Consumption of Tlayudas (Tortillas) Containing Standardized Extracts from Mexican Medicinal Mushroom *Ganoderma lucidum* (Agaricomycetes) Increases the Expression of Antioxidant Genes in C57BL/6 Mice Fed a High-Cholesterol Diet

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ABSTRACT: Maize is relevant part of Mexican food and culture. Tlayudas are a regional variant of tortillas from Oaxaca region. Tlayudas (T) containing functional properties of standardized hydroalcoholic extracts of the medicinal mushroom *Ganoderma lucidum* from Mexico were studied [*Gl*-1: extracts from basidiocarps cultivated on the control substrate; *Gl*-2: extracts from basidiocarps cultivated on substrate treated with acetylsalicylic acid (ASA, 10 mM)]. Antioxidant properties of these tlayudas (T+*Gl*-1, T+*Gl*-2) were assessed using an *in vivo* model (C57BL/6 mice) fed with a high-cholesterol diet (HC). The consumption of T+*Gl*-1 or T+*Gl*-2 decreased serum levels of cholesterol, triglycerides, LDL-c, and glucose. The expression of antioxidant genes increased in mice groups consuming tlayudas plus *Gl*-1 or *Gl*-2 extracts, namely SOD1 (37.3–72.7%), SOD2 (28.3–29.6%), CAT (29.3–59.4%), and GPX1 (62.7–66.8%), respectively, compared with the HC group. There was a differential effect of *Gl* extracts, the mice group consuming tlayudas plus the *Gl*-2 extract showed further increase of expression of SOD1, CAT, and GPX1 genes, compared with the group administered with tlayudas plus the *Gl*-1 extract. This effect was associated with a higher content of antioxidant compounds [β-glucans, total polyphenols, antioxidant capacity (ORAC)] in tlayudas containing the *Gl*-2 extract. We showed that new functional food products of high added value (T+*Gl*-1, T+*Gl*-2) can be developed from Mexican traditional foods highly consumed, such as tlayudas, in order to promote healthier diets for preventing cardiovascular and chronic degenerative diseases associated to oxidative stress.

KEY WORDS: antioxidants, *Ganoderma lucidum*, gene expression, tlayudas

ABBREVIATIONS: CAT, catalase; ***Gl***, *Ganoderma lucidum*; ***Gl*-1**, extracts from basidiocarps cultivated on the control substrate; ***Gl*-2**, extracts from basidiocarps cultivated on substrate treated with acetylsalicylic acid (ASA, 10 mM); **GPX1**, glutathione peroxidase 1; **SOD1**, superoxide dismutase 1; **SOD2**, superoxide dismutase 2

I. INTRODUCTION

Maize is a staple food in Mexico, and its nixtamalized product, the tortilla, is normally eaten by the population. Daily intake of tortillas has been estimated 79.5 kg per capita in rural areas, while 56.7 kg per capita

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in urban areas. They can be considered as the main source of nutrients in the Mexican diet, particularly rich in protein, carbohydrates, fiber, calcium, and vitamin E.[1] The nutritional value of tortillas can be improved by mixing them with other foods. García-Rojas et al.[2] increased the functional properties of blue maize tortillas by adding milled basidiocarps from the edible mushroom *Pleurotus agaves*. Similar case is the production of tlayudas, traditional maize tortillas from Oaxaca region in Mexico, containing standardized extracts from the ligzhi or reishi medicinal mushroom *Ganoderma lucidum* (Curtis:Fr.) P. Karst. (Ganodermataceae, Agaricomycetes).[3] This species has been shown to possess immunomodulatory, hypocholesterolemic, anti-inflammatory, antidiabetic, antioxidant, anticancer, and prebiotic properties.[4–6] Diverse bioactive compounds have been linked to these properties, such as α-glucans, β-glucans, terpenes, polyphenols, and other antioxidants.

Oxidative stress and hypercholesterolemia have been linked to a higher risk of cardiovascular disease.[7] The antioxidant activity of *G. lucidum* extracts has been shown to inhibit the human low-density lipoprotein (LDL) oxidation *in vivo*, as well as to increase antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase), to lower plasma levels of total cholesterol, triacylglycerol, and LDL cholesterol, and to increase high-density lipoprotein (HDL) cholesterol *in vivo*.[8]

Studies by our group have shown antioxidant, antibacterial, hypocholesterolemic, anti-inflammatory, prebiotic, and anticancer properties of standardized extracts of a Mexican genotype of *G. lucidum*, known as "repisas" (brackets) by local indigenous and peasant communities. These extracts are safe and show no adverse effects at the effective dose using *in vitro* and *in vivo* models, as well as exploratory clinical trials. We have also used the acetylsalicylic acid (ASA) added to the cultivation substrate, as a novel strategy to modulate the functional and medicinal properties of *G. lucidum*.[4–6,9–11] In this research, we studied the antioxidant properties of the new functional food product previously developed, based on tlayudas containing standardized extracts of *G. lucidum*.[3] The effects of consuming these functional tlayudas on the expression of antioxidant genes [SOD1 (superoxide dismutase 1), SOD2 (superoxide dismutase 2), CAT (catalase), GPX1 (glutathione peroxidase 1)] were assessed in the liver of an *in vivo* model (C57BL/6 mice) with hypercholesterolemia. These important genes are involved in the antioxidant defense system at cellular level. SOD1 and SOD2 convert O_2^- into the reactive oxygen intermediate H_2O_2, CAT detoxifies H_2O_2, and GPX1 catalyses the breakdown of H_2O_2 and lipid hydroperoxides into nontoxic products.[12] A highly consumed food, such as traditional tlayudas, containing antioxidant properties from the medicinal mushroom *G. lucidum* can be a potential strategy for the prevention of diseases associated to oxidative stress in the population.

II. MATERIALS AND METHODS

A. Preparation and Composition of *Ganoderma lucidum* Extracts (*GI*-1, *GI*-2)

The Mexican strain (CP-145) of *G. lucidum* sensu lato is deposited at the Centre of Biotechnology of Medicinal, Functional, and Edible Mushrooms (CB-HCFM), CP, Mexico. The gene sequence of the ITS1-5.8S-ITS2 rDNA from the CP-145 strain is deposited at the GenBank (www.ncbi.nlm.nih.gov/genbank/) under accession number LN998989.

