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Immunomodulatory Impact of Herbs and Probiotics in Type 2 Diabetic Rat Model

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ABSTRACT

Diabetes mellitus exerts many complications, irregular immune responses is more serious one which potentially increases infections. This work aimed to assess, the immunomodulatory effect of cardamom, ginger, and cinnamon without/with probiotics in type2 diabetic rat model (T2DRM). Seventy-two adult male, Wistar rats were assigned to nine groups. Eight rats were kept as healthy control. Sixty-four rats were used to induce T2DRM. One group was offered to 0.2 ml multi-strain probiotics orally. The rest of T2DRM were offered to 100 mg/kg aqueous extract of cardamom, ginger, or cinnamon without/with 0.2 ml multi-strain probiotics orally. Oral glucose tolerance test (OGTT), serum insulin, and C-peptide were determined. Total leucocyte count (TLC), neutrophil (N%), lymphocyte (L%) were calculated. Spleen cellular viability%, phagocytic index (*vitro, vivo*) and glucose consumed with lymphocytes were estimated. Pancreatic tissue glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) activities were assayed. INF-γ, IL-5, and IL-17 cytokines were determined. Groups fed cardamom or ginger with probiotic showed significant ($P<0.05$) improvement in OGTT, serum insulin, spleen cellular viability%, phagocytic index (*vitro, vivo*) and glucose consumed with lymphocytes compared to T2DRM. Besides, they recorded significant amelioration in INF-γ IL-5 and IL-17. Ginger without/with probiotic recorded significant increase ($P<0.05$) in TLC, L%, GSH-Px, SOD and CAT. Cardamom without/with probiotic exerted significant increase ($P<0.05$) in GSH-Px and CAT. The probiotic group showed significant increase ($P<0.05$) in GSH-Px. However, cinnamon had a moderate immunomodulatory effect. This study indicated that administration of ginger or cardamom with probiotics success to achieve immunomodulation of T2DRM through antioxidant and anti-inflammatory effects.

Keywords: Cardamom, Cinnamon, Ginger, Immunomodulatory, Probiotics, Type 2 diabetes.

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INTRODUCTION

Diabetes mellitus (DM) becomes rapidly increased in Eastern societies as in the Kingdom of Saudi Arabia (KSA), due to the modern lifestyle [1]. KSA among the top ten countries worldwide with a high prevalence of diabetes [2]. DM exerts many complications; infections are more common and serious one in patients which potentially increases their morbidity and mortality [3-4]. Patients with DM are reported to experience 21% more infection than the non-diabetic population [5]. Diabetes is characterized by immune dysfunction therefore altered levels of certain cytokines and chemokines [6]. Consequently, some type of infection occurs more frequently in patients with DM including soft tissue, urinary tract, and respiratory tract infections [4, 6]. Hyperglycemia usually accompanied by oxidative stress that believed to increase the levels of pro-inflammatory proteins with infiltrated macrophages secreting inflammatory cytokines which leads to local and systemic inflammation [7]. Patients with DM, showed chronic hyperglycemia, leading to irregular neutrophil synthesis, phagocytosis defects, and acidosis, which limit the activity of the immune system [8]. DM caused oxidative damage to cell membranes due to excessive lipid peroxidation. These oxidative stresses required sufficient antioxidants to eliminate reactive oxygen species (ROS) to stop oxidative damage and trigger inflammation [9-10].

Immunomodulation is a modification of the immune responses using natural or synthetic chemical substances can regulate the immune function. Phytochemicals, the active constituents in herbs have a long history of medicinal uses that have immunomodulatory beneficial effects for fighting, preventing, and treating infectious as well as non-infectious diseases [11]. It is noteworthy that the people who consume spices are less susceptible to the development of chronic diseases. In comparison to the synthetic drugs, the herbs recorded fewer side effects. They are also inexpensive and show better patient tolerance [12-13].

The most common herbs used in KSA, as an Arabian coffee mix that show biological activities are cardamom, ginger, and cinnamon. Cardamom (*Elettaria cardamomum*) exerts immunomodulatory roles and anti-cancer activities, through its anti-inflammatory roles [14-15]. Cardamom

has anti-inflammatory and immunomodulatory, which has been largely attributed to its major active, eucalyptol. Recent research on Cardamom essential oil has shown evidence of its antimicrobial [16], antioxidant [17], and anti-inflammatory properties [18]. It usually used as a treatment for throat infection, lung congestion, and pulmonary tuberculosis [19].

With regard to ginger (*Zingiber officinale*), it contains many bioactive substances, including antioxidants that are responsible for its many health-related conditions. It is widely used for the treatment of many conditions as inflammation of the respiratory system [20]. Ginger can treat many inflammation-related diseases due to its anti-inflammatory and antioxidant properties [21]. An aqueous ginger extract has the ability to deal with the innate and acquired immunities, it has the potential to improve the birds' antioxidant capacity, enhance immune function, and reduce the inflammatory response [22].

Cinnamon (*Cinnamomum Verum*) uses as herbal medicine, the major active component of it is made up of cinnamaldehyde [12]. Cinnamaldehyde may help fight many types of infection as respiratory tract infections [23]. Certain antioxidant phytochemicals that have been found in cinnamon may reduce oxidative stress in tissue [24] demonstrated that the antioxidant effect of cinnamaldehyde have potential for treating skin disorders on human keratinocytes. *Cinnamomum* has beneficial effects on the immune system in the animal that it improves humoral, cellular, and innate immunity. Besides, it showed immunomodulatory activity through normal and infection-related immuno-compromised conditions [10, 25].

