverage

Many fruits contained bioactive component which have many beneficial effect on bone health namely flavonoids and phenols. As mentioned in methods section, antioxidants were determined by two methods, i.e. DPPH and ABTS. The obtained resulted in Table 4 reveled that DPPH resulted in 69.19 ± 1.56%, 87.01 ± 1.98% for T1, T2. After storage period it was 68.95 ± 1.52% and 44.88 ± 1.04% for T3 and T4 respectively. Meanwhile, using ABTS method, resulted in 32.6 ± 1.10, 39.07 ± 0.98, 36.666 ± 0.14 and 41.99 ± 0.95% for T1, T2, T3 and T4 respectively. It worth to mention that DPPH and ABTS resulted showed increase in antioxidant activity of kiwi beverage due to refrigeration while it decreases for fig beverage. It was shown that fig fermented beverage contains total phenols and flavonoid more than kiwi beverage with significant differences in fresh samples was 17.32, 16.89 mg/100 g as Gallic acid and 459.5, 156.5 mg/100 g as catachin. During refrigerated storage, a gradual decrease in all treatments was found which was 16.48, 15.72 mg/100 g as Gallic acid, 265.75, 132.12 mg/100 g as catachin for fig and kiwi respectively. In this respect, Atallah [16] and Rizk [36] whom reported that the biologically active components and antioxidant activity were significantly decreased ($P \leq 0.05$) during refrigeration due to their low stability. The antioxidant reacts with free radical which produced by aerial oxygen and depleted. So the concentration of phenols, flavonoids and ascorbic acid decreased during storage, even though stored at refrigerator, which was in parallel with the present results.

Table 4 Antioxidant activity of produced beverage

Treatments	DPPH %	ABTS %	Total phenols (mg/100 g)	Total flavonoids (mg/100 g)
T1	69.19 ± 1.56^{b}	32.60 ± 1.10^{d}	16.89 ± 0.12^{b}	156.55 ± 0.42^{c}
T2	87.01 ± 1.98^{a}	39.07 ± 0.98^{b}	15.72 ± 0.18^{c}	132.12 ± 0.85^{d}
T3	68.95 ± 1.52^{b}	36.66 ± 0.14^{c}	17.32 ± 0.22^{a}	459.56 ± 0.35^{a}
T4	44.88 ± 1.04^{c}	41.99 ± 0.59^{a}	16.48 ± 0.14^{b}	265.75 ± 0.78^{b}

T1 (fresh functional beverage with Kiwi Fruit), T2 (functional beverage with kiwi after cold storage), T3 (fresh functional beverage with fig fresh), T4 (functional beverage with Fig after cold storage)

a, b, c letter for significant differences between treatments

Standard division (±)

Table 5 Microbiological evaluation of functional beverage

Treatments	T.C X10^2	M&Y	Coliform	LAB X 10^6
T1	$16^c \pm 0.242$	ND	ND	$20^c \pm 2.756$
T2	$30^a \pm 0.132$	ND	ND	$60^a \pm 3.156$
T3	$30^a \pm 0.122$	ND	ND	$15^d \pm 3.043$
T4	$25^b \pm 0.351$	ND	ND	$45^b \pm 4.235$

T1 (fresh functional beverage with Kiwi Fruit), T2 (functional beverage with kiwi after cold storage), T3 (fresh functional beverage with Fig Fresh), T4 (functional beverage with Fig after cold storage)

a, b, c letter for significant differences between treatments

Standard division (±)

3.7 Microbiological evaluation of produced beverage

Table 5 showed the microbial counts of the produced beverage. The data reveled that an increase in lactic acid bacteria from 20 ± 2.7 for fresh kiwi beverage to 60 ± 3.156 cfu/ml for the refrigerated ones this effect is related for the presence of dietary fiber in fig and kiwi fruits that stimulate and help probiotic growth. Similar trend was found due to fig beverage (15 ± 3.0 vs 45 ± 4.23 cfu/ml). On the other hand, Kiwi Fresh beverage, total count decreased from 16 ± 0.24 to 30 ± 0.13 which was about two fold as that of fresh ones. Meanwhile showed total count bacteria (T.C) of fig beverage resulted in 30 ± 0.12 for fresh decreased to 25 ± 0.35 for refrigerated ones. It good to mentioned that mold and yeast and coliform not detected. This can be attributed to the hygienic conditions granted during process and storage. The obtained results are in accordance with Rizk [36].