Cultivation and harvesting of *G. lucidum* (*Gl*) has been described previously.[9] Dry oak (*Quercus acutifolia* Née) sawdust (1000 g) was introduced into polypropylene plastic bags (51 × 21 cm) with a microfilter of 0.5 μm (3 × 8 cm) for allowing gas exchange (CB-HCFM, Mexico). Distilled water (1300 mL) was added to each control bag, while a solution of acetylsalicylic acid (ASA, 10 mM; Sigma-Aldrich, USA) in distilled water (1300 mL) was added to every treated bag. All bags (2 kg wet substrate per bag) were sterilized at 121°C for 15 min (Sterivap HP IL, BMT, Brno, Czech Republic). After cooling, under aseptic conditions, they were inoculated, incubated, and fruited. Mature basidiomata from each bag were harvested,

cut into slices (ca. 1–2 cm), dried at 40°C in a forced air-drying oven (SMO28-2, Shel Lab, USA) for 5 d, and stored at –80°C inside plastic bags until use.

Chopped mushroom slices (10 g) were placed in a filter paper (8 μm) bag, macerated during 24 h, and hydroalcoholic extracts, 32% v/v (alcohol : water), obtained according to a previous patent (MX322035-B).[10] Mushroom extracts were concentrated in a rotary evaporator (Buchi R-220, Switzerland) at 28–30°C, 90–110 rpm, filter-sterilized (0.45 μm, Merck Millipore, Mexico), and stored at 4°C until use. Standardized extracts of *Gl* cultivated on the control substrate were labeled as *Gl*-1, whereas those cultivated on the treated substrate (ASA, 10 mM) were labeled as *Gl*-2. The standardized extracts of *Gl* were prepared at concentration of 25 mg/mL.

Standardized extracts of *G. lucidum* (*Gl*-1, *Gl*-2) were previously analyzed according to standard protocols.[4] *Gl*-1 and *Gl*-2 contain carbohydrates (0.58%), glucans (15.96–17.01%, w/w), total protein (0.315–0.365%), total dietary fiber (0.10–0.15%), fat (0.01%), vitamins (B_1, B_2, B_3, B_6, B_{12}, D), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium, zinc), several organic acids, and an energy content of 4 kcal/100 g extract. There are differences in the nutrient profiles between *Gl*-1 and *Gl*-2,[4,9] including total protein, total dietary fiber, vitamins (B_1, B_2, B_3, B_{12}) and minerals (copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc). These differences are due to the addition of ASA (10 mM) to the substrate, as all other variables remained constant during mushroom cultivation (*i.e.*, species, strain, substrate and environmental conditions). Interestingly, there were differences in the total content of polyphenols and in the antioxidant capacity of both extracts.

B. Preparation of Tlayudas

Preciado Iñiga[3] previously described the method used for the elaboration of tlayudas. Briefly, the traditional process of nixtamalization and elaboration of maize dough for tlayudas was carried out at the CB-HCFM facilities. A thermo-alkaline cooking of maize (*Zea mays* L.) was performed using a 1% (w/v) $Ca(OH)_2$ solution and water in a 2 : 1 (w/v) ratio for 89 min. After cooking, the mixture was allowed to rest for 13 h. The water derived from the nixtamalization process (nejayote) was discarded, and the cooked maize (nixtamal) was washed with 3 L of water. The nixtamal was ground in a mill having two circular stones of 35 cm diameter for obtaining the maize dough, which was finally prepared on a stone base (metate). Thereafter, 5 mL of each *G. lucidum* extract (*Gl*-1 or *Gl*-2) was immediately added to the dough, and mixed homogeneously for subsequent pressing and cooking at *ca*. 400°C for 6.40 min. The effect of *G. lucidum* extracts was determined according to the following cooked experimental products: (1) TC (control tlayudas) = 100 g of control traditional maize dough + 5 mL of water; (2) T+*Gl*-1 (tlayudas *Gl*-1) = 100 g of traditional maize dough + 5 mL of the *Gl*-1 extract (25 mg/mL); (3) T+*Gl*-2 (tlayudas *Gl*-2) = 100 g of traditional maize dough + 5 mL of the *Gl*-2 extract (25 mg/mL).

C. Bromatological Analysis and Bioactive Compounds of Tlayudas

Calories, calories from fat, total fat, saturated fat, monounsaturated fat, polyunsaturated fat, trans fat, cholesterol, moisture, ash, sodium, total carbohydrate, dietary fiber, total sugars, added sugars, protein, calcium, iron, potassium, and vitamin D were determined in experimental tlayudas (TC, T+*Gl*-1, T+*Gl*-2).[3] The methodology from AOAC 2000 was followed to determine the content of ash, fat, protein, and fiber, using triplicate samples.[3]

Bioactive compounds were determined in hydroalcoholic extracts, 32% v/v (alcohol : water), obtained from experimental ground tlayudas (TC, T+*Gl*-1, T+*Gl*-2) according to a previous patent (MX322035-B),[3,10] as described above.

D. Determination of Total Phenolic Content, Antioxidant Capacity, and β-Glucan Analysis

Standard protocols were followed for these analyses.[3] The concentration of phenolic compounds in hydroalcoholic extracts from tlayudas, expressed as gallic acid equivalent (GAE), was measured using the Folin and Ciocalteu's phenol reagent, as previously described by the authors.[11] The antioxidant capacity of hydroalcoholic extracts from tlayudas was determined according to the oxygen radical absorbance capacity (ORAC) assay.[11] The analysis of β-glucans (1→3, 1→4 linkage) in samples of ground tlayudas was performed following the colorimetric AOAC no. 995.16 method, using the K-BGLU kit (Megazyme, Ireland), according to the manufacturer's instructions.[4]