Concerning probiotics, it has been found that the most important properties are regulation of host immune response thereby, they are able to treat infectious and non-infectious diseases in animal models [26-27]. The probiotics usually balance and control pro-inflammatory and anti-inflammatory cytokines [28]. Noteworthy, the immunomodulatory effects of probiotics has been documented by many researches. *L. Plantarum ZDY* 2013 pretreatment have an immunomodulatory response and can prevent gastric mucosal inflammation [29].

From our knowledge, many studies have been conducted to study the effect of various herbs or probiotics separately, as an immunomodulatory agents in the nondiabetic hosts, but no work was performed to study the impact of herbs without/with probiotics in a diabetic animal model. Therefore, the current work aimed to assess, for the first time, the immunomodulatory, anti-inflammatory, and antioxidants effect of the common herbs used in KSA (cardamom, ginger, and cinnamon) without/with probiotics in type 2 diabetic rat model (T2DRM).

Materials and Methods

Animal

Adult male Wistar rats (weight 225 ±25 g) were obtained from the King Saud University laboratory center, Riyadh, KSA. The animals were transported to suitable housing rooms at the College of Agriculture and Veterinary Medicine, Qassim University, KSA. For adaptation to the new environment, rats were kept in cages at constant room temperature (22 ± 2◦ C) under a photoperiod 12-h light/dark cycle with free access to water and feed. The commercial diet obtained from General Company of Feed Silo and Powder Mint. The diet was formulated to furnish the requirements of NRC [30]. The experimental conditions carried according to the guidelines for animal care and ethics as recommended by Scientific Research Dean. After 1 week of acclimatization, the animals were prepared for the T2DRM.

Induction of T2DRM

After the period of acclimatization, the animals were offered to high-fat diet mixed in Faculty Lab. to induce obesity. High-fat diet composted of casein 23.31%, L-cystine 0.35%, corn starch 8.49%, maltodextrin 11.63%, sucrose 20.13%, cellulose 5.82%, sunflower oil 2.91%, beef tallow 20.72% and Mineral Vitamin Mix 6.64%. Therefore, it furnished 45% of calories as fat as suggested by Research Diets. Inc. [31]. After 2 weeks from a received the high-fat diet, overnight-fasted animals were dosed intravenously once with 50 mg/kg streptozotocin (STZ) as recorded by Skovsø [32]. STZ was obtained from Santa Cluz, Germany). A total of 3 days after STZ treatment, rats that had reached an elevated blood glucose plateau were included in the study.

Herbs

Herbs seeds: cardamom (*Elettaria cardamomum L. Maton*), ginger (*Zingiber officinale Roscoe*), and cinnamon (*Cinnamomum Verum J. Presl*) were purchased from the local market, Qassim, Saudi Arabia. The voucher specimens were deposited at Janaki Ammal Herbarium, IIIM, Jammu under G. No.14. Accession number, 2751 for *Elettaria cardamomum L. Maton*, G. No.15. Accession number, 2753, for *Zingiber officinale Roscoe*, and G. No.16. Accession number, 2483 for *Cinnamomum Verum J. Presl*. The dried herb's seeds were ground separately into a fine powder and kept for analysis and preparation of the aqueous extract.

Gas Chromatography- Mass Spectral analysis (GC/MS)

Herbs methanol extracts were prepared by soaking 20 gm. of each herb in 100 ml of methanol 99.9%. After 24 hours, was filtered and dehydrated by sodium sulfate. GC/MS analysis of methanol extracts of the plants were analyzed according to Soumya et al. [33] using Agilent Gas Chromatography (Model 6890N coupled to 5973 Mass Selective Detector (MSD), (USA). Gas chromatograph was interfaced to a Mass Spectrophotometer equipped with Elite -5MS (5% diphenyl / 95% Dimethyl poly siloxane), 30 x 0.25 mm x 0.25 µm df. The total GC/MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas using software adopted to handle mass spectra and chromatograms (Turbo Mass Version 5.2) (Table 1).

Antioxidants activity of herbs

For the preparation of plant extracts, 0.1 g of each herb was stirred for 3 minutes in 10 ml 50% aqueous ethanol at 25,000 rpm using a homogenizer (IKA, Germany). Samples were then centrifuged at 3500 rpm 10 min and the supernatants were used for further analyses [34].

The total phenolic content (TPC) was estimated using the Folin-Ciocalteu method using gallic acid as the standard. A 100-µL aliquot of plant extract was oxidized with diluted Folin-Ciocalteu reagent (500 µL). After 5 min, the mixture was neutralized with 1 ml sodium carbonate (7.5%, w/v), and incubated for 120 min before reading absorbance at 765 nm. The total phenolic content (TPC) is expressed as mg gallic acid equivalents (GAE) per 100 g dry weight of samples [35].

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) modified assay method of Brand-Williams et al. [36] was used to

determine antioxidant activity. 2 ml methanolic DPPH solution (40 mg/L) was mixed with 100 µl sample extract. Samples were incubated in the dark at room temperature for 30 min and then the absorbance of the solution at 517 nm was measured.

2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) radical scavenging activity assay modified method of Re et al. [37] was used to determine antioxidant activity using Trolox as the standard. ABTS radical cations were generated by oxidizing 7 mM ABTS with 2.45 mM potassium persulfate, and the mixture was kept in the dark at room temperature for 12 h before use. The ABTS solution was diluted with distilled. For assays, 1 ml ABTS cation solution was mixed with 100 µL sample extract, and the decrease in absorbance at 734 nm was measured.

Preparation of herbs aqueous extracts

Air-dried powder of each herb (10 g) was mixed well in 100 ml distilled water and kept at room temperature for 24 h. The solution was filtered using a muslin cloth. The filtrate was then centrifuged at 5000 rpm for 15 min. The supernatant was filtered through Whattman filter No. 1 and the filtrate was collected in a pre-weighed test tube [38]. Aqueous extracts were prepared in a final concentration of 100 mg/ml.

