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Research Article

Physiological, Histological, and Molecular Evaluation of Enriched Flavored Pasteurized Milk with Tamarind Kernel Powder on Rats Model

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Abstract:

Tamarind Kernel Powder (TKP), *Tamarindus indica* L, as an enrichment addition to pasteurized milk in different concentrations was evaluated. The evaluation procedure involved physiochemical, microbial, and sensory properties of flavored pasteurized milk as well as an in vivo experiment using albino model rats (*Sprague dawley*). In vivo experiment involved the determination of associated changes in physiological, Biochemical, and histological parameters and changes in the transcription patterns of some stress and metabolic correlated genes: Copper-Zinc/superoxide dismutase, (*SOD-1, SOD-3*), Glutathion peroxidase (*GPX*), Glutathion transferase (*GST*), Heme oxygenase (*HO*) and one of proteinase inhibitors; Alpha-2-Macroglobulin (*α 2-MG*) with administration of pasteurized and TKP enriched milk. The obtained results concluded that adding TKP improved the antioxidant properties of pasteurized milk which positively improved pasteurized milk's anti-oxidant properties, which positively affected its physiochemical and sensory properties. And it also caused an increase in the protein content of enriched milk treatments. Additionally, TKP led to a decline in the number of viable total bacteria and yeast & mold compared with control milk treatment. *In vivo* experiments also revealed the good impact of TKP addition on body weight as well as liver and kidney function. These improvements were associated with the capability of TKP to regulate the transcription of some stress and metabolic correlated genes. Thus pointing to their potential application as an immune modulator at cellular and genetic levels in some therapeutic products.

1. Introduction

Milk is a dairy product that combines significant natural compounds such as protein, fat, carbohydrates, vitamins, minerals, and other compounds that make milk a complete diet (Haug et al., 2007). As a result of these components, milk is considered an ideal culture medium for micro-organisms to grow and increase (Soomro et al., 2002). Various temperature and heat treatments were used to make milk and dairy products safe from food-borne diseases and to remove pathogenic organisms which led to raising the shelf-life of dairy products (Dash, et al., 2022). Pasteurized milk has different physio-chemical, and microbial characteristics which reflect its high quality and keep the natural value of milk (Woldemariam and Asres, 2017).

Additives have been broadly acclimated in aliment industries to advance concrete properties such as smell, color, and texture and prevent the aliment artifact from microbial attacks. Nowadays, aliment additives are acclimated to access the flavor, texture, emulsification, and stabilization of the aliment at the time of accomplishment and processing of the artifact at the automated level. An accretion can be accustomed or synthetic, depending on the compound's actinic blueprint and complication. Additives can be categorized as preservatives, colorants, antioxidants, flavoring agents, thickeners, humectants, emulsifying agents, antifoaming agents, and stabilizers, which are about acclimated in dairy-based products (Harshika et al., 2022).

To safeguard against oxidative damage and extend the longevity of dairy supplement products, the inclusion of antioxidants is necessary. (Abdel-Hameed et al., 2014). Natural antioxidants from plant sources could control the generation of ROS in food products and avoid their hazardous effects such as liver damage and various carcinogenesis (Meenakshi et al., 2009). Induction of antioxidant activity through natural agents has been verified as a promising strategy to face multistage carcinogenesis in either experimental animals or clinical trials (Sheweita, 2000). Finding more potent inducers of antioxidants and other protection pathways is considered to improve food products to mitigate the increase in oxidative stress sources and their hazardous effects.

Tamarindus indica L. has nutritional and high health promotion compounds to be used as food additives in different foods (Taksene and Belay, 2024). It contains polyphenol and flavonoid compounds responsible for high ROS scavenging activity (Soong et al., 2004; Bonin et al., 2023). These Phenolic compounds have various health benefits for cardiovascular and immunological health as well as their precise roles as anticancer and anti-microbial agents to deal with numerous human health hazards (Assiri et al., 2023).

Tamarind seed kernels powder (TKP) have various

phenolic content such as epicatechin, 3,4-dihydroxyphenyl acetate, methyl 3,4-dihydroxybenzoate, and 2-hydroxy-3', 4'-dihydroxyacetophenone (Tsuda et al., 2004; Natukunda et al., 2016). Antioxidant components proliferate the shelf-life of food products, improve fat stability, and promote sensory and nutritional quality (Taksene and Belay, 2024). Phenolic compounds in TKP also have many biological effects that enhance health (Shams et al., 2022). Lessening the oxidative destruction associated with aging, mutagenesis and carcinogenesis in living systems is a significant effect of the addition of TKP in food products (Sudjaroen et al., 2005; Taksene and Belay, 2024).

Due to the expected valuable implications of food polyphenols for human health, great interest was paid to it as an essential part of diets consumed by humans or animals. Polyphenols or phenolic compounds of TKP react with reactive oxygen species (ROS), resulting in great ROS scavenging activity leading to decreasing the risk of cancer and various diseases (Assiri et al., 2023). Phenolic compounds display great chemo-protective properties in vitro and modulate antioxidant activity to alleviate oxidative cardiac disorders (Vulapalli et al., 2002; Venardos and Kaye, 2007). It can modulate the immune system (Patil et al., 2023) and impact gene transcription patterns (Rathee et al., 2024). Regulation of antioxidant and metabolic activity is documented as beneficial effects of food supplements in vivo (Zhang et al., 2024).

Therefore, the present investigation was carried out to illustrate the potential impact of Tamarind kernel powder (TKP) as an enrichment additive on physio-chemical characteristics, and sensory properties of flavored pasteurized milk. Moreover, to illustrate the physiological and histological changes associated with its use on the albino model rats (*Sprague dawley*) considering its effect on the transcription pattern of some stress and metabolic-related genes to understand the underlying mechanism for its impact.

2. Materials and Methods

This study was approved by the Animal Experiment Ethics Committee, Faculty of Agricultural Tanta University.

2.1 Materials

Fresh cow's milk (moisture 83.45%, fat 2.5%, total protein 2.6%, ash 0.77, total solid 11.55%, pH 6.8, and acidity 0.18%) was obtained from a local farm in Tanta, Egypt. Tamarind kernel powder (TKP) (*Tamarindus indica* L.) was obtained as a commercial product from a local market. Carboxy Methyl Cellulose (CMC) utilized in the research was obtained from Misr Food Additives Company (MIFAD), Egypt. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich (Germany). The chemicals used in the physiochemical and sensory determination were obtained from El-Naser Pharmaceutical Chemicals, Egypt.

2.2. Methods

2.2.1. Preparation of Flavored Pasteurized Milk

Fresh cow's milk was heat treated at 65 °C for 30 min low-temperature long time (LTLT) according to Lewis and Deeth (2009)**,** and then divided into four treatments. Control pasteurized milk without any additives Control (Milk), the second portion was milk fortified with 0.2% (w/v) Tamarind kernel powder (TKP) then pasteurized (TKP-1), and the third portion was milk fortified with 0.3% (w/v) Tamarind kernel powder then pasteurized (TKP-2), Tamarind kernel powder (TKP-3) was added to the fourth portion of milk at 0.4% (w/v) then pasteurized. All treatments were cooled to 10 °C then 7% sugar and 0.02 % carboxymethyl cellulose were added for stabilization, and chocolate essence was added.

Each milk treatment was packed in 100 ml sterilized plastic bottles. Bottles of milk treatments were kept at 4±1 °C and analyzed at 0, 7, and 14 days of storing period sterilized 100 ml plastic bottles. The bottles were kept at 4±1 °C and analyzed at 0, 7, and 14 days of storage.

2.2.2. Antioxidant activity

Antioxidant activity of extract DPPH free radical scavenging activity stock solution of DPPH (33 mg/l) was prepared in methanol. Stock solution (5 ml) was added to 1 ml of solution of TKP at different dilutions. After 30 min, absorbance was measured spectrophotometrically at 517 nm and concentration was calculated from the standard calibration curve. Scavenging activity was expressed as the percentage inhibition computed using the following formula:

$$\text{Inhibition (\%)} = A_0 - A_1 / A_0 \times 100$$

Where A_0 = absorbance of the control and A_1 = absorbance of the extract as described by (Li et al., 2015).

2.2.3. Physio-chemical analysis

Moisture content, Total protein (TP), Fat, and titratable acidity as lactic acid were identified (A.0.A.C, 2005). pH values were determined with a pH meter (JENCO, model 1671, USA).

2.2.4. Microbiological analysis

2.2.4.1. Yeast and mold count

Yeast and mold enumeration was detected using Oxytetracycline Glucose Yeast Extract agar (O.G.Y.E agar, LAB, United Kingdom) following the procedure of (Difco, 1984). Suitable dilutions were prepared in peptone saline solution (0.85%) and then plated on oxytetracycline glucose yeast extract agar. Plates were incubated at 25°C for 5 days in aerobic conditions.