E. Animal Model

1. Animals and Treatments

The project was evaluated and approved by the Internal Bioethics Committee (CIB) of the CB-HCFM, Campus Puebla, College of the Postgraduates, through the registration number CIB-CB-HCFM-001. A total of 48 C57BL/6 male mice, 5 weeks old, were obtained from the Salvador Zubirán National Institute of Medical Sciences and Nutrition (INCMNSZ). The animals were housed in SmartFlow ventilated racks (Tecniplast S.p.A., Italy) at 23 ± 2°C, a relative humidity of 45% to 55%, and a daily cycle of 12 h light/12 h darkness. Mice were assigned to six different treatments ($n = 8$): (1) Ctrl: Control diet (AIN-93);[13] (2) HC: High-cholesterol diet (0.5% cholesterol, Sigma-Aldrich, New Zealand); (3) HC+T: High-cholesterol diet (0.5%) + Control tlayudas (60%); (4) HC+T+*Gl*-1: High-cholesterol diet (0.5%) + Tlayudas containing the *Gl*-1 extract (60%); (5) HC+T+*Gl*-2: High-cholesterol diet (0.5%) + Tlayudas containing the *Gl*-2 extract (60%); and (6) HC+T+AT: High-cholesterol diet (0.5%) + Control tlayudas (60%) + Atorvastatin drug (0.03 g/100 g, Almus, Spain). Each group was fed *ad libitum* for 45 d. Food consumption by mice was recorded daily, while body weight was recorded twice a week, using an Ohaus Adventurer H-5276 analytical balance (Ohaus Corporation, NJ, USA). At the end of the study, mice were deprived of food and water for 9 h. Mice were sacrificed by cervical dislocation. Blood was collected via the portal vein. The serum was obtained by centrifugation at 1000 × *g* for 10 min and stored at –70°C until analysis. The liver was rapidly excised, weighed, frozen in liquid N_2, and stored at –70°C. All researchers involved had advanced training in animal care and good laboratory practices. The composition of the different diets is shown in Table 1. Diets including tlayudas were elaborated by mixing homogeneously all ingredients per treatment plus ground tlayudas.

TABLE 1: Composition of six diets studied and administered to the experimental groups of C57BL/6 mice

Ingredients (g/kg)	Ctrl	HC	HC+T	HC+T+*Gl*-1	CH+T+*Gl*-2	CH+T+AT
L-Cystine	3.0	3.0	1.2	1.2	1.2	1.2
Choline	2.5	2.5	1	1	1	1
Vitamins	10.0	10.0	4	4	4	4
Cellulose	50.0	50.0	21.98	21.98	21.98	21.98
Minerals	35.0	35.0	14	14	14	14
Soybean oil	70.0	70.0	28	28	28	28
Starch	397.5	397.5	159	159	159	159
Dextrin	132.0	132.0	52.8	52.8	52.8	52.8
Saccharose	100.0	100.0	40	40	40	40

TABLE 1: (*continued*)

Casein	200.0	200.0	80	80	80	80
Cholesterol	—	5.0	5.0	5.0	5.0	5.0
Atorvastatin	—	—	—	—	—	0.3
Tlayudas Control	—	—	600	—	—	600
Tlayudas *Gl*-1	—	—	—	600	—	—
Tlayudas *Gl*-2	—	—	—	—	600	—

Ctrl: Control diet (AIN-93); HC: High-cholesterol diet (0.5% cholesterol); HC+T: High-cholesterol diet (0.5%) + Control tlayudas (60%); HC+T+*Gl*-1: High-cholesterol diet (0.5%) + Tlayudas containing the *Gl*-1 extract (60%); HC+T+*Gl*-2: High-cholesterol diet (0.5%) + Tlayudas containing the *Gl*-2 extract (60%); HC+T+AT: High-cholesterol diet (0.5%) + Control tlayudas (60%) + Atorvastatin drug (0.03 g/100 g).

2. Serum Biochemical Parameters

Serum concentrations of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-c) and glucose were measured using a COBAS C111 Chemistry Analyzer (Roche Diagnostics Ltd, Basel, Switzerland).[3] Standard analytical methods use absorption photometry for determining the amount of absorbance of a fluid, which is then used to calculate the concentration in the sample solution.[14]

3. Gene Expression

a. RNA Extraction

Total RNA was isolated from the frozen mouse liver using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. The quantification of RNA was performed using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

b. Quantification of Gene Expression by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was reverse transcribed to cDNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, USA). For the real-time PCR analyses of cDNA from different samples, amplifications were performed using TaqMan probes in a Light Cycler 96 thermal cycler system (Roche Diagnostics Ltd, Switzerland). The TaqMan probes used were: SOD1 (Mm01344233_g1), SOD2 (Mm01313000_m1), CAT (Mm00437992_m1), GPX1 (Mm00656767_g1) (ThermoFisher 29 Scientific, Massachusetts, USA), and the beta-actin (β-actin) (Mm02619580_g1) was used as a housekeeping gene. Expression values were obtained as the relative expression of the target gene minus the constitutively expressed β-actin gene [relative expression = 2 − (Ct, Target gene—Ct, Reference gene)]. Assays for each gene were performed in triplicate.

F. Statistical Analysis

Statistical analysis was performed using SAS (Statistical Analysis 21 System, 2022). The results are expressed as the mean ± standard error of the mean. Statistical significance was determined by one-way analysis of variance followed by Tukey's multiple comparisons test. Differences were considered significant at $P < 0.05$.

III. RESULTS AND DISCUSSION

A. Bromatological Analysis and Bioactive Compounds of Tlayudas

The composition of studied tlayudas (TC, T+*Gl*-1, T+*Gl*-2) varied in total fat and carbohydrate, polyunsaturated fat, calcium, sodium, ash, caloric and moisture content (Table 2).[3] The total polyphenol content was higher (8.2–14.2%) in tlayudas containing extracts from *G. lucidum* (T+*Gl*-1, T+*Gl*-2), compared with control tlayudas (TC), as shown in Fig. 1A. The antioxidant capacity by ORAC test was also greater in T+*Gl*-1 and T+*Gl*-2, compared with control tlayudas (Fig. 1B). The addition of *Gl* extracts to tlayudas increased significantly the content of β-glucans from 0.12% (TC) to 0.14% (T+*Gl*-1) and 0.24% (T+*Gl*-2:), as can be seen in Fig. 1C.