3.8 Effect of addition of produced functional beverages on the biological parameters

3.8.1 Weight gain, feed intake and feed efficiency ratio

The mean values of body weight gain (BEG), feed intake (FI), and feed efficiency ratio (FER) of the osteoporosis rats fed on the replacement of basal diet with 20, 30, and 40% of either kiwi or fig beverage was reported in Table 6. All treated groups resulted in decrease in weight gain compared with the negative control. Similar trend was found concerning feed consumption which affected the decrease in weight gain. On the other hand, feed efficiency rates ranged between 0.128 and 0.415. The major feed efficiency ratio was found in the positive control (0.415). The results can be attributed to the satiating effect of fruits fiber content or due to the presence of permeate in highly amounts which leads to decrease feed intake and parallel weight gain as mentioned by Smith [41]. It is worth mentioning that treatments with beverage increased weight gain, feed intake and feed efficiency ratio compared with positive

Table 6 Effect of different levels Functional fresh beverages (Permeate: fig or kiwi, 1:1) of diet on body weight gain, feed intake, and feed efficiency ratio of rats with osteoporosis

Groups	Weight gain (g/day)	Feed intake (g/day)	Feed Efficiency Ratio (FER)
(G1)	$65.20^a \pm 8.62$	$14.56^a \pm 2.64$	$0.224^a \pm 0.035$
(G2)	$17.33^f \pm 2.65$	7.21 ± 2.05	$0.415^c \pm 0.102$
(G3)	$34.97^e \pm 4.56$	$7.76^{de} \pm 0.62$	$0.218^a \pm 0.030$
(G4)	$48.29^{bc} \pm 6.05$	$10.89^{bc} \pm 1.07$	$0.226^{ab} \pm 0.024$
(G5)	$42.19^{cde} \pm 7.65$	$9.44^{cde} \pm 2.54$	$0.221^a \pm 0.020$
(G6)	$35.84^{de} \pm 4.87$	$10.08^{cd} \pm 2.99$	$0.287^{ab} \pm 0.075$
(G7)	$53.02^b \pm 5.78$	$12.89^{ab} \pm 0.88$	$0.293^b \pm 0.032$
(G8)	$44.28^{cd} \pm 6.07$	$11.56^{bc} \pm 0.54$	$0.220^a \pm 0.021$
LSD	8.667	2.808	0.069

Permeate mixed with fig or kiwi at ratio 1:1 and replaced with 20, 30, and 40% of rat diet

Each value in the Table is the average of 4 rats from each group and followed by ± standard deviation

Means at the same column bearing different superscript letters are different significantly ($P < 0.05$)

Table 7 Effect of different levels Functional fresh beverages (Permeate: fig or kiwi, 1:1) of diet on ash, calcium, and phosphorous contents in femur bone of rats with osteoporosis

Groups	Ash/bone (g)	Calcium /ash (mg/g)	Phosphorous/ash (mg/g(
(G1)	$0.78^{a} \pm 0.02$	$11.3^{a} \pm 2.2$	$7.3^{a} \pm 1.33$
(G2)	$0.55^{c} \pm 0.08$	$8.11^{d} \pm 1.76$	$6.41^{b} \pm 1.21$
(G3)	$0.6^{c} \pm 0.04$	$9.2^{cd} \pm 2.12$	$6.62^{ab} \pm 0.99$
G4)	$0.7^{b} \pm 0.12$	$10.5^{ab} \pm 0.92$	$7.06^{ab} \pm 0.23$
G5)	$0.62^{c} \pm 0.11$	$9.71^{bc} \pm 3.11$	$6.94^{ab} \pm 0.87$
(G6)	$0.7^{b} \pm 0.04$	$9.63^{bc} \pm 1.72$	$6.62^{ab} \pm 0.67$
(G7)	$0.74^{ab} \pm 0.03$	$10.7^{ab} \pm 1.67$	$7.08^{ab} \pm 1.07$
(G8)	$0.72^{ab} \pm 0.01$	$10.01^{bc} \pm 0.82$	$6.98^{ab} \pm 0.23$
LSD	0.073	1.279	0.732