2.2.4.2. Total plate Count

A dilution of milk samples was transferred onto a sterile plate and Platt's agar media was poured following the procedure outlined in (Difco, 1984). Resulting colonies after plates incubation at 37°C for 48 hours were counted to determine the total bacterial count.

2.2.5 Sensory evaluation

The evaluation of the sensory characteristics of the flavored pasteurized milk primarily relies on the sensory evaluation method introduced by Fresh and stored

samples. The assessment was carried out by a panel of ten arbitrators who are experts in sensory arbitration according to (Kailasapathy, 2006).

2.2.6 HPLC Analysis of Tamarind Kernel Powder (TKP) Compounds

The aqueous extract of the TKP was used for detecting flavonoids and phenolic compounds using HPLC (Agilent Technologies 1100 series, Santa Clara, CA, USA) according to Kim et al., (2006). Three different wavelengths: 280, 320, and 360 nm were used for peak detection, and then compared with standards (El-Magd et al., 2022).

2.2.7. In vivo experiment

2.2.7.1. Animal treatments and collecting the sample

Forty-two male albino model rats, (*Sprague dawley*), with average body weight (170 ± 10 g) were retained in cages. A standard rat diet ad libitum with free access to water was applied. Experimental rats were kept at optimal laboratory conditions (12 h light/12 h dark and 23 ± 2 ºC) for twelve days before starting the experiment as an acclimatization period. Animals were allocated randomly to 6 groups (7 rat /group). In the control group (C), rats were given a diet without any additives, and in the second group (Milk), rats were given by stomach tube with pasteurized milk without any additives at a dose of 2 ml. TKP, rats group were treated with two ml of tamarind kernel powder aqueous solution (0.4 mgkg-1 bw). TKP-1, the rat's group was given 2 ml of pasteurized milk with 0.2%TKP. While TKP-2, the rats group was given 2 ml of pasteurized milk with 0.3% TKP. Rats were given two ml of pasteurized milk with 0.4% TKP (TKP-3). All animals in the experiment were given a 12-hour fast before ending the experiment and recording experimental parameters. Blood samples were collected from the hepatic portal vein. Under diethyl ether anaesthesia. The serum was separated using under-cooling centrifugation and then stored at -20 ºC until analysis. Liver and kidney functions were detected using Jenway spectrophotometer 6715 (Staffordshire, UK) following kit instructions (Amany et al., 2021). Organs were immediately removed and weighed. Samples of kidney and liver tissues of each treatment were frozen immediately in liquid nitrogen and then stored at -80 ºC for molecular analysis.

2.2.7.2. Physiological, biochemical and histological analysis

2.2.7.2.1 Body and organs weight

Throughout the experiment, the body weights of the rats were recorded weekly. The percentage of body weight gain (BWG %) was calculated according to Farag et al. (2022). At the end of the experiment, the weight of organs was detected for all tested animals, and the average of organ weight was calculated.

2.2.7.2.2 Hematological evaluation

Blood samples of each rat group were collected after anesthetizing the rats with diethyl ether at the end of the experiment for blood parameters determination. Rats were sacrificed with the approval of the ethical committee.

Blood samples were collected for hematological analysis in EDTA and plain tubes to determine the plasma lipid profile represented as triglycerides level, serum low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Also, liver enzymes: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and Alkaline phosphatase (ALP) as well as Kidney function tests (Creatinine, urea level and total protein) were detected.

2.2.7.2.3. Histopathology analysis

Histological examination of kidney tissue was carried out in fixed kidney specimens according to standard procedures (Mohamed et al., 2021; Vangaveti et al., 2021). Examining procedures were carried out for three samples/groups and as ten fields/slides (at 40×).

2.2.7.3. Analysis of gene expression

2.2.7.3.1. RNA extraction and cDNA synthesis

About 100 mg of frozen tissues at -80 ºC were used for extraction of total RNA using easy-spin TM [DNA free] Total RNA Extraction (iNtTRON Biotechnology, Korea) according to the manufactory procedures. The purity and concentration of extracted RNA were determined using a nano-drop spectrophotometer (BioDrop ULite, Wales, and England). For integrity check, extracted RNA was separated on 1% agarose gel electrophoresis. Samples with RNA purity of more than 1.9 were considered for gene expression analysis. One µg of extracted RNA was used for cDNA synthesis using the HiSenScriptT MRH cDNA synthesis Kit (iNTRON Biotechnology, Cat. No: 25087) according to the manufactory techniques.

2.2.7.3.2. Quantitative time real-time PCR analysis (qRT-PCR)

Changes in the transcript amount of selected genes were detected using quantitative real-time PCR (qRT-PCR). The reactions were out using SYPER green stain (PerfectStartTM Green qPCR Super Mix, Beijing, China) in 20 µL reaction volume used for qRT-PCR reactions. Step One PlusTM Time Real-Time PCR system (Applied BiosystemTM 4376600) was used for running the reactions. Gene's specific primers were designed for selected genes using available data for albino rats (*Sprague dawley*) at the National Center on Biotechnology Information (NCBI) using the online software of primer 3 and Blast (Table 1). The GAPDH gene (accession no. M17701.1) was used as a reference gene for relative expression calibration with control treatment for all reactions. The reaction was conducted using standard conditions with variflex option to allow multiple annealing temperatures on the same plate for different genes.

Table 1. Names, functions, accession numbers, the primer sequences, and annealing temperatures of used primes in the analysis of gene expression.

Gene	Gene function	Accession no. /Reference	Primer seq. 5' to 3'	Ta °C
SOD-1	Cu/Zn-superoxid-dismutase	XM_021185540	5- GCAGAAGGCAAGCGGTGAAC-3 5- TAGCAGGACAGCAGATGAGT-3	60
SOD-3	Cu/Zn-superoxid-dismutase	AH002084	5-AGGATTAACTGAAGGCGAGCAT-3 5- TCTACAGTTAGCAGGCCAGCAG-3	56
GPX	Glutathione peroxidase	AK_010999	5- CTCTCCGCGGTGGCACAGT-3 5- CCACCACCGGGTCGGACTTAC -3	60
GST	Glutathion transferase	L06047.1	5-GCTGGAGTGGAGTTTGAAGAA -3 5-GTCCTGACCACGTCAACATAG -3	55
HO	Heme oxygenase	Kant et al., 2015	5- AGAGTCCCTCACAGACAGAGTTT-3 3-5- CCTGCAGAGAGAAGGCTACATG	55
α 2-MG	proteinase inhibitor	Alkhedaide et al., 2017	5-GCTCCTGTCTGTTTCCTTAGTT-3 5-ATTGGCCTTTCGTGGTTTAG-3	55
GAPDH	Used as reference gene	M17701.1	5-AGATCCACAACGGATACATT -3 5-TCCCTCAAGATTGTCAGCAA -3	5

Calibrated value with the endogenous gene and control treatment as $2^{-\Delta\Delta ct}$ was used to calculate the relative expression (RQ) according to Livak and Schmittgen (2001). Three biological replicates for each cDNA sample were used to run the reaction.

2.2.8. Statistical analysis

For physiochemical and sensor analysis of enriched flavored milk, a completely randomized design (CRD) with three replicates per treatment was used in designing the experiment. Analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) was used as a post hoc analysis to compare means ($p< 0.05$). For the In-vivo experiment, The SPSS statistical software package for analysis of variance (ANOVA) and Duncan's test (SPSS 20 for Windows, SPSS INC., IBM, New York) was used.

3. Results and Discussions

3.1. Antioxidant activity of Tamarind kernel powder

The function of an antioxidant is to eliminate free radicals by providing hydrogen to a free radical causing a removal of the electron responsible for radical reactivity and converting it to a less reactive species (Abou–Elalla, 2009). DPPH radical scavenging activity indicated that TKP solution has a notable antioxidant activity (Figure 1). The antioxidant activity of tamarind kernel powder (TKP) measures the capacity of TKP to neutralize free radicals. The dose-dependent response observed in Figure 1 demonstrated a positive correlation between TKP concentration and % inhibition of DPPH radicals. The linear with an R2 value of 0.997. The IC_{50} value, calculated as 0.543 mg/ml, is the concentration at which TKP achieves 50% inhibition of DPPH radicals. This IC_{50} value suggests that TKP has a moderate antioxidant capacity, as lower IC_{50} values are generally indicative of stronger antioxidant effects. The findings support the potential of TKP as a natural antioxidant source, which could be beneficial for food fortification or nutraceutical applications where radical-scavenging activity is desired. The TKP's antioxidant activity revealed its polyphenol compounds such as pyrogallol catechol and other compounds. As illustrated as the concentration of tamarind powder increases the antioxidant activity increases. This result is in agreement with Khan et al. (2007) and El-Gindy et al. (2015).