B. Animal Model

1. Intake and Weight Increase

The average food intake was significantly higher HC+T (6.44 g/d) and HC+T+*Gl*-1 (6.18 g/d) mice groups (Table 3). However, the weight gain was significantly higher in the HC+T+AT mice group, while

TABLE 2: Bromatological analysis of tlayudas studied

Analysis (100 g final product)	TC	T+*Gl*-1	T+*Gl*-2
Calories	378	377	371
Calories from fat	49	48	43
Total fat	5.40 g	5.38 g	4.81 g
Saturated fat	18%	17%	17%
Monounsaturated fat	39%	39%	39%
Polyunsaturated fat	43%	44%	44%
Trans fat	0 %	0%	0%
Cholesterol	0 mg	0 mg	0 mg
Moisture	6.40 g	7.21 g	7.95 g
Ash	1.47 g	1.59 g	1.56 g
Sodium	9.94 mg	12.8 mg	13.0 mg
Total carbohydrate	77.83 g	77.13 g	77.07 g
Dietary fiber	8.8 g	7.3 g	7.7 g
Total sugars	0.6 g	0.6 g	0.6 g
Added sugars	0.6 g	0.6 g	0.6 g
Protein	8.90 g	8.69 g	8.60 g
Calcium	179 mg	250 mg	231 mg
Iron	3.33 mg	3.29 mg	3.02 mg
Potassium	277 mg	278 mg	273 mg
Vitamin D	0 µg	0 µg	0 µg

Average of three replicates. TC = Control tlayudas. T+*Gl*-1 = Tlayudas containing 5 mL of the *Gl*-1 extract (25 mg/mL). T+*Gl*-2 = Tlayudas containing 5 mL of the *Gl*-2 extract (25 mg/mL).

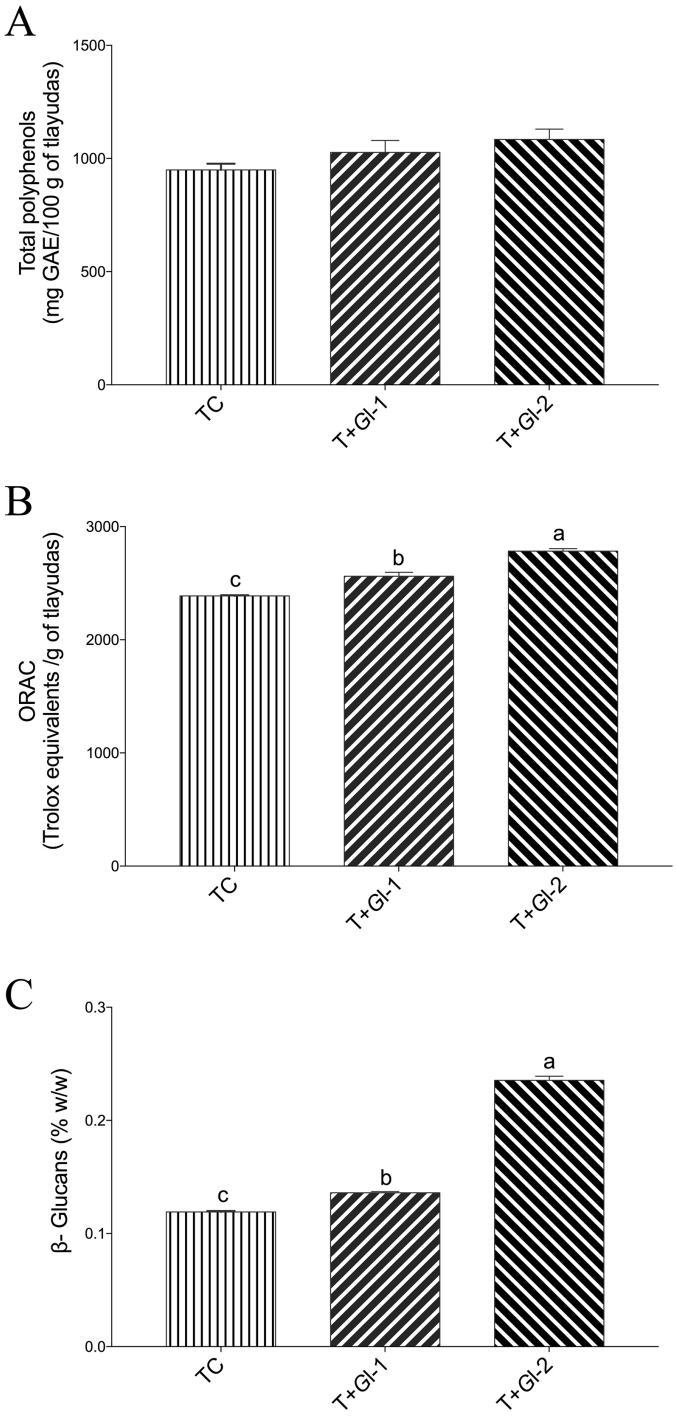

FIG. 1: The content of bioactive compounds in tlayudas studied.[3] (A) Total polyphenols. (B) Antioxidant capacity by the ORAC assay. (C) β-Glucans. Average from five replicates. TC = Control tlayudas. T+*Gl*-1 = Tlayudas containing 5 mL of the *Gl*-1 extract (25 mg/mL). T+*Gl*-2 = Tlayudas containing 5 mL of the *Gl*-2 extract (25 mg/mL). Means in a column showing differing letters indicate statistically significant difference, $P < 0.05$. Values correspond to one-way ANOVA from the Tukey's multiple comparisons test. The analysis compared the mean of each group against the means of every other group.

TABLE 3: Average data of intake, weight gain and serum biochemical parameters from experimental groups studied of C57BL/6 mice fed with a high-cholesterol diet during 45 d

Parameters	Ctrl	HC	HC+T	HC+T+*Gl*-1	HC+T+*Gl*-2	HC+T+AT
Intake (g/d)	4.72 ± 0.14^{b}	5.27 ± 0.11^{b}	6.44 ± 0.35^{a}	6.18 ± 0.12^{a}	5.44 ± 0.21^{b}	4.93 ± 0.22^{b}
Weight gain (g)	3.60 ± 0.97^{ab}	3.45 ± 0.50^{ab}	3.84 ± 0.75^{ab}	4.18 ± 0.59^{ab}	2.07 ± 0.76^{b}	5.22 ± 0.52^{a}
Cholesterol (mg/dL)	71.81 ± 7.80^{b}	101.90 ± 4.15^{a}	91.16 ± 9.17^{a}	80.39 ± 4.07^{a}	73.88 ± 3.23^{b}	92.93 ± 3.33^{a}
Triglycerides (mg/dL)	73.26 ± 4.83^{a}	81.01 ± 6.76^{a}	72.42 ± 2.42^{a}	68.79 ± 2.42^{a}	60.68 ± 3.68^{b}	77.3 ± 7.42^{a}
LDL-c (mg/dL)	11.24 ± 1.15^{c}	35.15 ± 2.51^{a}	21.85 ± 1.14^{b}	15.67 ± 1.26^{bc}	13.12 ± 1.11^{c}	20.27 ± 1.87^{b}
Glucose (mg/dL)	148.30 ± 16.78^{ab}	173.70 ± 11.97^{a}	168.3 ± 14.72^{a}	155.7 ± 9.35^{a}	119.40 ± 12.65^{b}	143 ± 7.83^{a}