Permeate mixed with fig or kiwi at ratio 1:1 and replaced with 20, 30, and 40% of rat diet

Each value in the Table is the average of 4 rats from each group and followed by ± standard deviation

Means at the same column bearing different superscript letters are different significantly ($P < 0.05$)

Table 8 Effect of different levels of functional beverages with kiwi and fig on organs weight of rats with osteoporosis

Heart weight (g)	Kidney weight (g)	Liver weight (g)	Groups
(G1)	4.08 ± 0.08^{b}	0.43 ± 0.01^{b}	0.53 ± 0.05^{b}
(G2)	4.48 ± 0.23^{a}	0.56 ± 0.01^{a}	0.74 ± 0.04^{a}
(G3)	4.32 ± 0.08^{a}	0.54 ± 0.06^{a}	0.70 ± 0.05^{a}
(G4)	4.22 ± 0.04^{b}	0.46 ± 0.01^{b}	0.55 ± 0.08^{b}
(G5)	4.24 ± 0.20^{a}	0.50 ± 0.07^{a}	0.66 ± 0.07^{a}
(G6)	4.34 ± 0.08^{a}	0.53 ± 0.11^{a}	0.65 ± 0.06^{a}
(G7)	4.04 ± 0.13^{b}	0.44 ± 0.01^{b}	0.54 ± 0.08^{b}
(G8)	4.28 ± 0.08^{a}	0.49 ± 0.03^{a}	0.60 ± 0.05^{a}
LSD	0.24	0.09	0.18

Each value in the Table is the average of 4 rats from each group and followed by ± standard deviation

Means at the same column bearing different superscript letters are different significantly ($P < 0.05$)

control because the functional beverage contains a lot of nutrients like carbohydrate, protein, mineral and vitamin beside probiotic bacteria that stimulate the gut function and digestion.

3.8.2 Effect of different level of produced functional beverages on the calcium and phosphorus contents of osteoporotic femur bone

From the results in Table 7, it could be found that the ash content ranged from 0.55 to 0.78 g/100 g bone. The major amount was resulted in negative control (0.78 g/100 g) while the minimum amount found in positive group. Regarding calcium and phosphorus in negative control resulted in maximum amounts being 11.3 ± 2.2 and 7.3 ± 1.33 mg/g ash, respectively. The results showed that Ca and P in all treatments are near to G- and higher than positive control. The treatment with 30% fig beverage, 20, 30 and 40% kiwi beverage were the best treatments. The obtained results confirmed by the mineral content of fig and kiwi and the produced beverage using permeate (Table 1). In this respectively, Lobo- Alexander et al. [42] and Contreras- Padilla et al. [43] reported that fig dietary fiber composition can influences short chain fatty acids formation through fiber fermentation cased colonic bacterial in large intestine. The short chain fatty acids decrease the intestinal pH which intern dissolve insoluble mineral salts particularly Ca, Mg and which increasing their absorption. Kiwi exceeds most other fruits in its content of key micronutrients including K, P, Mg, Fe and folate. It is also contained exceedingly high level of ascorbic acid which increase the bioavailability of nonheme Fe and Ca [44].

3.8.3 Effect of different levels of produced functional beverage on the organs weight

From the results in Table 8, it could be noticed that all treatments affected the organ weight which showed an increase that of negative control. The increase in organs weight may be due to fruit and permeate in produced functional beverages which contained minerals in organic structure which help the maintenance of bone mineral density (BMD) and prevent internal organs from damage [43, 44].

3.8.4 Effect of different levels of produced functional beverages on serum Ca, PTH, Protein and Vit. D of osteoporotic rats