3.2. HPLC analysis of TKP

HPLC analysis illustrated that TKP had various compounds, about 24 flavonoids and alkaloid compounds such as: Pyrogallol (24.28 mg/kg), Quinol, Gallic acid, 3-Hydroxytyrosol, Catechol (13.24 mg/kg), p-Hydroxy benzoic acid, Catechin, Chlorogenic, Vanillic acid, Caffeic acid, Syringic acid, p- oumaric acid, Benzoic acid, Ferulic acid, Rutin, Ellagic, o-Coumaric acid, Resvertol, Cinnamic acid, Quercitin, rosemarinic, Neringein, Myricetin, Kampherol as shown in Figure (2). It is worth noting that a lot of antioxidants have aromatic 1,2-diols (catechols) structure, which accounts for their easy oxidation (Ortega-Moo et al., 2016).

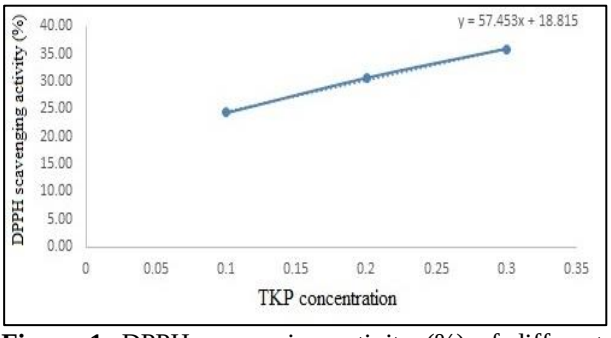

Figure 1. DPPH scavenging activity (%) of different concentrations of Tamarind kernel powder (TKP)

3.3. Physiochemical, microbial, and sensory properties of TKP flavored pasteurized milk

The basic compositions of flavored pasteurized milk treatments were determined in samples for 14 days.

3.3.1. Moisture content of pasteurized milk

Figure (3) revealed the effect of enriched flavored pasteurized milk with Tamarind kernel powder TKP on moisture. On observing the moisture content of flavored

pasteurized milk treatments, it could be concluded that there were significant differences in moisture content among all flavored pasteurized milk treatments at fresh and throughout the storage period ($p < 0.01$) as affected by TKP. The fresh control sample had the lowest moisture content. This might be due to the minimal incorporation of TKP with pasteurized milk. In contrast, (TKP-3) pasteurized milk with 0.4 % of Tamarind kernel powder had the highest moisture content after 14 days of storage compared with other treatments. The increased moisture of enriched milk samples might be due to the water-retention capacity of TKP's polysaccharides, which bind and hold water, leading to higher moisture content (Ajayi and Fadeyi, 2016).

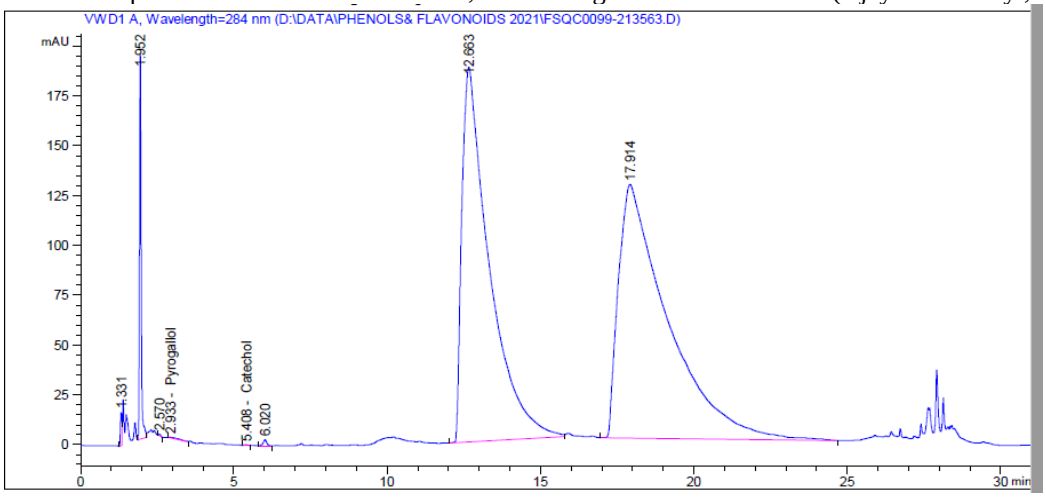

Figure 2. HPLC chromatograms of aqueous extract of Tamarind kernel powder (TKP) compounds

Also, the data reveals that the moisture content generally increases with the storage period for treatments TKP-1, TKP-2, and C, especially noticeable from fresh to 7 days and again from 7 days to 14 days. This trend indicates that longer storage periods may positively affect the performance of these treatments. TKP-3 had the highest moisture content while the control sample was the lowest after the 14-day storage period, with means of 83.77 and 83.57, respectively.

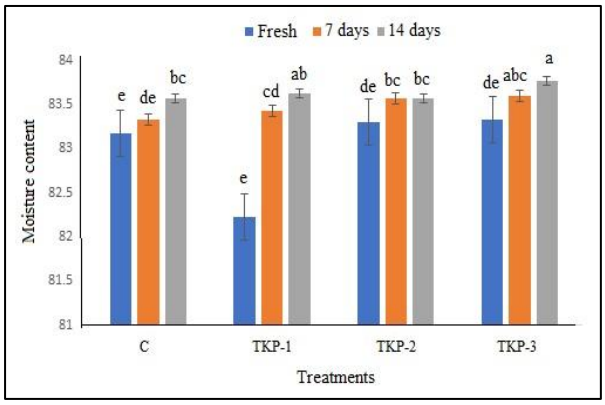

Figure 3. The effect of Tamarind kernel powder TKP on the moisture content of flavored pasteurized milk

3.3.2. Protein content of pasteurized milk

Data illustrated in Figure (4) showed the effect of adding TKP to pasteurized milk on protein content at fresh and throughout the storage period ($p < 0.01$). It could be observed that there were significant different in protein content among all treatments. Pasteurized milk with 0.4% TKP had the highest protein content at fresh while the control sample had the lowest protein content. At the end of the storage period, pasteurized milk with 0.3% TKP had the highest protein content, and the lowest protein content was recorded in a control sample. This may be due to the effect of adding TKP. TKP is derived from the seeds of the tamarind fruit and contains moderate protein levels, typically around 15–20 %. In addition to protein, it has high carbohydrate content, including polysaccharides that may aid in the texture and viscosity of food products. When incorporated into fortified foods, TKP can add nutritional value, including protein, dietary fiber, and some essential minerals. However, it's typically combined with other higher-protein ingredients to achieve substantial protein fortification (Jain and Khare, 2008). On the other hand, the protein content decreased during the storage period in all milk treatments. This was more observed in a control sample.

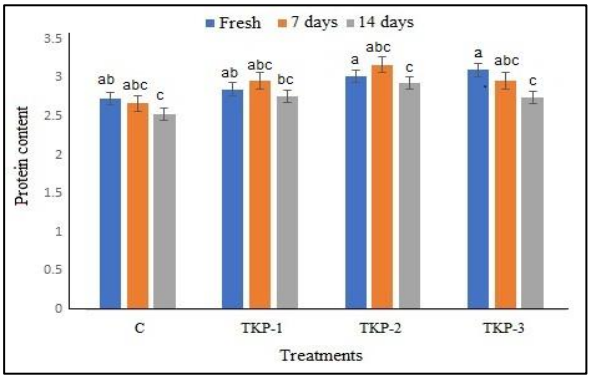

Figure 4. The changes in the protein content of pasteurized milk and enriched pasteurized milk with different concentrations of TKP.

3.3.3. Fat content of pasteurized milk

There were significant differences in fat content during two weeks in all milk treatments (Figure 5). All treatments C, TKP-1, TKP-2, and TKP-3 were exhibit identical fat content at fresh. Control and TKP-2 had a low fat content whereas TKP-1 and TKP-3 had the highest fat content. This trend was much more pronounced in TKP-1 and TKP-2, which had the lowest fat percentage compared with other treatments. As storage periods increase, there is a noticeable decline in fat content across all treatments. After 7 days, the fat con-

tent decreased significantly, while treatment TKP-3 maintained the highest fat content among all treatments. This decline continues into 14-day storage period where the fat for all treatments. This may be due to the TKP being defatted and purified, there was not much influence on the fat levels of the final products (Abiraami et al., 2021).