Ctrl: Control diet (AIN-93); HC: High-cholesterol diet (0.5% cholesterol); HC+T: High-cholesterol diet (0.5%) + Control tlayudas (60%); HC+T+*Gl*-1: High-cholesterol diet (0.5%) + Tlayudas containing the *Gl*-1 extract (60%); HC+T+*Gl*-2: High-cholesterol diet (0.5%) + Tlayudas containing the *Gl*-2 extract (60%); HC+T+AT: High-cholesterol diet (0.5%) + Control tlayudas (60%) + Atorvastatin drug (0.03 g/100 g). Data are presented as the mean ± SEM. Means in a column showing differing letters indicate statistically significant difference, $P < 0.05$. Values correspond to one-way ANOVA from the Tukey's multiple comparisons test. The analysis compared the mean of each group against the means of every other group.

significantly lower in the HC+T+*Gl*-2 group (Table 3).[3] Meneses et al.[4] reported no significant differences in food intake among experimental groups, after administering *Gl* extracts directly added to the mice diet (0.5% and 1.0%). However, they also reported the lowest significant value of weight gain in the mice group consuming the high dose (1.0%) of the *Gl*-2 extract.

2. Serum Parameters

There were antihyperlipidemic effects on C57BL/6 male mice consuming tlayudas containing *Gl* extracts studied, T+*Gl*-1 and T+*Gl*-2. Total cholesterol (TC) levels decreased in HC+T+*Gl*-1 and HC+T+*Gl*-2 groups, compared with the HC group, as shown in Table 3. This was so also for serum levels of triglycerides (TG), as HC+T+*Gl*-1 and HC+T+*Gl*-2 groups showed lower levels than HC and Ctrl groups (Table 3). Significant lower levels of LDL-c were recorded for all experimental mice groups, compared with the HC group ($P < 0.0001$), as can be seen in Table 3. The levels of glucose were significantly lower in the HC+T+*Gl*-2 group (Table 3), compared with the HC group ($P = 0.0306$). These results showed the hypolipidemic properties of *Gl* extracts administered to *in vivo* models of hypercholesterolemia.[3–5] Cholesterol, triglycerides, and LDL-c decreased consistently after the consumption of *Gl* extracts, involving the expression of genes linked to cholesterol metabolism, the decrease of lipogenesis (Srebp1c, Acaca, Fasn), and reverse cholesterol transport (abcg5 and abcg8), while inhibiting the rate-limiting enzyme for cholesterol biosynthesis, Hmgcr (3-hydroxy-3-methylglutaryl-CoA reductase), as well as exerting important prebiotic effects on the gut microbiota.[4] Triterpenoids from *G. lucidum* have shown inhibitory capacity on the Hmgcr rate-limiting enzyme.[15]

3. Gene Expression

The expression of the SOD1 gene increased in HC+T, HC+T+*Gl*-1, HC+T+*Gl*-2, and HC+T+AT groups, compared with the HC group, being significantly different in two mice groups (HC+T+*Gl*-2, HC+T+AT).

The proportion of increase in SOD1 expression was 72.7% for the HC+T+*Gl*-2 group, compared with the HC group, followed by 52.1% for the HC+T+AT group, 37.3% for the HC+T+*Gl*-1 group, and 20.6% for the HC+T group. A differential effect between *Gl*-1 and *Gl*-2 extracts was evident, as a further increase was recorded in the expression of the SOD1 gene from the HC+T+*Gl*-2 group (Fig. 2A). Interestingly, the HC group showed a lower expression of the SOD1 gene, compared with the Ctrl group.

The expression of the SOD2 gene was higher in the HC+T, HC+T+*Gl*-1, HC+T+*Gl*-2, and HC+T+AT groups compared with the HC group, being significantly different the HC+T+AT group only ($P = 0.0067$). Gene expression of these mice groups was not significantly different compared with the Ctrl group. The