Table 9 exhibits serum Ca, P, Protein, Vit. D and parathyroid hormone content of the osteoporotic rats treated with produced beverage. Serum Ca content ranged between 9.3 ± 0.02 mg/dl for the negative control to 14.56 ± 0.23 mg/dl for the positive one significantly. P increased also and ranged from 3.5 ± 0.94 to 4.5 mg/dl with nonsignificat differences between treatments. On the contrary, PTH, Protein and Vit. D resulted in significant decrease than these of negative control. Worthy, treatments cased on slight increase concerning all treatments compared with positive control. The most effective formulas were G3 and G6 for Ca, G4 and G7 for PTH, G3 and G6 for P, G4 and G7 for protein and G4 and G7 for Vit. D., respectively. Consumption of the phytoestrogen and is flavones precursors found in kiwi, metabolic conversion occurs in the gastrointestinal tract resulting in the formation of heterocyclic phenols that are similar to estrogen structure. As steroidal estrogen is use in preventing osteoporosis [45]. *Ficus carica* was chosen for dietary source of Ca. fig has also been reported to modulate bone remodeling. The mechanism of bone remodeling is composed of balance between bone resorption phase regulated by osteoclast and bone formation phase regulated by osteoblast [46]. Fig can act as potent inhibitor of osteoclastogenesis in receptor activator of nuclear factor kappa- B ligand (RAN KL) pathway of relates expression of osteoblast specific genes such as bone morphogenetic protein 2 (BMP-2), osteoprotegerin (OPG) and osteocalcin (OCN) [46].

3.8.5 Radiography of bones by X-ray

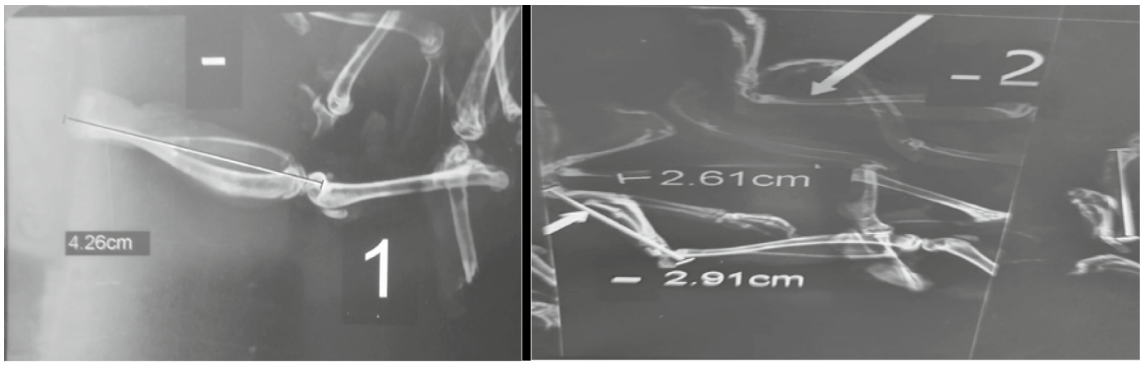
The positive control group recorded Subchondral sclerosis and the emergence of common space (Narrow joint space) compared to negative control group with significant differences. Where the lowest incidence was recorded with 2 followed by 4. The bone composition was significantly altered by drugs: the mineralization degree decreased as period increased. Histomorphometry of the growth plates of lead-exposed rats shows defective remodeling, altered growth plate thickness due to the loss of proliferating cells, and disorganization of the growth plate architecture. Our study was concluded in Fig. 2 and found that histomorphometric measures of bone density (i.e., bone volume, trabecular number, and trabecular thickness) were lower in positive control rats than treatment groups. Positive control rats had lower DEXA-based BMD than normal control group. Osteoporosis defined by decreasing bone density can be appreciated by decreased loss of bony trabeculae in the early stages in radiography. Bones like the vertebra, long bones (proximal femur), calcaneus and tubular bones are usually looked at for evidence of osteoporosis. Figs are a good source of both

Table 9 Effect of different levels of produced functional beverages on serum calcium, phosphorous, protein, Vit. D and PTH of rats with osteoporosis