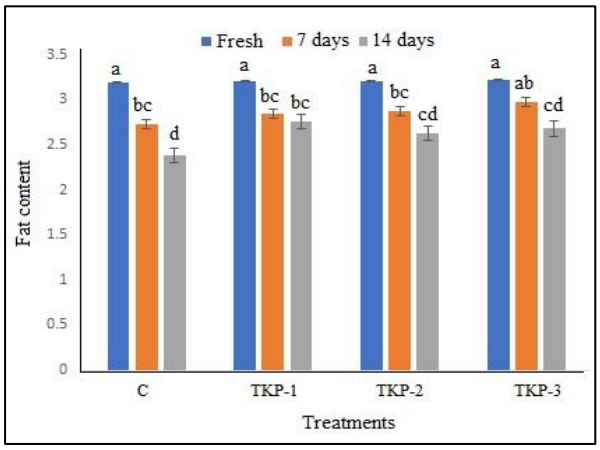

Figure 5. The effect of Tamarind kernel powder TKP on the fat content of flavored pasteurized milk.

3. 3.4. Acidity and pH Values of pasteurized milk

Throughout the storage period, there were significant differences in acidity and pH values ($p < 0.01$) across all treatments (Figures 6 A and B). There was r acidity development and a decrease in pH values in all pasteurized milk after 14 days compared with fresh samples. It was noticed that TKP-3 milk had the highest acidity at the end of 14-day storage periods. In contrast the other treatments C, TKP-1, and TKP-2 all showed lower acidity% had the lowest acidity %. Across all treatments, there is a noticeable increase in acidity % from fresh to the 7-day and then 14-day storage periods. While It had a reverse approach in pH values. Both acidity percent and pH values positively correlated with the storage period. The acidity increased throughout the storage period for all pasteurized milk treatments and vice versa for pH values. These changes in acidity and pH values of enriched flavored pasteurized milk with TKP may be because *Tamarindus indica* has a balanced combination of tartaric acid and reduced sugar content (Kumar and Bhattacharya, 2008).

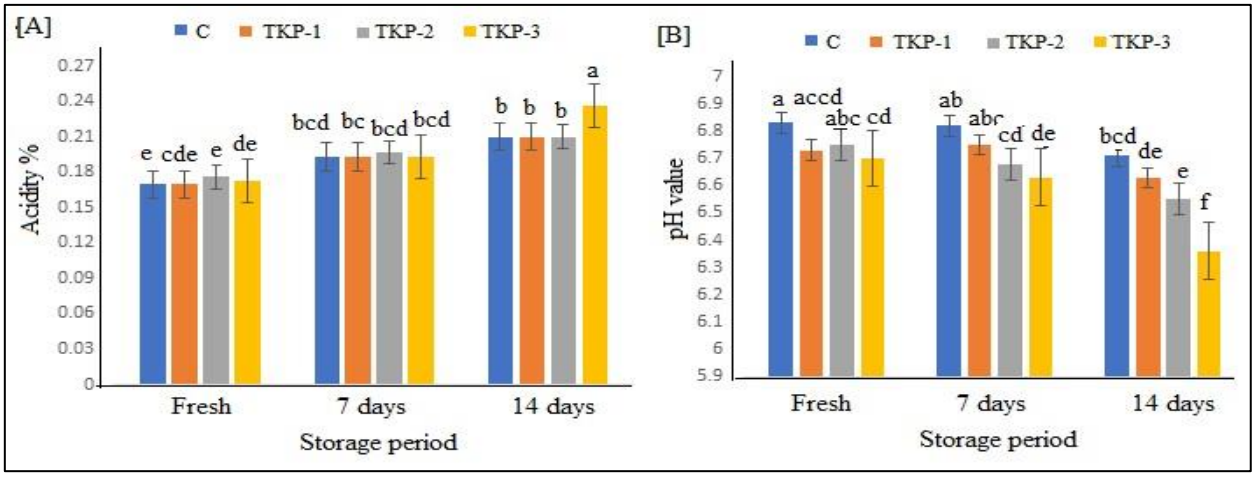

Figure 6. A; The effect of Tamarind kernel powder TKP on the acidity %t, B, The effect of Tamarind kernel powder pH values TKP on the flavored pasteurized milk

3.3.5. Microbiological properties of flavored pasteurized milk

Data shown in Table (2) revealed the effect of TKP at different concentrations on the total plate count (T.P.C), yeast, and mold (Y&M) count as shown in Table (2). The results showed significant differences in both T.P.C and Y&M count at fresh and throughout 14 days of storage ($p < 0.01$) across all treatments. There was a gradual rise in the microbial count during the storage period this was pronounced in the control sample (C) compared with other milk treatments. It could be noticeable that pasteurized milk (C) had the highest microbial count, while pasteurized milk enriched with TKP had the lowest microbial count. That may be because TKP is the final product of the tamarind seed industry, which causes an improvement in nutritional content and increased shelf life due to its potent antioxidant and anti-microbial properties (Chakraborty et al., 2016).

Table 2. The counts of total plate and yeast & mold of enriched pasteurized milk with TKP

Treatments	Total plate count (log cfu/ml)			Yeast & mold count (log cfu/ml)		
	Fresh	7 days	14 days	Fresh	7 days	14 days
C	3.65 ^{b}	6.42 ^{a}	7.21 ^{a}	0.00 E	4.10 ^{a}	3.77 ^{a}
TKP-1	2.31 ^{d}	2.57 ^{cd}	3.25 ^{bc}	0.00 E	0.77 ^{d}	2.90 ^{b}
TKP-2	2.27 ^{d}	2.45 ^{cd}	2.98 ^{bcd}	0.00 E	0.00 ^{e}	2.12 ^{bc}
TKP-3	2.10 ^{d}	2.98 ^{d}	2.42 ^{d}	0.00 E	0.00 ^{e}	1.43 ^{cd}

Data are means ± S.E. C: Control pasteurized milk without any additives; TKP-1: enriched pasteurized milk with 0.2% TKP, TKP-2: enriched pasteurized milk with 0.3% TKP; TKP-3: enriched pasteurized milk with 0.4% TKP.

3.3.6. The sensory properties of flavored pasteurized milk

Figure (7) illustrates the effect of adding TKP to flavored pasteurized milk on sensory properties at fresh and throughout 14 days of storage. It could be notable that there were no significant differences in obtained sensory scores through the 9 points. The acceptability with appearance was slit decreased by rising the percentage of TKP. This may be due to TKP causing a darker appearance compared with the control sample. The flavored and viscosity scores didn't have significant differences compared with the control. Also, the overall scores indicated that enriched pasteurized milk with TKP didn't cause unacceptable changes in sensory properties which was almost similar to the sample. This agrees with Abiraami et al. (2021) who mentioned that fortified yogurt with TKP didn't cause observed changes in consumer acceptability.

Supporting our results, Soradech et al., (2016) illustrated that *Tamarind indica* seeds are a good health supplement, nutraceutical as well as a food preservative because of the occurrence of flavonoids, phenols, and tannins, the extracts from the seed coat possess several activities such as lipid peroxidation, diminution, antimicrobial, antihyperlipidemic, antidiabetic, antityrosinase, collagen stimulating, and anti-inflammatory.

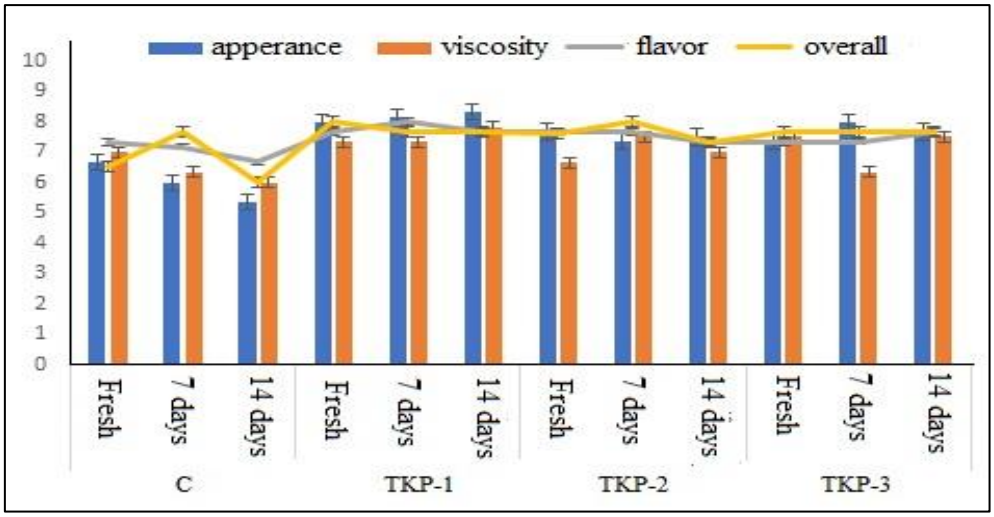

Figure 7. The sensory properties of enriched pasteurized milk with TKP

3.4. In-vivo experiment

3.4.1. Physiological, biochemical and histologic study

3.4.1.1 Body and organ weights of the experimental rat

The obtained results for the changes in body and organ weights of model rats throughout the experiment period (Table 3) showed that there aren't significant differences between treatments in body weights. The liver and spleen weight was recorded as high in control treatment (C). TKP treatment recorded an obvious decrease, whereas TKP-1 treatment recorded a low value for liver and spleen weights.

