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Research Article

Rice Bran Oil Influences Atherosclerosis

Dietary Njavara (*Oryza Sativa* Linn.) Rice Bran Oil Regulates Immune Response by Regulating Th1/Th2 Lineage Balance in High Fat Diet Induced Atherosclerosis

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Abstract: When considering inflammation, $CD4^+$ T cells play a crucial role in the pathogenesis of atherosclerosis. The hypocholesterolemic effects of Njavara (*Oryza sativa* Linn.) rice bran oil (NjRBO) in the treatment of atherosclerosis has been previously explored. However, no evidence regarding understanding Th1/Th2-cell lineage differentiation in atherosclerotic rats has been studied. Hence the objective of this study was to investigate the regulatory effects of Njavara rice bran oil in modulating $CD4^+$ T cell imbalance in the Sprague–Dawley rat model. Following *in-vivo* research for two months, splenocytes isolated were cultured *ex-vivo* and treated with Concanavalin A (1.5-3µg/ml medium), followed by incubation with anti-CD3e/CD28 plate-bound antibodies. Initially, body weight and biochemical parameters, including serum and hepatic lipid profile, were analyzed, and the results revealed suppression of the pro-inflammatory biochemical markers upon supplementation of NjRBO. Further evaluation of the effects of Njavara rice bran oil on Th1/Th2 lineage development of $CD4^+$ T cells under anti-CD3/anti-CD28 antibody stimulation was studied. Results revealed that NjRBO significantly suppressed the amount of Th1 cytokines demonstrating its broad suppressive effect on Th1-related pro-inflammation, while an upregulation in the production of Th2 cytokines was noticed. Furthermore, significant attenuation in the mRNA expression pattern of Th1/Th2-related transcription factors and co-stimulatory expression in splenic $CD4^+$ T cells were further examined. Hence these data indicate that NjRBO incorporation selectively alters Th1/Th2 Cell-mediated inflammation patterns and provides the pharmacological basis for its applications.

Keywords: Atherosclerosis, Njavara rice bran oil, $CD4^+$T cell, T helper cells, High-fat diet

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1. INTRODUCTION

Atherosclerosis is a progressive pathological condition characterized by chronic arterial wall inflammation. Much inflammation in atherosclerosis is mediated by cells of the adaptive immune system, where $CD4^+$ T cells play crucial roles. T cells get activated upon engagement of the T-cell receptor (TCR) by antigenic peptides presented by MHC molecules on the surface of APCs. Both TCR and CD28 co-stimulatory signals result in the vigorous proliferation of T lymphocytes and ultimately drive them to produce effector cytokines that mediate immune responses [1]. Depending on the type of cytokines secreted, they are divided into mainly Th1 and Th2, which differentiate from naive T lymphocytes (Th0) [2]. Th1 effector cells produce characteristic cytokines interleukin (IL)-2, interferon IFN-γ and tumour necrosis factor (TNF)-α, which primarily mediate cellular immunity. Th2 effector cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 to augment immune responses against extracellular pathogens [3]. Th1/Th2 cell types are characterized by lineage-specific transcription factors T-bet and GATA3 [4,5]. It is widely recognized that Th1-cell-secreted cytokines, IFN- γ and TNF-α, enhance inflammatory responses *in-vivo*. In contrast, Th2-cell-secreted cytokines, particularly IL-4, IL-5, and IL-10, inhibit the synthesis of other cytokines and immune cell functions during the late inflammation phase [6] and potentially inhibit the prevalence of Th1 cell types. Hence, Th1 and Th2 cytokine secretion profiles may reflect the Th1/Th2 immune balance [7]. The effect of various nutraceuticals and therapeutics on maintaining Th1/Th2 balance has been extensively studied[7-9]. Hence, regulation of Th1/Th2 immune cell balance, using potential dietary components, may reduce the risk factor for atherosclerosis. Co-stimulation plays an essential role in regulating T Cell immune response [10,11]. CD28 is expressed on naive and activated T cells and engages with ligands B7-1 (CD80) and B7-2 (CD86). This secondary signal mediated by CD28 is required for full T cell activation and cytokines production [12]. In contrast. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4, CD152) is induced after the initial steps of T cell activation. CTLA-4 gets expressed on activated T cells and plays an inhibitory role in T cell proliferation [13]. Furthermore, CTLA-4 regulates the production of cytokines by Th1 and Th2 cells and has diverse effects on disease progression. 'Njavara' (*Oryza sativa* L.) is a staple medicinal rice endemic to south India and is an immune booster in relieving inflammatory ailments. Rice bran oil (RBO) is extracted from rice's germ and inner husk. It is rich in bioactive compounds such as gamma-oryzanol, tocotrienols, B-complex vitamins and β-sitosterol[14], which confer anti-inflammatory and antioxidant properties. Studies demonstrated that intake of rice bran oil (RBO) resulted in a decreased plasma level of total cholesterol (TC) and low-density lipoprotein (LDL) in hyperlipidaemia [15,16]. To add, previous studies conducted in our laboratory have elucidated that supplementing a diet with NjRBO decreased atherosclerotic complications by modulating genes involved in lipid metabolism [17] and regulating inflammatory responses in hypercholesterolemic rats. [18] However, the atheroprotective effect of Njavara rice bran oil supplementation on Th1/Th2 balance has not yet been proven regarding atherosclerotic diseases, hence the objective of this study aimed is to examine the immunomodulatory effects of Njavara rice bran oil (NjRBO) on the dysregulated immune activation in the context of the T cell proliferation and differentiation in experimentally induced atherosclerotic rats. To evaluate the immunomodulatory effects of NjRBO on T-cell functionality, we stimulated the rat primary splenocytes with concanavalin A (ConA), a T-cell mitogen, to stimulate cytokine production. Splenocytes comprise antigen-presenting cells, B cells, and various types of T cells and have been widely used as primary cell models for studying the functionality of T cells [19-21]. Therefore, the present study focussed on the study of various Th1/Th2 cell components to determine the effects of this oil on Th1/Th2 functionality.