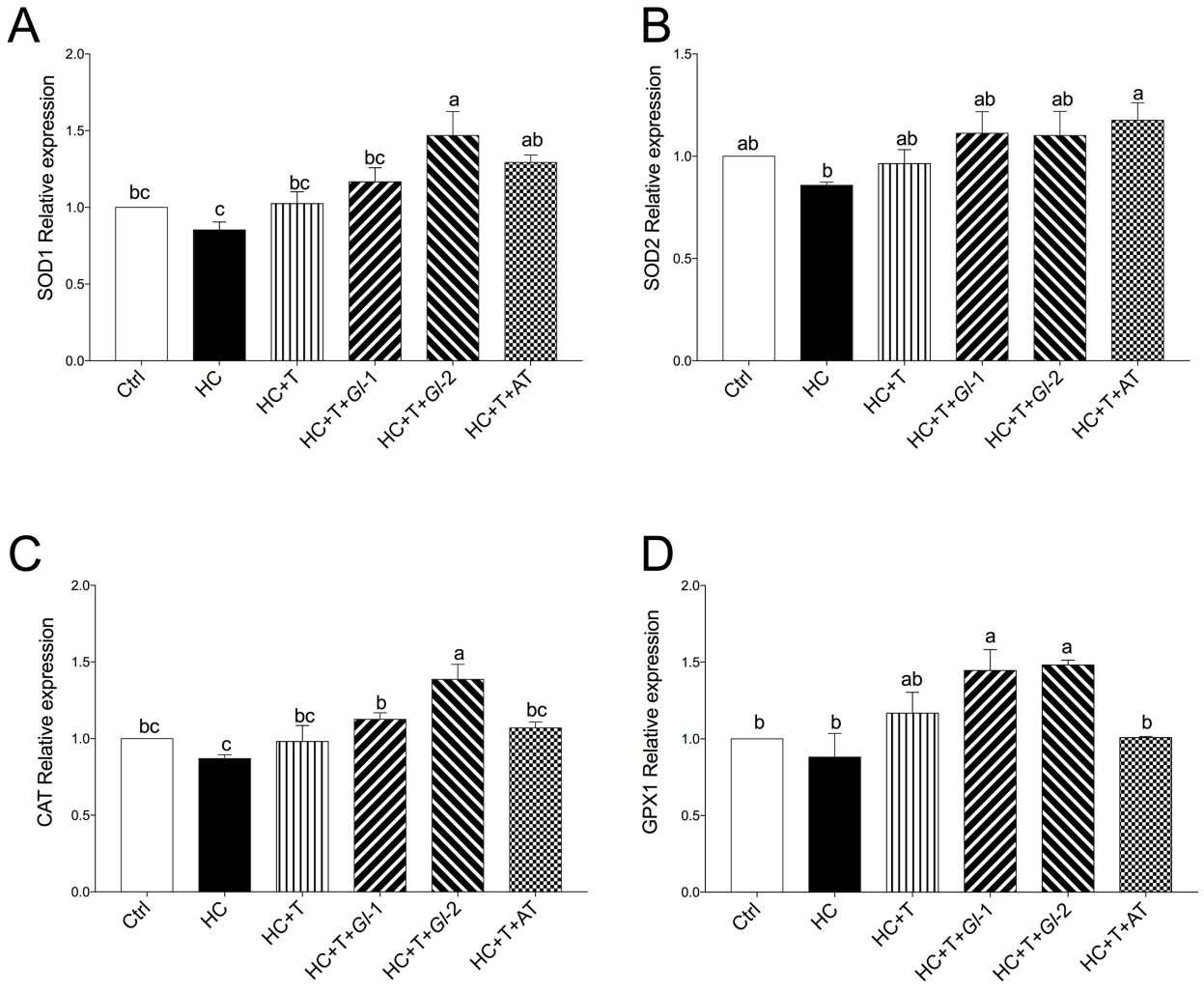

FIG. 2: Average data of liver gene expression from experimental groups studied C57BL/6 mice fed with a high-cholesterol diet for 45 d. (A) SOD1. (B) SOD2. (C) CAT. (D) GPX1. Ctrl: Control diet (AIN-93); HC: High-cholesterol diet (0.5% cholesterol); HC+T: High-cholesterol diet (0.5%) + Control tlayudas (60%); HC+T+*Gl*-1: High-cholesterol diet (0.5%) + Tlayudas containing the *Gl*-1 extract (60%); HC+T+*Gl*-2: High-cholesterol diet (0.5%) + Tlayudas containing the *Gl*-2 extract (60%); HC+T+AT: High-cholesterol diet (0.5%) + Control tlayudas (60%) + Atorvastatin drug (0.03 g/100 g). Data are presented as the mean ± SEM. Means in a column showing differing letters indicate statistically significant difference, $P < 0.05$. Values correspond to one-way ANOVA from the Tukey's multiple comparisons test. The analysis compared the mean of each group against the means of every other group.

expression of SOD2 gene increased 37.1% for the HC+T+AT group, followed by 29.6% for the HC+T+*Gl*-1 group, 28.3% for the HC+T+*Gl*-2 group, and 12.4% for the HC+T group, compared with the HC group (Fig. 2B). There was no differential effect between *Gl*-1 and *Gl*-2 extracts on the expression of the SOD2 gene. The HC group showed a lower expression of the SOD2 gene, compared with the Ctrl group.

The expression of the CAT gene significantly increased in HC+T+*Gl*-1 and HC+T+*Gl*-2 groups, compared with the HC group ($P < 0.0001$). The proportions of increase in CAT expression for mice groups HC+T, HC+T+*Gl*-1, HC+T+*Gl*-2, and HC+T+AT was 12.6%, 29.3%, 59.4%, and 23%, respectively, compared with the HC group (Fig. 2C). The CAT gene had a significant further expression in the HC+T+*Gl*-2 group, compared with the HC+T+*Gl*-1 group. The HC group showed a lower expression of the CAT gene, compared with the Ctrl group.

The expression of the GPX1 gene was higher in HC+T, HC+T+*Gl*-1, HC+T+*Gl*-2, and HC+T+AT experimental groups compared with the HC group, being significantly different in two mice groups (HC+T+*Gl*-1 and HC+T+*Gl*-2; $P <.0001$). The expression of GPX1 gene increased 66.8% for the CH+T+*Gl*-2 group, followed by 62.7% for the HC+T+*Gl*-1 group, 31.4% for the HC+T group, and 13.5% for the HC+T+AT group, compared with the HC group (Fig. 2D). There was no differential effect between *Gl*-1 and *Gl*-2 extracts on the expression of the GPX1 gene. The HC group showed a lower expression of the GPX1 gene, compared with the Ctrl group.

Pharmacological studies have shown that main bioactive compounds from *G. lucidum* possessing antioxidant activity are polysaccharides, polyphenols, terpenoids and sterols. They have been shown to exert antioxidant activity against various pathologies, such as obesity.[16] Krishna et al.[17] reported that polysaccharides from *G. lucidum* show high antioxidant and free radical elimination activity. Furthermore, *G. lucidum* polysaccharides are also capable of reducing the production of oxygen free radicals, slowing the aging process.[18] These polysaccharides have positive effects on the synthesis of SOD, CAT and GPX genes, inducing also hyperlipidemia-reducing effects.[19] Polysaccharides from *G. lucidum* have shown anti-free radical and anti-superoxide capacity, as well as reduction of lipid peroxidation.[20] Wong et al.[21] found that *G. lucidum* showed antioxidant effects on lipid peroxidation and superoxide scavenging activity providing a cardioprotective effect, depending on the dose administered. Cao et al.[22] reported that the administration of *G. lucidum* increased N-acetylcysteinamide and pyruvate in the liver, which are ROS sequestrants, as well as levels of betaine improving antioxidant activity by the upregulation of the expression of SOD and CAT genes. This synergistic effect of SOD and CAT genes eliminates free radicals and H_2O_2. It has been reported that the type of substrate on which mushrooms grow can modify the antioxidant bioactive compounds.[23]