For brain weight, the results showed that the high weight of rats was noticed in the TKP treatment and these results were similar to TKP-1 while the other treatments were similar to the C treatment. The TKP treatment increased the weight of the brain in comparison with the C treatment; it caused a decrease in the weight of the kidney. For testes weight, all treatments recorded a significant decrease compared with the C treatment. This might be because TKP didn't have any side effect on digestibility in the experimental rats. Also, it causes increases in body weight because of a high protein content as a result of the absence of fiber in TKP (Mansingh et al., 2021).

Table 3. The effect of pasteurized and TKP-enriched milk on the body and organs' weight of the experimental rats

Treatments	body weight(g)	Liver(g)	Spleen (g)	Brain(g)	Kidney(g)	Tests(g)
C	267.00±24.05	9.14±0.95^{a}	1.40±0.31^{a}	1.35±0.05^{b}	0.84±0.07^{a}	1.62±0.13^{a}
Milk	268.80±12.35	7.99±0.92^{ab}	1.34±0.37^{ab}	1.31±0.10^{b}	0.84±0.10^{a}	1.45±0.21^{b}
TKP	266.71±21.07	7.42±1.44^{bc}	0.93±0.29^{bc}	1.62±0.12^{a}	0.72±0.05^{bc}	1.42±0.13^{b}
TKP-1	267.42±21.77	6.38±1.29^{c}	0.84±0.31^{c}	1.45±0.28^{ab}	0.75±0.19^{abc}	1.36±0.24^{b}
TKP-2	261.00±14.65	8.08±1.11^{ab}	1.32±0.39^{ab}	1.28±0.20^{b}	0.83±0.08^{ab}	1.38±0.09^{b}
TKP-3	271.42±18.89	7.80±1.33^{abc}	1.14±0.26^{abc}	1.33±0.14^{b}	0.72±0.13^{c}	1.32±0.17^{b}
Sig.	0.955	0.016	0.029	0.027	0.019	0.000

Data are means ± S.E. C: Control pasteurized milk without any additives; TKP1: enriched pasteurized milk with 0.2% TKP, TKP2: enriched pasteurized milk with 0.3% TKP; TKP3: enriched pasteurized milk with 0.4% TKP.

3.4.1.2. Liver's enzymes and total protein

The data in Table (4) showed the effect of feeding on TKP on the liver enzymes. TKP caused a significant decrease in the enzymes in comparison with the C treatment. Also, the results illustrated that milk affected the liver enzyme more than the aqueous solution of TKP, it decreased the liver enzymes. The treatment TKP-3 restores the normal value of the ALT enzyme. For ALP, the Milk treatment had the lowest value compared with the other treatments. In contrast, in the other treatments, there were no significant differences in comparison with the C treatment. Furthermore, re-

sults of total protein (T.P) showed that there were significant differences among treatments in comparison with the C and Milk treatments. While TKP-3 treatment restores the normal value of T.P in the blood serum.

Liver disorders are a worldwide medical issue due to the main role of the liver as a principal detoxifying organ and maintaining metabolic homeostasis. Numerous compounds that produce free radicals are metabolized in the liver. Oxidative stress occurs when the liver oxidative/antioxidative balance is interrupted, thus leading to harmful processes in the liver and producing liver disorders. Therefore, restoring antioxidants is required to maintain homeostasis (Casas-Grajales and Muriel, 2015). The majority of consumer goods are required to be presented with respectable aesthetics to improve acceptability, particularly those related to colors and taste (Thomas and Adegoke, 2015). Many color additives are not safe.

Therefore, the selection of color additives is more of tumors or any disease. The results indicated that pasteurized milk enriched with TKP reduced the liver enzymes important to avoid any side effects related to the induction in the blood in comparison with the C treatment. These results illustrated that TKP contains healthy substances that improve the health of the liver and reduce the cell damage that releases enzymes from damaged hepatocytes into the blood after hepatocellular injury or death (Sulava et al., 2017). The obtained results agree with (Ibrahim et al., 2017) who reported that camel milk has hepatoprotective action against acetaminophen-induced hepatotoxicity. The amelioration of rising serum enzymes in substance toxicity by camel milk may be a result of the inhibition of the leakage of intracellular enzymes by its membrane stabilizing activity. The mechanism by which camel milk lowered liver enzymes may be referred to as their ability to maintain liver cell integrity (Ahmed and Khater, 2001). Using TKP improved liver health, this may be the presence of phenolic compounds such as pyrogallol and catechol that possess markedly high activity to scavenge free radicals (Alavi Rafiee et al., 2018).

Table 4. Effect of Tamarind kernel powder (TKP) on the liver's enzymes and total protein

Treatments	AST(iu/L)	ALT(iu/L)	ALP(iu/L)	T.P. (iu/L)
C1	64.83±9.88^{a}	71.50±11.20^{a}	135.50±18.76^{ab}	3.63±0.12^{c}
Milk	30.00±10.12^{c}	27.80±4.76^{c}	68.20±7.36^{c}	3.70±0.18^{c}
TKP	44.33±4.58^{b}	49.83±7.22^{b}	118.66±10.98^{b}	4.03±0.15^{ab}
TKP-1	34.83±7.25^{bc}	45.83±9.47^{b}	118.66±12.45^{b}	4.16±0.16^{a}
TKP-2	44.60±11.45^{b}	66.00±10.86^{a}	123.40±12.03^{ab}	3.84±0.27^{bc}
TKP-3	31.80±4.20^{c}	33.20±9.83^{c}	141.20±26.98^{a}	4.08±0.23^{ab}
Sig.	0.00	0.00	0.00	0.00

Data are means ± S.E. C: Control pasteurized milk without any additives; TKP1: enriched pasteurized milk with 0.2% TKP, TKP2: enriched pasteurized milk with 0.3% TKP; TKP3: enriched pasteurized milk with 0.4% TKP

3.4.1.3. Kidney functions and cholesterol levels

Results presented in Table (5) revealed the effect of different concentrations of TKP on kidney function and cholesterol levels in the blood serum of experimental rats. TKP-3 and TKP-4 treatments cause an increase in the value of urea, whereas TKP treatment causes a reduction in the level of urea in serum in comparison with all other treatments. The low value of creatinine was recorded in TKP-1 and TKP-3 treatments. Also, it could be clear that TKP didn't cause any significant differences in cholesterol levels in the rat's blood serum. This indicated that combining TKP with pasteurized milk increased urea and cholesterol levels in the blood serum compared with TKP only. This good effect may be due to the protective effect of TKP against toxicity due to its content of bioactive compounds such as procyanidins with free radical scavenging activity (Ameeramja et al., 2016). Additionally, milk dietary pattern is associated with better kidney function parameters (Syauqy et al., 2020).

Furthermore, there is a correlation between increasing the concentration of TKP in pasteurized milk and the increase in the urea concentration. The protective effects of TKP due to its content of polyphenols like pyrogallol and catechol have been recognized not only for their essential antioxidant activity but also for their role in the modulation of cell signaling pathways, such as mitogen-activated protein kinase cascades, which regulate the responses of oxidative stress (Demir et al., 2017).

3.4.1.4. Lipid peroxidation

Lipid peroxidation is an indicator of oxidative stress induced due to ROS generation. This process has significant implications for cell biology and influences various physiological and pathological conditions (Ayala et al., 2014). Data shown in Figure (8) illustrates the impact of TKP on lipid peroxidation, assayed as malondialdehyde (MDA) content, in experimental rats. Milk treatment caused a significant increase in MDA content meanwhile TKP and other milk TKP-enriched treatments (TKP-1 to TKP-4) restored MDA values. Milk consumption is associated with upregulated metabolism pathways of carbohydrates, lipids, and amino acids (Zhang et al., 2024).