2. MATERIALS AND METHODS

2.1 Extraction of Njavara rice bran oil

Njavara rice bran oil (NjRBO) used for the study was isolated from the bran of Njavara rice (Poaceae). Authentic Njavara black (NB) samples were collected from the certified farm 'ECO FARM' karukamanikalam at Chittoor, Palakkad, Kerala. Plant specimens were verified and confirmed by Dr Maya C. Nair, Department of Botany, Government Victoria College, Palakkad- 678001, identical to the specimen sample IC 539968 deposited at National Bureau of Plant Genetic Resources, (NBPGR), New Delhi, India, against a collection (voucher) No. MS004/05. Stabilized rice bran of Njavara black (NBA) was defatted with 800 ml of the petroleum diethyl ether solvent (60-80°C) in a Soxhlet extractor for 16 hr. Then, the extract was evaporated on a rotary evaporator (Laborota 4000-Heidolph, Germany) at 60°C to remove the solvent. The crude extract obtained was used as Njavara rice bran oil (NjRBO) for further studies.

2.2 *Chemicals and Reagents*

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), SRL chemicals, Mumbai, India. Kits used for estimation were purchased from Agape Diagnostics and Thermo Fisher Scientific. Antibodies were purchased from Abcam (UK), Cell Signaling Technologies (USA), Invitrogen (USA), and Santa Cruz Biotechnology Inc. (USA).

2.3 *Animal experiments*

Adult male Sprague–Dawley rats (150–200g) were used for the study. Rats were bred in the department animal house and were housed in cages provided with controlled temperature (24–26°C), humidity (55–60 %) and photoperiod (12 h light–12 h dark cycle). Based on the formulations of the AIN-93M diet,[22]experimental diet is prepared, as shown in Table 1. Tap water was provided *ad libitum*. All experimental animal procedures were conducted per the current guidelines approved by Institutional Animal Ethics Committee (Ethical code: IAEC-KU-7/2015-'16-BC-AH (31)).

Table 1: Composition of experimental diet

Ingredient (g/kg diet)	Normal	High-fat diet	High-fat diet+ NjRBO	High-fat diet+ Atorvastatin
Corn starch	156	110	110	110
Casein	140	140	140	140
Sucrose	325	175	175	175
Njavara rice bran oil	-	-	100*	-
Cholesterol	-	1.5**	1.5**	1.5**
Cellulose	50	50	50	50
Mineral mix	25	18	18	18
Vitamin mix	12	13.78	13.78	13.78
L-Cystine	1.7	1.7	1.7	1.7
Choline bitartrate	2.5	2.5	2.5	2.5
DL-Methionine	1.94	1.94	1.94	1.94

**** -mg/kg body weight, **-per cent***

2.4 *Experimental design*

Animal's grouping was as follows. First, rats were divided into four groups of 6 rats each.

Group I - Normal (Standard diet given)
Group II - High-fat diet (HFD comprising 0.5% cholesterol and 1.5% choline)
Group III - HFD +NjRBO (100mg/kg body weight)
Group IV - HFD +Atorvastatin (10mg/kg body weight)

Njavara rice bran oil (NjRBO) crude extract was mixed with AIN-93M standard diet formulations and given at a concentration of 100mg per kg body weight of animals. The dosage for Njavara rice bran oil was selected from a previous study. [17] Standard drug Atorvastatin at a dose of 10mg per Kg body weight was administered along with the standard diet. Food intake and body weight were monitored weekly during the experiment. In addition, spilt food was collected and compensated in adjusting the calculation of food intake. The duration of the study was 60 days.