Probiotic

Eight strains of probiotics bacteria were established in this study as follows: *Lactobacillus acidophilus, Bifidobacterium lactis, Bifidobacterium longum, Lactobacillus rhamnosus, Bfidobacterium breve, Lactobacillus casei, Lactobacillus plantarum, and Lactobacillus salivarius.* The bacterial strains were obtained from iHerb Company, Danisco, USA as a mixture of lyophilized strains. The activity of strains was $1x10^9$ cfu per rat as oral intake. The lyophilized mixture strains were added to sterilize phosphate buffer saline (PBS) and were daily administrated by oral gavage in 0.2 ml of PBS [39].

Experimental design and sampling

Seventy-two rats were divided into nine groups eight per each. Animals received the treatment as follows; Group (1): healthy control rats fed a commercial diet with no supplementation, Group (2): type 2 diabetic rats' model (T2DRM) kept as a positive control. Group (3): T2DRM received 0.2 ml multi-strain probiotics in PBS orally. Group (4, 5): T2DRM gavaged with cardamom aqueous extract 100 mg/kg without/with 0.2 ml multi-strain probiotics in PBS orally respectively. Group (6, 7): T2DRM gavaged with ginger aqueous extract 100 mg/kg without/with 0.2 ml multi-strain probiotics in PBS orally, respectively. Group (8, 9): T2DRM gavaged with cinnamon aqueous extract 100 mg/kg without/with 0.2 ml multi-strain probiotic in PBS orally, respectively. Animals were subjected to the treatment for 12 weeks; through it herbs extract aqueous were dissolved in water and administered orally once a day.

At the end of the experiment, rats were anesthetized by diethyl ether, bleed, and sacrificed. Part of blood samples was placed in tubes with EDTA for counting the total leucocyte count (TLC) and differential leucocytes count. Serum was prepared, labeled, and stored deep-frozen (-20°C) until used for cytokines analysis. Three animals from each group were killed, pancreas and spleen were gently removed. Pancreatic tissue was used to estimate antioxidant enzymes activity. Spleen tissue was used to detect spleen lymphocytes viability %.

Measurements

Oral glucose tolerance test (OGTT)

Fasting blood glucose levels (FBG) was carried out at 2, 4, 8, 10, and 12 weeks. The animal was starved for 16 h, and then blood was collected via the tail vein to measure FBG. After starvation, the animal has received glucose (1.0 g/kg) solution by oral administration. After 2 h, the blood samples were collected via tail vein and taken for the measurement of postprandial 2 h blood glucose levels (PB2). Glucose levels were measured with Blood Glucose Monitoring System Glucometer (One-Touch Basic; MedNet GmbH 48163 Munster, Germany) in rat tail vein blood [40].

Serum insulin and C-peptide

Serum insulin level was done using ELISA Kits. (SE120069-1KT. Lot No. INS4565, Sigma Aldrich, USA). Quantitative determination of serum C-peptide level was performed using ELISA Kits (SE120040-1KT. Lot No. CPT4779, Sigma Aldrich, USA).

Leucogram

Blood samples with EDTA were used for counting the TLC according to Coles [41]. The differential leucocytes count for neutrophil (N %), lymphocyte (L %) and N/L ratio within one hour after collection by cross-sectional method [42].

Spleen lymphocytes viability %

At the end of the experiment, the spleen was used for the preparation of splenocytes [43]. The spleen specimens were suspended in ice-cold RPMI culture medium (Sigma-Aldrich), then engaged with tweezers to release the splenocytes. The released cells were washed 2 times in culture medium. Red cells were lysed with warm isotonic ammonium chloride lysing solution [44]. After centrifugation, the cells were suspended in a culture medium. Viable cell number was calculated in percentage by trypan blue dye exclusion method [43].

Phagocytic activity

In vitro, the phagocytic activity of T cells in blood was used to estimate carbon clearance assay. Blood samples at the end of the experiment (1.5 ml) were mixed with 5ul Indian ink. Each mixed sample was divided into 3 equal parts. Each part was diluted with 2 ml saline and incubated for 20 and 40 min at 37°C, followed by centrifugation at 50 g for 4 min. The optical densities (OD) of supernatants were visualized by spectrophotometer at 535 nm, with a serum ink-free sample as a blank. Phagocytic indices were calculated by converting the ODs to log2 scale per hour [45].

In vivo, at the end of the experiment, 3 animals from each group were selected for intravenously injected with10 ml/kg body weight of Indian ink dispersion. Then blood samples were collected at an interval of 2 min and 10 min after the injection of ink [46-47]. Red cells were hemolysed by the addition of 4 ml of lysis buffer to each sample (NH_4Cl (0.155M), 90 gm, $KHCO_3$ (0.01M) 10 gm EDTA (0.1mM) 370 mg) dissolved in one liter of ddH_2O). OD was measured spectrophotometerically at 675 nm, after 10 min of blood collection. Rate of carbon clearance (K) and phagocytic index were calculated by using the following formula:

K = log OD of first reading – log OD of second reading / 10 min – 2 min

Glucose consumed with lymphocytes stimulated by PHA (Lymphocyte transformation test)

RBCs present in whole blood were removed to obtain leucocytes using lysis buffer. Filter was done through .22-micron filter. Buffer was diluted 1:10 in ddH2O before use. Whole blood (200 µl) was mixed with 2 ml of lysis buffer, incubated at room temperature for 5 minutes, and centrifuged at 300 x g in order to remove lysis buffer. Assay of lymphocyte transformation was done using a glucose consumption test as previously described by Kosti et al. [48]. Phyto-haemagglutinin-P (PHA, Sigma-Aldrich) was used as T cell mitogen. Lymphocytes were cultured in triplicate in 24-well culture plates. Each well enclosed 200 µl of culture suspension containing 2 × 10^6 cells with the addition of 5 µg/mL PHA. The plates were kept in CO2 incubator (CO2 5%) and 37◦ C for 3 days. Incubation media were separated for glucose estimation by using glucose assay kits at 500 nm. The lymphocyte activity was calculated as follow:

Quantity of glucose consumed (mg/dL) - the quantity of glucose concentration of cell culture media of control samples.