Table 5. The effect of Tamarind kernel powder (TKP)on kidney functions and cholesterol

Treatments	Urea (mg/dL)	Creatinine (mg/dL)	Cholesterol (mg/dL)
C	46.16±5.63^{bc}	1.54±0.28^{a}	122.83±13.96
Milk	51.40±8.29^{b}	1.36±0.33^{ab}	124.60±13.95
TKP	36.50±3.61^{d}	1.17±0.12^{b}	122.16±17.44
TKP-1	38.83±5.34^{cd}	0.61±0.08^{c}	134.33±10.23
TKP-2	60.80±6.61^{a}	1.54±0.11^{a}	134.80±6.94
TKP-3	59.60±8.50^{a}	0.63±0.12^{c}	131.60±8.20
Sig.	0.00	0.00	0.330

Data are means ± S.E. C: Control pasteurized milk without any additives; TKP-1: enriched pasteurized milk with 0.2% TKP, TKP-2: enriched pasteurized milk with 0.3% TKP; TKP-3: enriched pasteurized milk with 0.4% TKP.

Upregulation of such metabolic pathways is usually associated with ROS production, thus could explain the increase in MDA content after consuming pasteurized milk. The reduction in MDA content associated with receiving either TKP or TKP-enriched milk is supported by the increase in their antioxidant activity (Figure 1) and points to the antioxidant role of TKP components (Shams et al., 2022; Taksene and Belay, 2024).

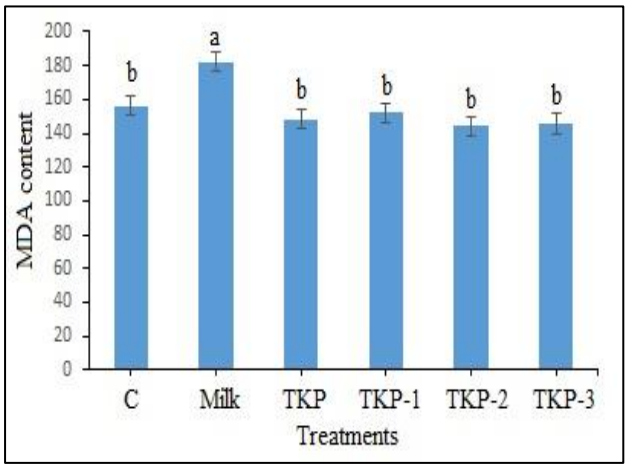

Figure 8. Changes in lipid peroxidation level as MDA content in rats received pasteurized milk enriched with different concentrations of TKP

3.4.1.5. Histopathological Examination

Histological examination of renal tissue sections of all treatments was carried out. Treatments with C, TKP-3, and TKP-1 groups showed the presence of normal glomeruli, Bowman's capsules, and tubules (Figure 9 A, C, and D). Meanwhile, the administration of Milk resulted in adhesion between the surface of the glomerular tuft and Bowmen's capsules (Figure 9 B). These changes are also shown in the group of TKP-2 (Figure 9 E). Also, the TKP-3 group showed slight dilatation in renal tubules (Figure 9 F). Obtained results revealed that the most changes appeared in Milk and TKP-2 treatments. These changes agree with the other gained results about metabolic activity with consuming pasteurized milk and pointed to the positive effect of TKP as an enrichment addition for posturized milk.

3.4.2. Quantitative analysis of gene expression

Molecular responses of albino rats (*Sprague dawley*) to TKP enrichment treatments for pasteurized milk were investigated using qRT-PCR. The investigation has detected the changes in the transcription level of some stress and metabolic-related genes in kidney and liver tissues under control and other tested fed conditions. Quality of gene expression analysis was assured by confirming the intact form of total RNA used in cDNA synthesis as well as a primer specification for selected genes (Figure 10). Intact extracted RNA appeared as two main bands in agarose gel electrophoresis (Figure 10 A). Amplification of studied genes using designed primers produced one main sharp specific band in expected molecular weight (Figure 10 B).

Studied genes included antioxidant enzyme encoding genes: Copper-zinc/superoxide dismutase, Cu-Zn/SOD (*SOD-1*, *SOD-3*), Glutathion peroxidase (*GPX*), Glutathion transferase (*GST*), Heme oxygenase (*HO*) and one of proteinase inhibitors; Alpha-2-Macroglobulin (*α 2-MG*) with using GADPH gene as an endogenous control. Obtained results showed that expression levels of *SOD-1* and *SOD-3* were increased significantly with consuming pasteurized milk in both of kidney (Figure 11: A, B) and liver (Figure 12: A, B) tissues excluding *SOD-1* in the kidney, where the increase was not significant (Figure 11A). On the other hand, consumption of TKP and the enriched pasteurized milk (TKP-1 and TKP-2) induced a significant reduction in the expression level of both *SOD-1* and *SOD-3* in both studied tissues. *SOD-1* and *SOD-3* are different isoforms of Cu/Zn-SOD encoded by two different genes, it involved in the scavenging of O_2 radicals (Weisiger and Fridovich, 1973; Mondola, et al., 2016). Proper expression and activity of both isoenzymes are needed for full protection from oxidative stress (khan et al., 2019).

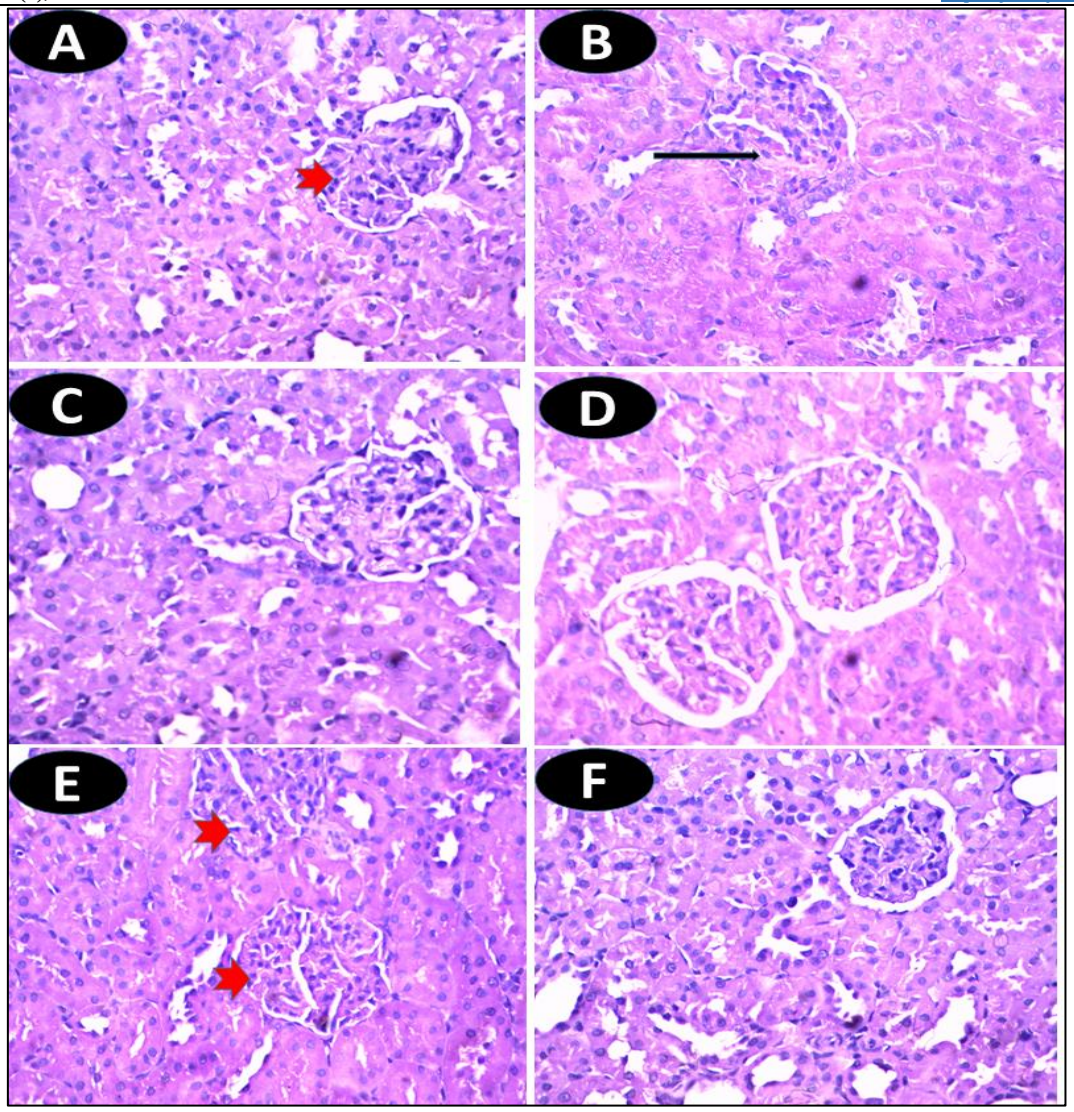

Figure 9. Microscopic pictures of HE-stained renal sections showing normal glomeruli (red arrowhead), tubules, and interstitial tissue from the control (A) groups; adhesion between the surface of the glomerular tuft and Bowmen's capsules (black arrow) in the milk group (B); normal appearance in both c and d groups (TKP-3 and TKP-1); adhesion between the surface of the glomerular tuft and Bowmen's capsules (red arrowhead) in TKP2 group (E); normal appearance with dilatation in renal tubules in TKP-3 group (F). H&E, × 400.