2.5 *Estimation of serum lipid profile and atherogenic index*

Serum total cholesterol (TC), triglycerides (TGs), LDL-C and HDL-C concentrations were determined using diagnostic kits from Agape Diagnostics, India, according to the manufacturer's instructions. The atherogenic index was calculated using the formulae:

AI= LDL-C/HDL-C

2.6 *Estimation of liver weight, total cholesterol and triglycerides in the liver*

The rats were euthanized at the end of the 2-month experimental period. The liver was removed and perfused with ice-cold physiological saline. The excised liver was blotted dry and then weighed. The hepatic lipids were extracted using the following procedure [23]. In brief, to the frozen liver samples, sufficient volumes of chloroform: methanol mixture (2:1 v/v) were added (15mL/g). The obtained solvent extracted fraction was filtered and dried to produce an exsiccated pellet to estimate the total hepatic lipid content. Total cholesterol and triglycerides were estimated using commercially available kits from Agape Diagnostics.

2.7 *Preparation of splenocytes*

After 60 days of animal treatments, rats were sacrificed. The procedure was adapted from a previous study with slight modifications.[24] Spleens were then excised under sterile conditions. Splenocyte suspensions were prepared by passing through stainless-steel sieves in RPMI 1640 medium (Cat no: R0883, Sigma Aldrich) supplemented with 10% Fetal bovine serum (Cat no: F2442, Sigma Aldrich), 40 µmol/ml 2-mercaptoethanol (Cat no: 83759, SRL Pvt Ltd), 100 µg/ml streptomycin, and 100 U/ml penicillin (Cat no: P7539, Sigma Aldrich). Lymphocyte suspension was further centrifuged (2000 rpm, 5 min), and the obtained lymphocyte pellet was resuspended in 4 ml RPMI–FBS. This suspension was layered on a 4ml Ficoll solution (Cat no: F5415, Sigma Aldrich) and centrifuged at 2500 rpm for 20 min. The lymphocyte band obtained at the interface was recovered, washed twice and resuspended in 4 ml RPMI–FBS.

2.8 *Lymphocyte proliferation assay*

Lymphocyte proliferation assay was done by the method described earlier[7] with slight modifications. Briefly, Splenocytes (4×10^5 cells/ml) at a concentration of 100 µl/well were seeded into 96 healthy plates. Next, 100 µl/well of Concanavalin A (ConA) (Cat no: 234567, Sigma Aldrich) dissolved into ten µg/ml with RPMI-1640, which was added to induce lymphocyte proliferation. The plates were further incubated at 37 °C for 48hr in a humid atmosphere of 5% CO_2. Next, 20 µl of MTT (Cat no: M5655, Sigma Aldrich, 5 mg/ml) was added into each well after 48hr of incubation, and the plates were incubated for another 4hr. Finally, the supernatant was removed, and 100 µl of DMSO (Cat no: 24075, SRL Pvt Ltd) was added to each well. After 5 min, a microliter enzyme-linked immunosorbent assay reader measured the absorbance at a wavelength of 470 nm (*A*470) of lymphocytes in each well. The mean *A*470 values were taken to indicate peripheral T-lymphocyte proliferation [25].

2.9 *Enzyme-linked immunosorbent assay of surface markers.*

The presence of CD28 (Cat no: ab-243228, Abcam), CD25 (Cat no: ab-PA546922, Invitrogen), CTLA-4 (Cat no:12-1522-

82, Invitrogen) was determined by ELISA[26] using specific antibodies. First, briefly isolated CD4+T cells were added to the microtiter plate cells precoated with respective antibodies. Following incubation at 37°C for two hrs, the samples were washed to remove unbound particles using PBS. Primary antibodies were then added and incubated for 2hr. After 2hr, secondary antibody IgG conjugated with HRP was added and incubated for another one hr at 37°C. Finally, the substrate solution and stop solution were added, and the reaction developed was measured with an ELISA reader at 450 nm. The samples were assayed in triplicate, and results were expressed in OD units/mg protein.

2.10 *Cytokine production*

Splenocytes (4×10^5 cells/ml) were stimulated with plate-bound anti-CD3 monoclonal Ab (Cat no: ab11089, Abcam) (coated overnight at 1.5 µg/ml) at 37 °C under 5% CO_2 for 72hr. In addition, culture supernatants were collected and were quantified for protein expression using rat IFN-γ (Cat no: sc-57208, Santa Cruz), IL-4 (Cat no: ab11524, Abcam), IL-5 (Cat no:ab-9624, Abcam), IL-2 (Cat no:ab-25104, Abcam), TNF-α (Cat no: ab-286149, Abcam), IL-10 (Cat no: ab-189392, Abcam) antibodies using enzyme-linked immunosorbent assay.