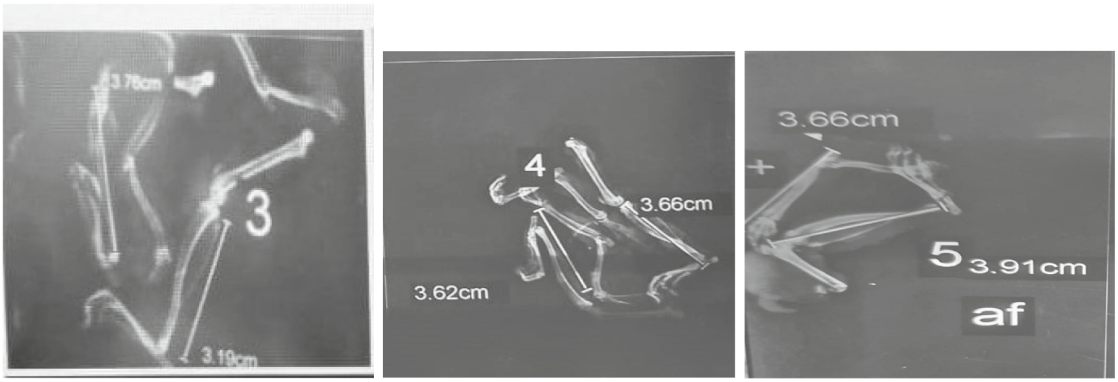
Groups	Serum profile				
	Serum Ca (mg/dl)	PTH (parathyroid hormone (pg/ml)	P (mg/dl)	Protein (g/dl)	Vit.D (ng/ml)
(G1)	9.31 ± 0.026^{d}	22.43 ± 0.21^{a}	3.5 ± 0.94^{b}	6.59 ± 1.97^{a}	30 ± 0.54^{a}
(G2)	14.56 ± 0.23^{a}	12.65 ± 0.66^{e}	4.5 ± 0.48^{a}	4.734 ± 0.78^{b}	13.55 ± 0.35^{d}
(G3)	13.76 ± 0.43^{a}	14.56 ± 0.60^{c}	4.43 ± 0.29^{a}	4.95 ± 0.96^{b}	17.87 ± 0.21^{c}
(G4)	11.54 ± 1.21^{c}	18.45 ± 0.76^{b}	4.10 ± 0.44^{a}	5.21 ± 0.66^{b}	20.87 ± 0.32^{b}
(G5)	12.78 ± 1.08^{b}	15.43 ± 0.88^{c}	4.22 ± 0.08^{a}	4.45 ± 0.45^{b}	18.98 ± 1.97^{c}
(G6)	13.98 ± 0.89^{a}	14.11 ± 2.75^{d}	4.41 ± 0.06^{a}	4.87 ± 0.32^{b}	17.44 ± 2.45^{c}
(G7)	11.09 ± 0.65^{c}	18.87 ± 2.56^{b}	4.01 ± 0.24^{a}	5.34 ± 0.65^{b}	23.76 ± 1.78^{b}
(G8)	13.02 ± 0.42^{b}	14.93 ± 1.03^{c}	4.19 ± 0.76^{a}	4.66 ± 0.27^{b}	17.22 ± 2.67^{c}
LSD	1.09	1.06	0.76	0.98	3.23

Each value in the table is the average of 4 rats from each group and followed by ± standard deviation

Means in the same column bearing different superscript letters are different significantly ($P < 0.05$)

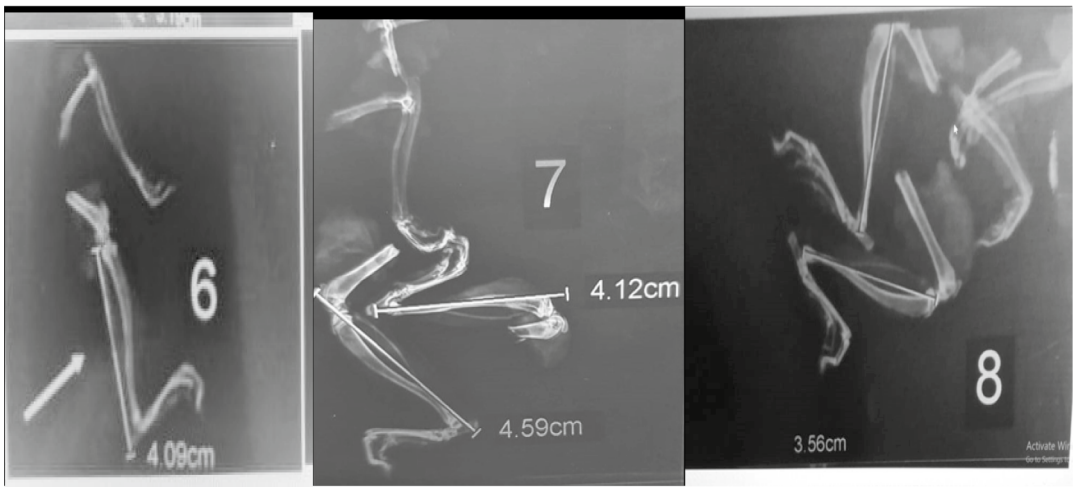


(a) Normal control group (b) Positive control



(c) (G3) (d) (G4) (e) (G5)