Expression patterns of GPX and GST were similar in the kidney (Figure 11; C, D) and liver (Figure 12; C, D) tissues. Consumption of milk caused a non-significant increase in the transcript amount of GPX in the kidney while this increase was significant in liver tissue. *GST* transcription level was highly up-regulated in kidney and liver tissues with milk consumption (Figure 11, 12: D). Meanwhile consumption of TKP or enriched milk (TKP-1 and TKP-2) induced a significant reduction in the transcription level of *GPX* and *GST* in both of kidney and liver. GPX is an important antioxidant enzyme that has protection and detoxification effects. It uses reduced GSH as an electron donor to catalyze the reduction of organic hydroperoxides (Yoshizawa et al., 1998; Zhou et al., 2023). Also, GST is considered one of the most efficient antioxidant species involved in the detoxification of many dangerous compounds (Mazzetti et al., 2015; Mannervik et al., 2021).

Milk is known with its antioxidant power due to bioactive proteins as well as naturally occurring non-enzymatic and enzymatic antioxidants (Usta and Yilmaz-Ersan, 2013). *SOD*, *GPX*, and *GST* are some of the main antioxidant enzymes in milk (Khan et al., 2019). Increasing the activity of SOD and GST in the livers of mice receiving camel milk was reported (Soliman et al., 2015; Wang et al., 2017). Increased expressions of antioxidant enzymes with milk consumption are correlated to the up-regulation of metabolic pathways of carbohydrates, lipids, and amino acids (Zhang et al., 2024). Up-regulation of such metabolic pathways is usually associated with ROS production. Production of the superoxide radical is unavoidable as it is closely associated with the metabolism of molecular oxygen in mitochondria and in cellular membranes (Damiano et al., 2019; Mondola et al., 2016). This could explain the increase in MDA content after consuming pasteurized milk (Figure 8) as well as histological changes in kidney tissues (Figure 9) due to non-significant changes in oxidative burden. Up-regulating the expression of different genes in mice who received camel milk was reported (Mannervik et al., 2021; Zhang et al., 2024).

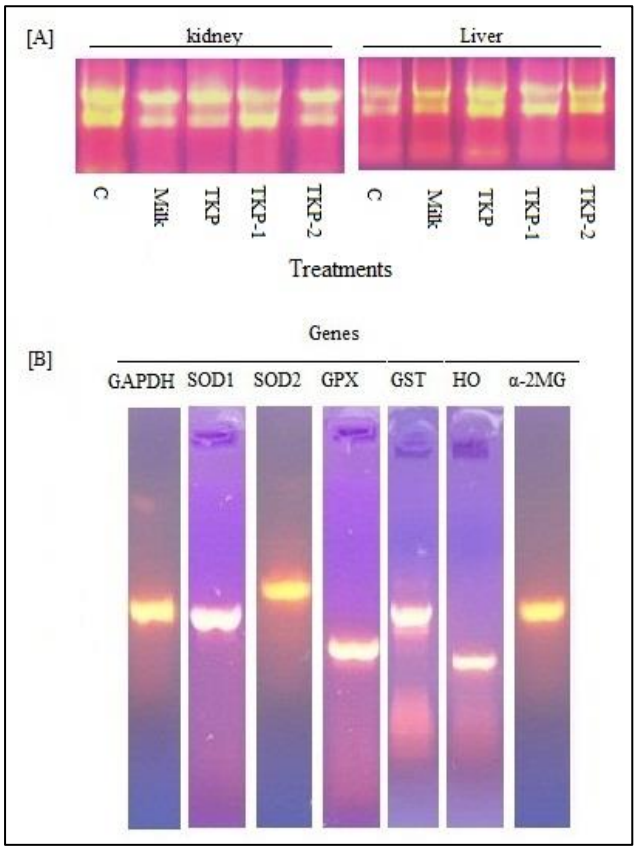

Figure 10. Verification of RNA integrity and primer-specification. [A] Electrophoresis analysis of total RNA extracted from kidney and liver tissues of rats at tested treatment on 1% agarose gel. [B] gel electrophoresis of primer-specific bands of all examined genes.

Treatments with TKP or Milk enriched with TKP (TKP-1 and TKP-2) were associated with significant downregulation of the expression of all tested antioxidant and detoxifying enzymes, *SOD-1*, *SOD-3*, *GPX*, and *GST*, in both kidneys (Figure 11: A, B, C, D) and liver's (Figure 12: A, B, C, D) tissues. Numerous components of TKP such as 2-hydroxy-3', 4'-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate, 3,4-dihydroxyphenyl acetate, and epicatechin have antioxidant activity (Tsuda et al., 2004). This improves nutritional quality and stabilizes the fat content of food products (Tsuda et al., 2004, Sudjaroen et al., 2005). This powerful antioxidant activity minimizes the accumulation of ROS produced during various metabolic activities. This could explain the reduction of studied antioxidant enzymes associated with receiving either TKP or TKP-enriched milk.

This investigation included also the determination of changes in the expression of the *HO-1* encoding gene. Interest in HO is driven by the contribution of this system in the regulation of renal hemodynamics and maintaining iron homeostasis that protects tissues against various injuries (Balla et al., 1992; Bae et al., 2013; Zou et al., 2000). Obtained results (Figure 11,12: E) showed that consumption any of pasteurized milk, TKP, or TKP enriched milk; upregulates the expression level of *HO* significantly compared with the control treatment. The increase in the expression level of *HO* was associated with the increase in TKP concentration. The capability of dietary phytochemicals with antioxidant properties in the induction of the transcription of *HO* is one of the protection mechanisms of these supplements (Halliwell et al 2005; Lotito et al., 2006). The upregulation effect of Protandim, an antioxidant food supplement, on the *HO* gene was reported in a mouse β-cell line and a human neuroblastoma cell line (Velmurugan et al., 2009). The presence of responsive elements sites in *HO* promoter is involved in their induction with receiving supplements with antioxidant activity. Also, their upregulation is constant with a number of these responsive element sites and the involvement of multiple transcription factors in different promoters (Hock et al., 2007; Velmurugan et al., 2009).

Expression analysis of the alpha-2-Macroglobulin (α 2-MG) gene showed similar expression patterns in both kidney and liver tissues. Rats who consumed milk showed a significant increase in the relative expression of α 2-MG (Figure 11,12: E). Meanwhile, others who received any of TKP or enriched milk with TKP showed downregulation of α 2-MG transcription. Alpha-2-Macroglobulin is a broad-spectrum proteinase inhibitor that could, through its molecular structure, block almost all kinds of proteinases that induce chronic inflammation (Carli et al., 2017; Wang et al, 2022). That is beside its central role as anti-coagulation and its capacity to limit fibrinolysis by sequestering fibrin (Atkinson et al., 2012; Carli et al., 2017). In the current study, downregulation of its expression could be associated with a reduction of oxidative burden as a result of increasing antioxidant capability of TKP and enriched milk with TKP as shown in (Figure 2).