Antioxidant activity of pancreatic tissue

The pancreas specimens were taken at the end of the experimental period and washed with saline in an ice bath and homogenized in the ratio 1:10 (w:v) with ice-cold Tris-HCl (0.1M, pH 7.4) [49]. The homogenates were stored at -80°C until use. The suspended mixture was centrifuged at 3000 r.p.m for 10 min at 4 °C in a refrigerated centrifuge. The resulting supernatant was used for the assay of antioxidant enzyme activities. Glutathione peroxidase (GSH-Px), Superoxide dismutase (SOD) and Catalase (CAT). GSH-Px (BIODIAGNOSTIC Kits, CAT. No 2524), SOD (BIODIAGNOSTIC Kits, CAT. No. 25 21), and CAT (BIODIAGNOSTIC Kits, CAT. No. 25 17). Protein content of the supernatants was determined [50]. Enzymes activities were expressed in terms of U/mg protein.

Serum Cytokines

Th1 pro-inflammatory cytokine, (INF-γ) determined using ELISA kits (Assaypro, 30 Triad South Drive, St Charles MO 63304, USA). Th2 anti-inflammatory cytokines including Interleukin-5 (IL5) and (IL17) were assayed by ELI-SA kit (Cusabio Biotech Co., Ltd. Lot: 004152651, Wuhan, China). The manufacturer's instructions were followed, and the color change was measured spectrophotometrically at a wavelength of 450 nm.

Statistical Analysis

Obtained data were calculated and statistically analyzed by SPSS 19 version for Windows. The differences between groups were determined with variance analysis (one-way analysis of variance [ANOVA]). When the differences were significant, Student-newman-kuels test was performed. All data were recorded on an individual basis. Significant differences were determined by Duncan,s New Multiple Range and used to distinguish between significant means at $P<0.05$ [51]. Data were expressed as means ± SE.

Ethical approval

The protocol for this study was approved under the number "cavm-2018-1-14-S-3478" and was recommended by the Deanship of Scientific Research, Qassim University, KSA.

Table 1: The main active compounds detected by area% of varies herbs used in the current experiment

Cardamon		Ginger		Cinnamon	
Name of compound	Area %	Name of compound	Area %	Name of compound	Area %
Terpineol	47.10	Zingiberene	25.47	Cinnamaldehyde	62.47
Eucalyptol	20.93	α-Bergamotene	22.87	Linaloolb	21.68
Linalool	4.79	AR-curcumene	20.15	Limonene	6.54
		Methylcyclohexane	15.33	a-Terpineolb	5.74
		β-esquiphellandrene	10.89		

Table 2: Antioxidant activity of varies herbs used in the current experiment

Parameters / Herbs	TPC	DPPH	ABTS
Cardamom	293.03± 32.54	12.50838±0.41	11.93548±0.96
Ginger	1121.81±37.87	60.16097±6.19	37.25806±0.16
Cinnamon	1608.18±42.42	86.48558±0.99	92.25806±0.32

Total Phenolic Content (TPC) expressed as mg gallic acid equivalents (GAE) per 100 g, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) express percentage inhibition of the DPPH radical, and 2, 2'-Azino- Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS). Mean± Standard error (SE)

Table 3. Effect of herbs aqueous extract without/with probiotic on oral glucose tolerance test (OGTT), serum insulin and C-peptide levels in T2DRM.

Parameters / Groups	OGTT		Insulin (uU/ml)	C-peptide (ng/ml)
	FBG (mg/dl)	PB2 (mg/dl)		
Healthy control	89.95±3.41	120.24±9.53	16.82±1.59	7.86±0.74
T2DRM	237.63±8.50*	274.59±13.62*	21.57±1.02*	12.21±0.98*
T2DRM + probiotic	180.79±10.42	195.74±16.83	17.68±1.46	9.95±1.74
T2DRM + cardamom	134.64±9.68a	156.23±10.94a	17.76±1.54	8.34±1.53
T2DRM + cardamom+ probiotic	122.11±8.41a	149.58±8.12a	16.45±0.92a	8.33±1.32
T2DRM + ginger	132.16±8.53a	151.21±10.43a	17.21±0.89	7.49±1.11
T2DRM + ginger+ probiotic	115.11±7.63a	132.27±9.78a	16.11±0.64a	7.51±1.23
T2DRM+ cinnamon	181.85±10.43	205.45±15.45	17.36±1.58	8.12±1.52
T2DRM + cinnamon+ probiotic	176.32±10.32a	197.38±13.63	17.95±0.66	9.29±1.32

Values in the same column with the mark (*) of the T2DRM group were differ significantly from the value of healthy control at P<0.05. Values of the treated groups with the letter (a) were differed significantly from the value of the T2DRM group at P<0.05. Mean± Standard error (SE), T2DRM: type 2 diabetic rat model. FBG: Fasting blood glucose level, PB2: postprandial 2 h blood glucose level

Table 4. Effect of herbs aqueous extract without/with probiotic on total leucocytes count (TLC); Neutrophil (N %); Lymphocyte (L %) and N/L ratio in T2DRM.