Treated with produced functional beverage containing fig



(f) (G6) (g) (G7) (h) (G8)

Treated with produced functional **beverage containing kiwi**

Fig. 2 longitudinal section of femur bone corticocortocoid admistiratel rats: **a** and **b** control negative and positive **c**, **d**, **e** treated with beverage containing fig and permeate with replacement ratio of 20, 30 and 40%, respectively. **f**, **g** and **h** treated with beverage containing kiwi and permeate with replacement ratio of 20, 30 and 40%, respectively

calcium and potassium. These minerals can work together to improve bone density, which can, in turn, prevent conditions like osteoporosis [46, 47]. They suggested that a potassium-rich diet, in particular, can improve bone health and reduce bone turnover. Kiwi contained vitamin C which improve the absorption of calcium and vitamin K can not only

increase bone mineral density in osteoporotic people but also actually reduce fracture rates [45]. The effect of vitamin K on bone collagen content may be important for bone quality. The material properties of bone, degree of mineralization, and micro damage accumulation are all influenced by collagen cross-link formation. In particular, the Type-I collagen fibers wired with crosslinks fibers form the framework that binds matrix proteins and mineral crystals. If the arrangement of collagen fibers is altered and the mineral crystal remains immature, these changes in material properties can cause an impairment of bone elasticity [48].

4 Conclusion

From the obtained data, fruits such as kiwi and fig conserved a rich source of mineral such as ca, P and Mg. when these fruits mixed with fermented permeate to make a fermented beverage, it can be used as a thereby diet for humans suffered from osteoporosis.

Author contributions All authors read and approved the final manuscript. HA: methodology, biology, writing, original draft DF: methodology, prepared beverage, microbiology, original draft, writing IZ: chemical analysis, biology, original draft, writing. All authors review the manuscript.

Funding The research paper supported and funding by agriculture Research Center, Egypt.

Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate All experimental animal conduct in this study was approved by the National Institute of the healthy guide for laboratory animal care and used NIH (publication No. 8023 revised 1978 and updated 2011). Animal experiments strictly complied with the legal requirements or guidelines in the country and/or state or province for the care and use of animals including [Arrival guideline. 2.0 updated in July 2020].

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

References

1. Lorentzon M, Cummings SR. Osteoporosis: the evolution of a diagnosis. J Intern Med. 2015;277(6):650–61. https://doi.org/10.1111/joim.12369.
2. Kanis JA, Melton LJ 3rd, Christiansen C. The diagnosis of osteoporosis. J Bone Miner Res. 1994;9(8):1137–41. https://doi.org/10.1002/jbmr.5650090802.
3. WHO. Guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report; 2002. www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf/.
4. Bartl C, Bartl P. Epidemiology of osteoporotic fractures. In: Bartl R, Bartl C, editors. The osteoporosis manual. Cham: Springer Nature Switzerland AG; 2019. p. 231.
5. Slupski W, Jawien P, Nowak B. Botanicals in postmenopausal osteoporosis. Nutrition. 2021;13(5):1609. https://doi.org/10.3390/nu13051609.
6. Brown EM. Control of parathyroid hormone secretion by its key physiological regulators. In: Bilezikian G, John P, editors. The parathyroid. 3rd ed. San Diego: Academic Press; 2015. p. 101–18.
7. Riggs BL, Parfitt AM. Drugs used to treat osteoporosis: the critical need for a uniform nomenclature based on their action on bone remodeling. J Bone Miner Res. 2015;20:177–84.