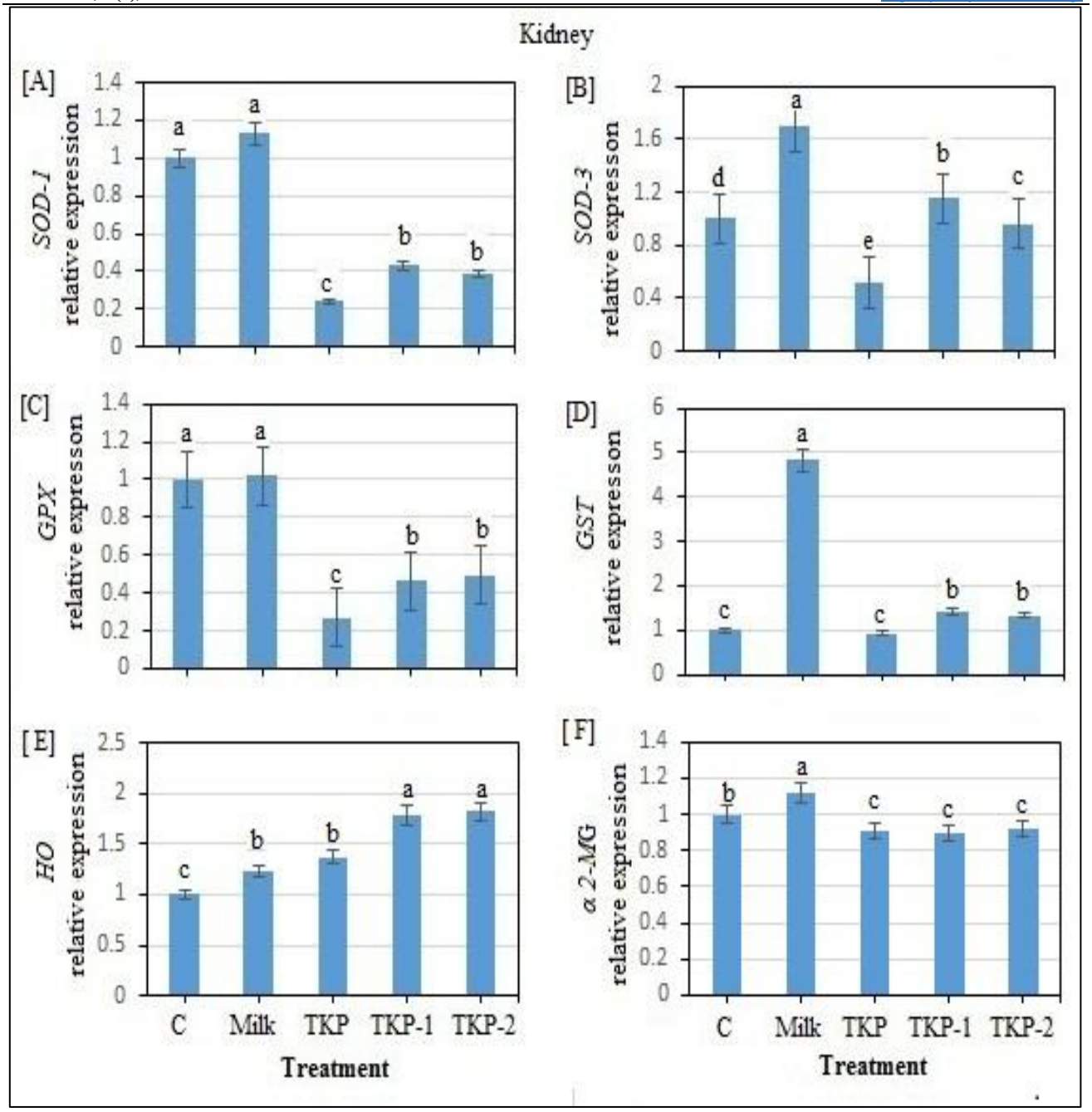

Figure 11. Changes in the expression level of tested genes in Kidney tissue of examined rats under control and other experimental conditions. C: control fed with pasteurized water; Milk: pasteurized milk; TKP: aqueous extract of 0.4% tamarind kernel powder TKP-1: pasteurized milk with 0.2% TKP; TKP-2 pasteurized milk with 0.3% TKP. Relative expression (RQ) was calculated as $2^{-\Delta\Delta ct}$ calibrated with reference gene (GAPDH gene accession no. M17701.1) and control treatment. Data are shown as the means ± SEs of relative expression for three biological replicates for every cDNA sample.

4. Conclusions

The current finding presents a physiological and molecular investigation of the role of Tamarind Kernel Powder (TKP) as an enrichment addition to pasteurized milk. Obtained results concluded that adding TKP improved the antioxidant properties of pasteurized milk which effect positively on body weight as well as both of liver and kidney function. These improvements were associated with the capability of TKP in regulation the transcription of some stress and metabolic responsive genes. Thus pointing to their potential application as immune-modulator at cellular and genetic levels in some therapeutic products.

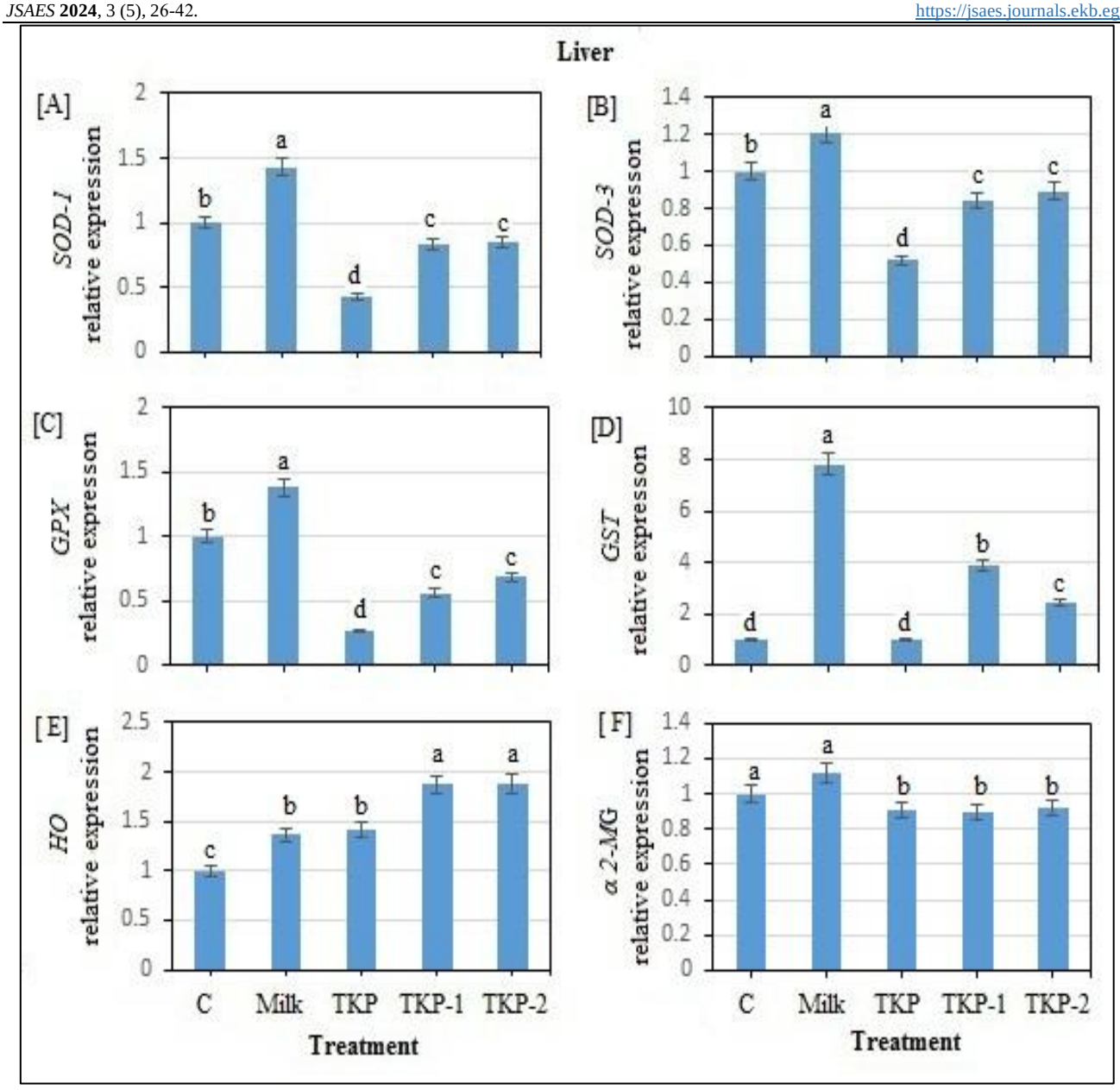

Figure 12. Changes in the expression level of tested genes in liver tissues of examined rats under control and other experimental conditions. C: control fed with pasteurized water; Milk: pasteurized milk; TKP: aqueous extract of 0.4% tamarind kernel powder TKP-1: pasteurized milk with 0.2% TKP; TKP-2 pasteurized milk with 0.3% TKP. Relative expression (RQ) was calculated as $2^{-\Delta\Delta ct}$ calibrated with reference gene (GAPDH gene accession no. M17701.1) and control treatment. Data are shown as the means ± SEs of relative expression for three biological replicates for every cDNA sample.

Author Contributions: Conceptualization, S.A.O; A.M.Z. and D.A.A. methodology, S.A.O; A.M.Z. and D.A.A.; software, S.A.O; A.M.Z. and D.A.A.; validation, S.A.O; A.M.Z. and D.A.A.; formal analysis, S.A.O; A.M.Z. and D.A.A..; investigation, S.A.O; A.M.Z. and D.A.A.; resources, S.A.O; A.M.Z. and D.A.A.; data curation, S.A.O; A.M.Z. and D.A.A.; writing—original draft preparation, S.A.O; A.M.Z. and D.A.A.; writing—review and editing, S.A.O; A.M.Z.; S.A.A .and D.A.A.; visualization, S.A.O; A.M.Z. and D.A.A.; All authors have read and agreed to the published version of the manuscript

Conflicts of Interest: The authors declare no conflict of interest."

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