Parameters / Groups	**TLC $10^3/mm^3$**	**N %**	**L %**	**N/L ratio**
Healthy control	8.84±1.01	53.86±1.09	59.76±1.11	0.91±0.03
T2DRM	5.97 ±0.11*	52.63 ±1.14	53.47 ±1.15*	1.02±0.04
T2DRM + probiotic	6.10 ±0.14	55.11 ±1.02	55.84 ±1.26	1.02±0.09
T2DRM + cardamom	6.95 ±0.12	56.34 ±0.18	56.74 ±1.63	1.03±0.04
T2DRM + cardamom+ probiotic	7.59 ±0.15	57.12 ±0.16	59.35 ±1.03a	0.95±0.05
T2DRM + ginger	8.11 ±0.13a	54.77 ±1.07	58.89 ±1.00a	0.99±0.05
T2DRM + ginger+ probiotic	8.94 ±0.17a	55.85 ±1.10	60.81 ±1.12a	0.92±0.04
T2DRM + cinnamon	7.12 ±0.11	54.71 ±0.18	53.23 ±1.91	1.01±0.06
T2DRM + cinnamon+ probiotic	7.80 ±0.16	56.52 ±1.06	55.65 ±1.37	1.02±0.05

Values in the same column with the mark (*) of the T2DRM group were differ significantly from the value of healthy control at P<0.05. Values of the treated groups with the letter (a) were differed significantly from the value of the T2DRM group at P<0.05. Mean± Standard error (SE), T2DRM: type 2 diabetic rat model.

Table 5: Effect of herbs aqueous extract without/with probiotic on Spleen cellular viability %, *in vitro* and *in vivo* phagocytic index in T2DRM.

Parameters / Groups	**Spleen cellular viability %**	***In vitro* phagocytic index**	***In vivo* phagocytic index**
Healthy control	97.19±1.14	0.0033 ± 0.0001	0.0066 ± 0.00003
T2DRM	83.18±1.12*	0.0018 ± 0.0001*	0.0031 ± 0.00003*
T2DRM + probiotic	91.48±1.16a	0.0025 ± 0.0002	0.0027 ± 0.00006
T2DRM + cardamom	87.24±1.12	0.0029 ± 0.0000a	0.0047 ± 0.00005a
T2DRM + cardamom+ probiotic	90.13±1.21a	0.0027 ± 0.0002a	0.0058 ± 0.00002a
T2DRM + ginger	88.23±1.19	0.0025 ± 0.0001	0.0033 ± 0.00004
T2DRM + ginger+ probiotic	92.54±1.13a	0.0031 ± 0.00003a	0.0057 ± 0.000006a
T2DRM + cinnamon	88.23±1.24	0.0026 ± 0.00002	0.0037 ± 0.000006
T2DRM + cinnamon+ probiotic	86.53±1.23	0.0032 ± 0.00002a	0.0059 ± 0.0002a

Values in the same column with the mark (*) of the T2DRM group were differ significantly from the value of healthy control at P<0.05. Values of the treated groups with the letter (a) were differed significantly from the value of the T2DRM group at P<0.05. Mean± Standard error (SE), T2DRM: type 2 diabetic rat model.

Table 6. Effect of herbs aqueous extract without/with probiotic on glucose consumed with lymphocytes stimulated by PHA in T2DRM

Parameters / Groups	Glucose in the medium (mg/dl) after 72hrs of incubation.		
	Without PHA	**With PHA**	**Glucose consumed**
Healthy control	38.43 ± 1.77	33.11 ± 1.26	5.34±1.05
T2DRM	37.27± 2.23	35.68 ± 2.47	2.12±0.17*
T2DRM + probiotic	40.21 ± 2.54	36.46 ± 1.47	4.31±0.95
T2DRM + cardamom	38.84 ± 2.92	33.84 ± 1.47	4.86±1.04
T2DRM + cardamom+ probiotic	38.68 ± 1.58	31.64 ± 2.47	6.87±0.33a
T2DRM + ginger	39.39 ± 1.27	35.14 ± 1.47	4.87±0.92
T2DRM + ginger+ probiotic	39.22 ± 1.17	34.17 ± 1.47	5.89±0.34a
T2DRM + cinnamon	38.39 ± 2.18	33.46 ± 1.47	4.66±0.29
T2DRM + cinnamon+ probiotic	39.46 ± 1.29	36.63 ± 2.47	3.16±1.65

Values in the same column with the mark (*) of the T2DRM group were differ significantly from the value of healthy control at P<0.05. Values of the treated groups with the letter (a) were differed significantly from the value of the T2DRM group at P<0.05. Mean± Standard error (SE), T2DRM: type 2 diabetic rat model; PHA: Phytohaemagglutinin-P.

Table 7. Effect of herbs aqueous extract without/with probiotics on pancreatic tissue glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) activities in T2DRM

Parameters / Groups	**GSH-Px (U/mg protein)**	**SOD (mU/mg protein)**	**CAT (U/mg protein)**
Healthy control	462.85±24.57	2.24±0.24	38.85±3.47
T2DRM	319.43±23.69*	1.07±0.16*	23.14±2.34*
T2DRM + probiotics	422.32±15.57a	1.55±0.23	32.67±2.82
T2DRM + cardamom	435.75±32.74a	1.38±0.56	39.38±2.38a
T2DRM + cardamom+ probiotics	514.83±33.58a	1.54±0.39	37.83±3.57a
T2DRM + ginger	485.39±34.12a	1.79±0.18a	38.95±4.11a
T2DRM + ginger+ probiotics	507.57±34.23a	1.82±0.27a	36.74±2.36a
T2DRM + cinnamon	424.54±63.69	1.48±0.22	33.53±3.48
T2DRM + cinnamon+ probiotics	425.85±72.41	1.52±0.46	34.86±4.89

Values in the same column with the mark (*) of the T2DRM group were differ significantly from the value of healthy control at P<0.05. Values of the treated groups with the letter (a) were differed significantly from the value of the T2DRM group at P<0.05. Mean± Standard error (SE), T2DRM: type 2 diabetic rat model.