Medicinal mushrooms are an important source of β-glucans and minerals (zinc, selenium, iron), which generate potent antioxidant activity.[24] Main compounds found in *Gl* extracts are α-glucans (*Gl*-1:14.19% w/w; *Gl*-2:15.14% w/w), β-glucans (*Gl*-1: 1.77% w/w; *Gl*-2: 1.87% w/w), zinc (*Gl*-1: 450 Ppb; *Gl*-2: 405 Ppb), selenium (*Gl*-1 and *Gl*-2: <100 Ppb), and iron (*Gl*-1: 200 Ppb; *Gl*-2: 335 Ppb). β-glucans are polysaccharides capable of scavenging radicals to terminate free radical-mediated oxidative stress.[24,25] In the antioxidant defence system, zinc assists as an important co-factor for enzymes, as well as induces the synthesis of metallothioneins, effective for reducing hydroxyl radicals and sequestering reactive oxygen species (ROS).[26] Selenium is essential for the formation of antioxidant selenoproteins, which are crucial for human health. Selenium, as a part of the enzyme glutathione peroxidase, detoxifies lipid peroxides and supplies shielding to cellular membranes against oxidative stress.[27,28] Iron is an important structural element of the antioxidant enzymes CAT, peroxidase, cytochrome oxidase, NADPH reductase, and it is critical for efficient antioxidant defenses.[29] Hydroalcoholic extracts have been reported to be efficient solvents for the extraction of antioxidant compounds in diverse samples due to their polarity.[30] The hydroalcoholic *Gl* extracts tested in this study (32% v/v, alcohol:water), retained main antioxidant compounds (α-glucans, β-glucans, zinc, selenium, iron), and acted synergistically for increasing gene expression of enzymes analyzed. A greater effect on the expression of antioxidant genes SOD-1, CAT and GPX1 was observed in the

mice group consuming tlayudas plus the *Gl*-2 extract, showing that it contains a higher amount of antioxidant bioactive compounds acting synergistically compared with the *Gl*-1 extract.

The results of the present study revealed that the consumption of tlayudas containing *Gl*-1 or *Gl*-2 extracts increase the expression of antioxidant SOD1, SOD2, CAT and GPX1 genes in the liver of C57BL/6 mice fed with a high-cholesterol diet. This is important considering that the high-cholesterol diet administered to the HC group consistently lowered the expression of SOD1, SOD2, CAT, and GPX1 genes, indicating different levels of inhibition compared with the Ctrl group. Under these conditions, *Gl*-1 and *Gl*-2 extracts strengthen the antioxidant defense system at cellular level, inducing the expression of SOD1, SOD2, CAT, and GPX1 genes. Bioactive compounds contained in *Gl*-1 and *Gl*-2 extracts associated to all these antioxidant effects are mainly α-glucans, β-glucans, total polyphenols, antioxidant capacity (ORAC), zinc, selenium, iron. The atorvastatin drug had a similar effect, but to a lesser extent in most cases.

IV. CONCLUSIONS

We showed that the new functional product developed by the authors, consisting of traditional tlayudas (widely consumed Mexican tortilla) containing hydroalcoholic extracts of a Mexican genotype from the medicinal mushroom *G. lucidum*, has potent antioxidant activity as detected by gene expression. Main bioactive compounds associated to this functional property [β-glucans, total polyphenols, antioxidant capacity (ORAC)], contained in *Gl*-1 and *Gl*-2 extracts studied, were remarkably not denatured by the heat treatment (~ 400°C for 6.40 min) of tlayudas cooking process and remained stable in the food matrix. The consumption of tlayudas containing *Gl* extracts increase the expression of antioxidant genes (SOD1, SOD2, CAT, GPX1) in the *in vivo* model of hypercholesterolemia (C57BL/6 mice), whereas decrease serum levels of cholesterol, triglycerides and LDL-c. New functional food products can be developed from highly consumed traditional foods, such as tlayudas, in order to promote healthier diets for preventing cardiovascular and chronic degenerative diseases associated to oxidative stress.

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REFERENCES

1. Pérez Ruíz RV, Aguilar Toalá JE, Cruz Monterrosa RG, Rayas Amor AA, Hernández Rodríguez M, Camacho Villasana Y, Herrera Pérez J. Mexican native maize: Origin, races and impact on food and gastronomy. Int J Gastron Food. 2024;37:100978.
2. García-Rojas DA, Meneses ME, Martínez-Carrera D, Figueroa-Cárdenas JD, Sánchez-Medina MA, Bonilla-Quintero M, Petlacalco-Sánchez B, Martínez-Gutierrez GA, Pérez-Herrera A. Effect of Pleurotus agaves mushroom addition on the physicochemical and sensory properties of blue maize tortillas produced with traditional and ecological nixtamalization. Food Funct. 2020;11:8768–79.
3. Preciado Iñiga G. Desarrollo de tlayudas con propiedades funcionales del hongo medicinal Ganoderma lucidum, su impacto potencial en la alimentación saludable de comunidades rurales de Oaxaca, México [dissertation]. San Pedro Cholula, Mexico: Colegio de Postgraduados, Campus Puebla; 2021.