8. El-Nawawy MA, EL-malkey WE, Aumara E. Production and properties of antioxidative fermented probiotic beverages with natural fruits juice. Annals Agric Sci. 2009;54(1):121–35.
9. Menrad M, Husing B, Menrasd K, Reib T, Beerr-Borst S, Zenger C, Functional Food. A 37/2000 Bern: 2000: Schweizerischer Wissenschafts und-Technnologierat
10. Fitzpatrick JJ, Ahrens MI, Smith SW. Effect of manganese on lactobacillus casei fermentation to produce lactic acid from whey permeate. Process Biochem. 2001;36(7):671–5.
11. Bayoumi HA, Mohamed AG, Sheikh M, Farrag H. Effect of ultrafiltration permeate on the quality of chocolate milk. J Am Sci. 2001;7(7):609–15.
12. Hattem HE, Einin EHA, Mehanaan NM. Utilization of milk permeate in manufacture of sports drinks. J Agric Tech. 2011;7(5):1247–54.
13. Vinson JA, Proch J, Zubik L. Phenol antioxidant quantity and quality in foods: cocoa, dark chocolate, and milk chocolate. J Agric and Food Chemi. 1999;47(12):4821–4. https://doi.org/10.1021/jf990312p.
14. Abd-Eltawab S, Ebid W. Production and evaluation of stirred symbiotic fermented milk fortified with fig fruit (Ficus carica L.). Egy J of Food Sci. 2019;47(2):201–12.
15. Aumara I E M. The use of bifidobacteria in manufacture of some dairy products. M. Sc. Thesis, Fac of Agric. Ain Shams Univ, Egypt. 2000
16. Atallah AA. Development of new functional beverages from milk permeate using some probiotic bacteria and fruits pulp. Egyptian J Dairy Sci. 2015;43(2):147–58.
17. AOAC. Official methods of analysis. 17th ed. Washington: Association of Official Analytical Chemists Inc; 2000.
18. IDF, Milk and milk products: Enumeration of Yeasts and Moulds (Colony count technique at 25°C). IDF: 94B 2003
19. APHA. Standard methods for the examination of dairy products. 16th ed. Washington: American Publ. Health Assoc Inc; 1992.
20. Ryan MP, Rea MC, Hill C, Ross RP. An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, Lacticin 3147. Appl Envirn Microbiol. 1996;62:612.
21. Liu SC, Lin JT, Wang CK, Chen HY, Yang DJ. Antioxidant properties of various solvent extracts from lychee (*Litchi chinenesis Sonn*.) flowers. Food Chemi. 2009;114:577–81.
22. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolonization assay. Free Radic Biol Med. 1999;26:1231–7.
23. Chang YL, Lin JT, Lin HL, Liao PL, Wu PJ, Yang DJ. Phenolic compositions and antioxidant properties of leaves of eight persimmon varieties harvested in different periods. Food Chemi. 2019;289:74–83. https://doi.org/10.1016/j.foodchem.2019.03.048.
24. Zielinski AAF, Braga CM, Demiate IM, Beltrame FL, Nogueria A, Wosiacki G. Development and optimization of HPLC- RI method for the determination of major sugars in apple juice and evaluation of the effect of the ripening stage. Food sci tech Campinas. 2014;34(1):38–43.
25. AIN. Purified diet for laboratory rodent. Final report ADHOC writing diet. J Nutr. 1993;123:1939–51.
26. Schemer S. The blood morphology of laboratory animals. Philadelphia: F. A. Davies Co; 1967. p. 359.
27. Chapman DG, Gastilla R, Campbell TA. Evaluation of protein in food. Method for the determination of protein efficiency ratio. Can J Biochem Physio. 1950;37:679–86.
28. Ismail AA, Stley P, Burr WA, Cawood M, Short F, Wakelin K, Wheeler MJ. The quntitiative measurement of estrogen, progesterone and tester on. Ann Clin Biochem. 1986;23:113–34.
29. Weatherburn MW, Baker PJ, Logan JE. Serum calcium methodology— a Canadian assessment based on the application of reference method. Clin Biochem. 1982;15:222–9.
30. Goseki MS, Omi N, Yamamoto A, Oida S, Ezawa I, Sasaki S. Ovariectomy decrease osteogenic activity in rate bone. J of nutr sci and vit. 1996;42:55–67.