Table 8. Effect of herbs aqueous extract without/with probiotic on (INF-γ, IL-5 and IL-17) Changes in T2DRM.

Parameters / Groups	**INF-γ (pg/ml)**	**IL-5 (pg/ml)**	**IL17 (pg/ml)**
Healthy control	13.38±0.78	16.26±0.59	15.48±0.47
T2DRM	17.36±0.59*	12.67±0.48*	11.28±0.16*
T2DRM + probiotic	15.46±0.95	15.86±0.33a	14.95±0.17a
T2DRM + cardamom	16.32±0.86	15.33±0.82	13.55±0.56
T2DRM + cardamom+ probiotic	13.14±0.27a	16.56±0.48a	15.39±0.17a
T2DRM + ginger	14.24±0.78	14.78±0.29	13.14±0.67
T2DRM + ginger+ probiotic	12.22±0.47a	16.97±0.74a	16.29±0.58a
T2DRM + cinnamon	15.15±0.54	14.38±0.74	13.46±0.87
T2DRM + cinnamon+ probiotic	15.74±0.67	14.89±0.66	14.01±0.96

Values in the same column with the mark (*) of the T2DRM group were differ significantly from the value of healthy control at P<0.05. Values of the treated groups with the letter (a) were differed significantly from the value of the T2DRM group at P<0.05. Mean± Standard error (SE), T2DRM: type 2 diabetic rat model.

RESULTS

Chromatographic analysis of herbs by GC–MS: The main active compounds of herbs extract detected by GC–MS were terpineol and eucalyptol for cardamom, zingiberene, α-bergamotene and AR-curcumene for ginger and cinnamaldehyde and linaloolb for cinnamon which are in agreement with many types of research (Table 1).

Antioxidants activity of herbs (TPC, DPPH and ABTS radical scavenging): TPC, DPPH, and ABTS were showed the lowest activities in cardamom, however, the highest activities were recorded in cinnamon followed by ginger (Table 2).

OGTT: Animals in the healthy control group had normal FBG levels after 16 h starvation (89.95±3.41) mg/dl at week 12. In contrast, T2DRM showed a significant rise in FBG level (237.63±8.50) mg/dl (P<0.05), whereas all experimental treatments recorded an improvement in FBG in a variable manner. Nevertheless, the improvement in FBG in groups received probiotic, or cinnamon alone was not significant. Groups offered to cardamom, or ginger without/with probiotics recorded a significant reduction in FBG if compared with T2DRM (P<0.05). The decline in blood glucose levels reached its highly significant level in T2DRM received ginger with a probiotic (P<0.05) (table 3). PB2 follows the same pattern of FBG. Animals received cardamom or ginger with probiotic suppressed PB2 by 45 and 52%, respectively as compared to T2DRM. However, rats fed sole cinnamon or probiotics had only 25 and 27% reduction in PB2 respectively relative to T2DRM (Table 2). In general, blood glucose level was improved in rats fed herbs with probiotics as compared to those fed herbs or probiotics alone.

Serum insulin and C-peptide: Serum insulin and C-peptide levels were found to be significantly higher in the T2DRM than the healthy control group (P<0.05). All experimental treatments showed an insignificant decrease in serum C-peptide comparable to T2DRM (table 3). Whereas animals received cardamom or ginger with probiotics recorded a significant reduction in serum insulin in comparison to T2DRM (P<0.05).

TLC, N %, L %, and N/L ratio: Table 4 revealed that T2DRM had a significant decrease (P<0.05) in TLC and L % in comparison to the healthy control group (5.97 ±0.11, 53.47 ±1.15 vs 8.84±1.01, 59.76±1.11). Whereas, L % showed significant increase in groups received cardamom or ginger with probiotics as well as group offered to ginger without probiotics. On the other hand, ginger without/with probiotics exerted significant (P<0.05) improvement in TLC comparable to T2DRM. It is clear that N % and N/L ratio did not show any significant difference between different experimental groups.

Spleen cellular viability %, *in vitro* and *in vivo* phagocytic indices: Spleen cellular viability % *in vitro* and *in vivo* phagocytic indices recorded significant (P<0.05) decrease in T2DRM comparable to the healthy control group (Table 5). Whereas those three parameters showed a significant increase (P<0.05) in groups received cardamom or ginger with probiotics comparison to T2DRM. There were significant (P<0.05) increase in spleen cellular viability % and in phagocytic indexes (*vitro, vivo*) in the group received probiotics, and group offered to cinnamon with probiotic respectively.

Glucose consumed with lymphocytes stimulated by PHA: The results of glucose consumed with lymphocytes stimulated by PHA were illustrated in table 6. It showed that there was significant (P<0.05) poorness in glucose consumed by lymphocytes in T2DRM in comparison to the healthy control group. Rats offered to cardamom or ginger with probiotic exerted a significant (P<0.05) improvement in glucose consumed. However, other experimental groups recorded the same improvement in glucose consumed by lymphocytes but not significant.