4. Meneses ME, Martínez-Carrera D, Torres N, Sánchez-Tapia M, Aguilar-López M, Morales P, Sobal M, Bernabé T, Escudero H, Granados-Portillo O, Tovar AR. Hypocholesterolemic properties and prebiotic effects of Mexican Ganoderma lucidum in C57BL/6 mice. PLoS One. 2016;11(7):e0159631.
5. Romero-Córdoba SL, Salido-Guadarrama I, Meneses ME, Cosentino G, Iorio MV, Tagliabue E, Torres N, Sánchez-Tapia M, Bonilla M, Castillo I, Petlacalco B, Tovar AR, Martínez-Carrera D. Mexican Ganoderma lucidum extracts decrease lipogenesis modulating transcriptional metabolic networks and gut microbiota in C57BL/6 mice fed with a high-cholesterol diet. Nutrients. 2021;13(1):38.
6. Martínez-Carrera D, Mayett Moreno Y, Maimone Celorio MR. Los Hongos Comestibles, Funcionales y Medicinales: Aportación a la Dieta, la Salud, la Cultura, al Ambiente, y al Sistema Agroalimentario de México. 1st ed. San Luis Huexotla, Texcoco, México: Editorial del Colegio de Postgraduados, AGRICULTURA, CONACYT, CB-HCFM; 2022.
7. Yang X, Li Y, Li Y, Ren X, Zhang X, Hu D, Gao Y, Xing Y, Shang H. Oxidative stress-mediated atherosclerosis: Mechanisms and therapies. Front Physiol. 2017;8:600.
8. Rahman MA, Abdullah N, Aminudin N. Evaluation of the antioxidative and hypocholesterolemic effects of lingzhi or reishi medicinal mushroom, Ganoderma lucidum (Agaricomycetes), in ameliorating cardiovascular disease. Int J Med Mushrooms. 2018;20(10):961–9.
9. Meneses ME, Martínez-Carrera D, González-Ibáñez L, Torres N, Sánchez-Tapia M, Márquez-Mota CC, Rendón G, Mitzi V, Morales A, Tello-Salgado I, Tovar AR. Effects of Mexican Ganoderma lucidum extracts on liver, kidney, and the gut microbiota of Wistar rats: A repeated dose oral toxicity study. PLoS One. 2023;18(4):e0283605.
10. Martínez-Carrera D, Pérez Armendáriz B, Mayett Y, Sobal M, Morales P, Ramírez P, Tello I, inventors. Propiedades Funcionales Agregadas al Tequila, Otros Mezcales y Destilados de Agave Convencionales, Derivadas del Extracto de un Hongo Comestible de uso Tradicional en México (Lentinula boryana). Mexico patent IMPI 322035. 2014 July 8.
11. Adebayo EA, Martínez-Carrera D, Morales P, Sobal M, Escudero H, Meneses ME, Avila-Nava A, Castillo I, Bonilla M. Comparative study of antioxidant and antibacterial properties of the edible mushrooms Pleurotus levis, P. ostreatus, P. pulmonarius, and P. tuber-regium. Int J Food Sci. Technol. 2018;53(5):1316–30.
12. Ni S, Yu Y, Zhang Y, Wu W, Lai K, Yao K. Study of oxidative stress in human lens epithelial cells exposed to 1.8 GHz radiofrequency fields. PLoS One. 2013;8(8):e72370.
13. Reeves G. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr. 1997;127:838–41S.
14. Bowling J, Katayev A. An evaluation of the Roche Cobas c111. Lab Med. 2010;41(17):398–402.
15. Zhang J, Ma K, Han J, Wang K, Chen H, Bao L, Liu L, Xiong W, Zhang Y, Huang Y, Liu H. Eight new triterpenoids with inhibitory activity against HMG-CoA reductase from the medical mushroom Ganoderma leucocontextum collected in Tibetan plateau. Fitoterapia. 2018;130(2):79–88.
16. Liang Z, Yuan Z, Li G, Fu F, Shan Y. Hypolipidemic, antioxidant, and antiapoptotic effects of polysaccharides extracted from reishi mushroom, Ganoderma lucidum (Leysser: Fr.) Karst., in mice fed a high-fat diet. J Med Food. 2018,21(12): 1218–27.
17. Krishna KV, Karuppuraj V, Perumal K. Antioxidant activity and folic acid content in indigenous isolates of Ganoderma lucidum. Asian J Pharmac Anal. 2016,6(4):213-15.
18. Wang J, Cao B, Zhao H, Feng J. Emerging roles of Ganoderma lucidum in anti-aging. Aging Dis. 2017;8(6):691–707.
19. Yu Y, Shen M, Song Q, Xie J. Biological activities and pharmaceutical applications of polysaccharide from natural resources: A review. Carbohydr Polym. 2018;183:91–101.
20. Cör D, Knez Ž, Knez Hrnčič M. Antitumour, antimicrobial, antioxidant and antiacetylcholinesterase effect of Ganoderma lucidum terpenoids and polysaccharides: A review. Molecules. 2018;23(3):649.
21. Wong KL, Chao HH, Chan P, Chang LP, Liu CF. Antioxidant activity of Ganoderma lucidum in acute ethanol-induced heart toxicity. Phytother Res. 2004;18(12):1024–6.
22. Cao YJ, Huang ZR, You SZ, Guo WL, Zhang F, Liu B, Lv XC, Lin ZX, Liu PH. The protective effects of ganoderic acids from Ganoderma lucidum fruiting body on alcoholic liver injury and intestinal microflora disturbance in mice with excessive alcohol intake. Foods. 2022;11(7):949.
23. Huang Q, Wang L, Zhang L, Hu B, Wang Q, Liang L. Antioxidant properties of triterpenoids isolated from bagasse-cultivated lingzhi or reishi medicinal mushroom, Ganoderma lucidum (Agaricomycetes), at different developmental stages. Int J Med Mushrooms. 2022;24(7):41–51.
24. Chang ST, Wasser SP. The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. Int J Med Mushrooms. 2012;14(2):95–134.
25. Wong WY, Ismail SM, Phan CW, Tan YS. Size matters: Influence of particle size on antioxidant, β-glucan, and anti-inflammatory potential in Pleurotus floridanus (Agaricomycetes). Int J Med Mushrooms. 2024;26(9):17–31.
26. Nascimento Marreiro DD, Clímaco Cruz KJ, Silva Morais JB, Batista Beserra J, Soares Severo J, Soares de Oliveira AR. Zinc and oxidative stress: Current mechanisms. Antioxidants. 2017;6(2):24.

27. Zoidis E, Seremelis I, Kontopoulos N, Danezis GP. Selenium-dependent antioxidant enzymes: Actions and properties of selenoproteins. Antioxidants. 2018;7(5):66.
28. Lubos E, Loscalzo J, Handy DE. Glutathione peroxidase-1 in health and disease: From molecular mechanisms to therapeutic opportunities. Antioxid Redox Signal. 2011;15(7):1957–97.
29. Zaka-Ur-Rab Z, Adnan M, Ahmad SM, Islam N. Effect of oral iron on markers of oxidative stress and antioxidant status in children with iron deficiency anaemia. J Clin Diagn Res. 2016;10(10):SC13–9.
30. Datta S, Sinha BK, Bhattacharjee S, Seal T. Nutritional composition, mineral content, antioxidant activity and quantitative estimation of water-soluble vitamins and phenolics by RP-HPLC in some lesser used wild edible plants. Heliyon. 2019;5(3):e01431.