31. Arjmandi BH, Khalil DA, Hollis BW. Isoflavone, a synthetic phytoestrogen enhances intestestinal calcium transport in vitro. Calcif tissue int. 2000;67(3):225–9.
32. Wang H, Yang Z, Zhou B, Yan X, Wang J. Fluoride-induced thyroid dysfunction in rats: roles of dietary protein and calcium level. Toxicol Ind Health. 2009;25(1):49–57. https://doi.org/10.1177/0748233709102720.
33. SPSS, SPSS for Windows. Release 10. Standard Version. Copyright SPSS Inc. 1999.
34. Nasir A, Jaafar FI, Muhammad AU, Matazu KI, Yaradua AL, Usman A, Suleiman ZA, Sulaiman H, Nasir NM. Proximate and mineral composition of the fruit of *Ficus polita*. J Appl Life Sci Int. 2020;23(8):7–13. https://doi.org/10.9734/JALSI/2020/v23i830178.
35. Ragab SS, Khader SA, Abd Elhamed EKS. Nutritional and chemical, studies on kiwi (*Actinidia deliciosa*) fruits. J Home Econ. 2019;29(2):4.
36. Rizk AE. Study of production functional beverages of milk permeate fortified with fruit and herbs. Middle East J Appl Sci. 2016;6(1):155–61.
37. Hashmi SI, Kadam S, Kale RV. Studies on effect of different varieties of date palm paste incorporation on quality characteristics of yoghurt. Elec J Environ Agric Food Chem. 2011;10:2371.
38. Zokaityte E, Cernauskas D, Klupsaite D, Lele V, Starkute V, Zavistanaviciute P, Ruzauskas M, Gruzauskas R, Juodeikiene G, Rocha JM, Bliznikas S, Viskelis P, Ruibys R, Bartkiene E. Bioconversion of milk permeate with selected lactic acid bacteria strains and apple by-products into beverages with antimicrobial properties and enriched with Galactooligosaccharides. Microorganisms. 2020;8:1182–204. https://doi.org/10.3390/microorganisms8081182.
39. Hossein MM, Ehsandoost E, Gholami P, Moshiri H, Nazemi M. Effect of permeate on growth and survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* for production of probiotic nutritive beverages. World Appl Sci J. 2012;18:1389–93.
40. Fan H, Mazza G, Liao X. Purification, composition and antioxidant activity of polysaccharides from wolfberry, cherry, kiwi and cranberry fruits. Croat J Food Sci Technol. 2010;2(1):9–17.
41. Smith ST. Sensory characteristics of whey and whey permeate from different sources and the use of permeates as salt substitutes. Master thesis, food science, North Carolina State University, Raleigh; 2016.
42. Lobo-Alexander R, Colli C, Filisetti MC. Fructooligosaccharides improve bone mass and biomechanical properties in rats. Nutr Res. 2016;26:413–20.
43. Contreras-Padilla M, Rivera-Muñoz EM, Gutiérrez-Cortez E, Del-Real-López A, Rodríguez-García ME. Characterization of crystalline structures in *Ficus carica*. J Biol Phys. 2015;41:99–112.
44. Drewnowski A. The nutrient rich foods index helps to identify healthy, affordable foods. Am J Clin Nutr. 2010;91(4):1095S-1101S. https://doi.org/10.3945/ajcn.2010.28450d.

45. Hafezi F, Rad HE, Naghibzadeh B, Nouhi A, Naghibzadeh G. *Actinidia deliciosa* (kiwifruit), a new drug for enzymatic debridement of acute burn wounds. Burns. 2010;36(3):352–5. https://doi.org/10.1016/j.burns.2009.04.02.
46. Mawa S, Husain K, Jantan I. *Ficus carica L*. (Moraceae): phytochemistry, traditional uses and biological activities. Evid Based Complement Alternat Med. 2013;2013:1–8. https://doi.org/10.1155/2013/974256.
47. Link TM, Guglielmi G, van Kuijk C, Adams JE. Radiologic assessment of osteoporotic vertebral fractures: diagnostic and prognostic implications. Eur Radiol. 2005;15(8):1521–32.
48. Azuma K, Inoue S. Multiple modes of vitamin K Actions in aging-related musculoskeletal disorders. Int J Mol Sci. 2019;20(11):2844. https://doi.org/10.3390/ijms20112844.