Antioxidant activity of pancreatic tissue: T2DRM showed significant (P<0.05) decrease in the activities of pancreatic GSH-Px, SOD and CAT antioxidants enzyme (319.43±23.69, 1.07±0.16 and 23.14±2.34) comparable to the healthy control group (462.85±24.57, 2.24±0.24 and 38.85±3.47) (table 7). All experimental groups exerted improvement in those antioxidants' enzyme, however, the group received ginger or cardamom without/with probiotics showed significant (P<0.05) increase GSH-Px and CAT if compared with T2DRM. Nevertheless, the activities of pancreatic SOD did not significantly affect in experimental groups except in groups offered to ginger without/with probiotics which showed significant (P<0.05) increase (1.79±0.18 and 1.82±0.27) respectively if compared to T2DRM. Cinnamon without/with probiotics exerts slight improvement in those three antioxidants enzyme

INF-γ, IL-5, and IL-17: To evaluate the immunomodulatory impact of the aqueous extracts of selective herbs (cardamom, ginger, or cinnamon) without/with probiotics production of the pro-inflammatory IFNγ, and anti-inflammatory IL-5, and IL-17 were estimated in serum. There were significant differences (P<0.05) of T2DRM in INF-γ, IL-5 and IL-17 comparable to the healthy control group (Table 8). The obtained results revealed that herbs (cardamom or ginger) with probiotic modified significantly (P<0.05) those three parameters to be nearly equal to the healthy control group, INF-γ: (13.38±0.78 vs 13.14±0.27, 12.22±0.47) IL-5: (16.26±0.59 vs 16.56±0.48, 16.97±0.74) and IL-17: (15.48±0.47 vs 15.39±0.17, 16.29±0.58) in cardamom or ginger with probiotic groups respectively. Sole administration of probiotics showed a significant increase (P<0.05) in anti-inflammatory cytokine, IL-5, and IL-17.

DISCUSSION

Hyperglycemia is one of the most complications of type 2 diabetes, it is associated with the risk of inflammation and oxidation that may compromise the immune system causing the immune-depressive conditions. The current study used anti-inflammatory and antioxidants herbs (cardamom, ginger, and cinnamon) without/with probiotics for immunomodulation of type 2 diabetes.

In the present study, groups offered to cardamom or ginger without/with probiotics recorded a significant reduction in FBG and PB2 caused by T2DRM. The obtained results are confirmed by many studies since the inclusion of ginger extracts led to a significant reduction in blood glucose [52]. The main component of ginger, gingerols could maintain glucose homeostasis and show anti-hyperglycemic effect in type 2 diabetic mice [53]. Regarding cardamom, its supplementation had glycemic control in type 2 diabetes [54]. The sole use of probiotics

or cinnamon alone exerted an improvement in FBG and PB2, but not reached to be significant. A moderate hypoglycaemic effect of probiotics was previously recorded [55]. Many studies concluded that fasting plasma glucose levels moderately reduced when diabetic patients received an aqueous cinnamon extract [56].

The significant increase in serum insulin level obtained in the T2DRM group may be attributed to type 2 diabetes is associated with obesity. Adipose tissue produces several hormone-like compounds that can increase insulin resistance [57]. Besides, the body began to secrete excessive insulin to maintain blood glucose at a normal level, leading to hyperinsulinemia [58]. Some treated groups showed insignificant decrease in serum insulin and C-peptide. Whereas animals received cardamom, and ginger with probiotics recorded a significant reduction in serum insulin comparable to T2DRM. Probiotics play a vital role in insulin resistance and type 2 diabetes by triggering low-grade inflammation [59].

The significant decrease in TLC and L % in T2DRM may be due to diabetes affects the damage of neutrophils function such as phagocytes [3]. Diabetic hosts have immunosuppression and deficit in neutrophil phagocytosis and microbial killing cells [60]. Immunosuppression in diabetic patients may be due to a reduction in phagocytosis as a result of hyperglycemia which accompanied by cytosolic calcium in polymorphonuclear leucocytes [8]. Groups received cardamom or ginger with probiotics showed a significant increase in L % which was parallel to the work of Mahassni and Bukhari [20] who concluded that the aqueous ginger extract had a significantly higher lymphocyte, lower neutrophil count that benefits to the immune system. With regard to cardamom, the active component of cardamom, eucalyptol might act as potent modulators of the immune response through the increased phagocytic ability of macrophages which are responsible for immunomodulation [61]. Concerning, probiotics many studies proved its vital role on immune responses, Azad et al. [62] found that *Lactobacillus* and *Bifidobacterium* exert beneficial impact on innate immunity by increasing the cytotoxicity of natural killer cells and phagocytosis of macrophages. On the other hand, *Lactobacillus* had a certain impact on innate immune and specific responses immune biomarkers through phagocytosis and pro-inflammatory cytokine production [63].

Cinnamon without/with probiotics showed a slight improvement in immune response biomarkers through neutrophil %; Lymphocyte % and N/L ratio. Contrary, Ose et al. [64] indicate that the anti-inflammatory effect of cinnamon might be exploited for fighting many diseases.

Spleen cellular viability % and phagocytic indices *in vitro* and *in vivo* recorded in the present study indicated a significant decrease in T2DRM which may be due to hyperglycemia and hyperinsulinemia recorded in the current study. Consequently, it may irregular the immune responses causing immunosuppressive [3]. The damage of neutrophils in *vitro* and in *vivo* recorded in T2DRM may cause a defect of phagocytes and immunosuppressive state of diabetes [6, 65]. This is justified that patients with diabetes have a high risk of some infections [4]. Groups received cardamom or ginger with probiotics recorded a beneficial effect in the immune responses through improvement in spleen cellular viability % and phagocytic indices *in vitro* and *in vivo* which are compatible with many studies on herbs and probiotics. Cardamom aqueous extracts in a dose-dependent significantly enhance the proliferation of splenocyte [14]. Many researchers indicated that cardamom possesses anti-inflammatory and immunomodulatory potency due to its active component, eucalyptol [15, 18]. Recently, it has been concluded that ginger contains many bioactive substances, including antioxidants that benefit to immune responses [20]. Likewise, an et al. [22] showed that ginger extract has the potential to improve the birds' antioxidant capacity, enhance immune function, and reduce the inflammatory response. The beneficial results obtained when cinnamon offered with probiotics through phagocytic indices *in vitro* and *in vivo* are compatible with previous work of Niphade et al. [66] who found that a high dose of cinnamon has an immunomodulatory effect due to an increase in the phagocytic index in the carbon clearance test and increased neutrophil adhesion. The essential oils including cinnamon affected the inflammatory signaling pathways, immune responses, and cell cycle control [15]. The group received probiotics only exerted a significant increase in spleen cellular viability %. Many studies have demonstrated that probiotics can modulate the immune responses in both humans and animals, and it has the ability to share in the treatment of diseases that are related to immune function, like a viral infection [29]. The administration of fermented milk containing probiotic increased the activity of spleen macrophages [26]. On the other hand, *Lactobacillus* and *Bifidobacterium* affect innate immunity by increasing the phagocytosis and cytotoxicity of natural killer cells [62]. Effect of herbs aqueous extract without/with probiotic on glucose consumed with lymphocytes stimulated by PHA is estimated in the present study. The significant poorness in glucose consumed in T2DRM recorded may be attributed to complications combined with diabetes. Hyperglycemia and/or hyperinsulinemia may compromise the immune system in diabetic patients [4]. All supplemented groups indicated that use neither herbs nor probiotic sole use successes to improve glucose consumption by lymphocytes significantly. Nevertheless, groups received herbs (cardamom, ginger) with probiotic showed a significant increase in glucose consumption by lymphocytes in comparison with T2DRM.

The significant decrease in the pancreatic activities of GSH-Px, SOD, and CAT enzymes recorded in T2DRM compared to the healthy control group may be due to oxidative stress which is the main pathogenesis of diabetic complications. Hyperglycemia induced oxidative stress leads to local and systemic inflammation associated with diabetes [67]. The obtained results indicated that cardamom and ginger without/with probiotics caused the highest antioxidant power in pancreatic tissue which compatible with much previous work. Cardamom essential oil has shown evidence of its antioxidant [17, 68]. Ginger contains many health-related effects because of its bioactive substances, including anti-inflammatory, and antioxidants [20, 22]. It is clear that cinnamon and probiotics have a moderate effect on pancreatic enzyme activities. Contrarily, many researches indicated that cinnamaldehyde which is the main active phytochemical of cinnamon possesses multiple functions, including anti-inflammatory and antioxidative power [10, 12].

Immunomodulation-related cytokines such as INF-γ, IL-5, and IL-17 were identified in the present study as targets to be used for amelioration of various immune illnesses. That there were significant differences of T2DRM in INF-γ, IL-5, and IL-17 comparable to the healthy control group. The significantly elevated level of INF-γ as a pro-inflammatory cytokine in T2DRM was compatible with the previous work of [6]. Hyperglycemia induced oxidative stress in the

diabetic patients was believed to increase the levels of pro-inflammatory proteins with infiltrated macrophages secreting inflammatory cytokines which led to local and systemic inflammation [4, 67]. Increase the secretion of the pro-inflammatory, INF-γ is always accompanied by insulin resistance which is the risk factor for the development of type 2 diabetes [69]

It is evident that groups offered to cardamom or ginger with probiotic ameliorate significantly pro-inflammatory Th1 (INF-γ) and, anti-inflammatory Th2 (IL-5 and IL-17). It has been proved that cardamom significantly suppresses, Th1 pro-inflammatory cytokine, conversely, triggers Th2 anti-inflammatory cytokine that is released by splenocytes [14]. With regard to ginger, [20, 22] showed that ginger extract not only can improve antioxidant capacity but also has a potential of reducing inflammatory response by its anti-inflammatory character thereby enhance immune function. The current work indicated that an administration of probiotics showed significant modulation for the immune responses through an increase in anti-inflammatory cytokine, IL-5, and IL-17. This result is in agree with many researches which indicated that when immunosuppressed animals offered to a mixture of probiotics might interact with Th1/ Th2, to modulate the adaptive immunity therefore reduce pro-inflammatory and enhance anti-inflammatory action [27, 62]. Other experimental groups showed insignificant modulation through INF-γ, IL-5, and IL-17.

Generally, from the overall results obtained in the current work, one can notice that groups received herbs (cardamom, ginger) with probiotic exert a significant immunomodulatory effect, might be due they manage type 2 diabetes, which expressed the synergistic effect between them as concluded by Ali et al. [70] who concluded that cardamom, saffron, and ginger enriched with multi-strain probiotics achieve a synergistic relationship for managing type 2 diabetes.

CONCLUSION

Conclusively, the current work indicated that hyperglycemia that leads to over inflammation and oxidation which are the most complication of type 2 diabetes may compromise the immune function causing the immune-depressive condition. In truth, herbs or probiotics solely caused a mild immune response but did not achieve the intended objective. Interestingly, an immunomodulation of type 2 diabetes achieved in the present work with anti-inflammatory and antioxidants herbs (ginger or cardamom) with probiotics. The lowest immune response resulted in cinnamon may be attributed to the species used. Finally, it can be concluded that ginger or cardamom with probiotics success to achieve immunomodulation of type 2 diabetes through elevation of circulatory lymphocyte, *in vivo* and *in vitro* phagocytic index, glucose consumed with lymphocytes, and anti-inflammatory IL-5 and IL-17 with a depression of pro-inflammatory cytokines INF-γ.

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CONFLICTS OF INTERESTS

The data and research results are honest, and the author reports no conflicts of interest in this work.

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