2.11 *Isolation of CD4+T cells*

CD4+T lymphocytes were isolated from spleen single cell suspensions by the FITC-conjugated CD4+ T cell negative selection kit (Stem Cell Technologies) using an Easy Sep purple magnet. Briefly, the spleen was disrupted in the recommended medium of PBS supplemented with 2% FBS (Fetal Bovine Serum) and one mM EDTA. Next, the cell suspension was passed through a 70 µm mesh nylon strainer and centrifuged at 120 x g for 10 minutes to remove aggregate particles. Next, the supernatant was removed and resuspended the cells at $5x10^7$nucleated cells/ml in the recommended medium. Next, CD4+ T cells were purified by immunogenic adverse selection.

2.12 *Quantitative real time PCR (q-PCR)*

RNA was extracted from CD4+ T cells using TRIZOL reagent (Sigma Aldrich) [27]. cDNA was synthesized, and qPCR was performed on (Eppendorf Thermocycler) using SYBR green Real-time PCR kit (Cat no: A46112, ThermoFisher Scientific) following manufacturer's instructions. Aliquots of 2µl template cDNA, 1µl forward and reverse PCR primers each and 10µl real-time PCR master mix were added, and the final volume was made up to 20µl with nuclease-free water. The reaction was run in an Eppendorf Master cycler after a pre-incubation at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 20 sec and annealing at 59°C for 15 sec. An incubation terminated the reaction at four °C. The relative quantitation was done using the $2^{-\Delta\Delta Ct2}$ method, where ΔC_t is the difference between the mean C_t value of triplicates of the sample and the endogenous GAPDH control. $\Delta C_t = C_t$ (a target gene) – C_t (a reference gene). The gene expression of transcription factors T-bet and GATA3 were analyzed using qPCR primer sequences. The PCR primers used for gene expression analysis are shown in Table 2.

Table 2: List of primer sequences for qPCR

S. No	Primer names	Primer sequences (5'-3')
1.	T-bet	Forward primer: 5'-CGGCTGCATATCGTTGAGGT-3'
		Reverse primer: 5'- GTCCCCATTGGCATTCCTC-3'
2.	GATA-3	Forward primer: 5'-TCATTAAGCCCAAGCGAAGG-3'
		Reverse primer: 5'-GTCCCCATTGGCATTCCTC-3'

2.13 Immunofluorescence

Cells ($5x10^4$ cells per chamber) were seeded into culture 96 well culture dishes (Axigen). The following day, cells were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde for 10 min at room temperature, followed by permeabilization using 0.1% Sodium Citrate plus 0.1% Triton X-100. The cells were further subjected to immunofluorescence staining with T-bet (1:500) (Cat no:14-5824-82, Invitrogen), GATA-3(1:500) (Cat no: PA5-105295, Invitrogen) antibodies for 2-4hr at room temperature. The cells were then washed with cold PBS three times for 3 min each and incubated with FITC-labelled anti-rabbit secondary antibody (1:900) (Cat no:7074P2, Cell signalling Technology) at room temperature for one hr. Nuclei were counterstained for 5 minutes with five mM DAPI stain. The cells were examined, and images were acquired using a fluorescence microscope (Zeiss, Life technologies).

2.14 Western blot

CD4+ T cells were lysed with the Protein Extraction Reagent (Merk, Millipore) at room temperature. Following cell lysis, the cell debris was removed *via* centrifugation at 15,000 rpm for 10 min at 4°C. Replicate aliquots of supernatants were then electrophoresed on SDS-10% polyacrylamide gel at 150 V for 2hr at room temperature. Proteins (15-50µg) resolved were transferred to nitrocellulose membranes (Millipore) at 100 V for 1.5 h. After blocking the non-specific sites, the membranes were developed with anti-CTLA-4 antibody (Cat no:12-1522-82, Invitrogen) and anti-CD28 antibody (Cat no: 16-0280-81, Invitrogen) at four °C overnight, followed by incubation with secondary antibody (1:2000 anti-rat IgG; Cat no: ab 6734, Abcam) for 1hr. The bands were finally visualized using the enhanced chemiluminescence (ECL) detection reagents (Pierce™ ECL Western Blotting Substrate, Cat no: 32109).

3. STATISTICAL ANALYSIS

Statistical analysis was done according to the method of Bennet and Franklin [28]. The results were analyzed using the statistical program SPSS version 11.5 (SPSS Inc, Chicago, USA). Comparison between the groups was performed by one-way ANOVA followed by Duncan's posthoc multiple comparison tests. The results were presented as mean (SEM) for six samples. Significance was defined at $p<0.05$.

4. RESULTS

4.1. *Body weight, food intake and lipid profile*

Following two months of experimental period in rats, the body weight was gained, and rats consumed considerably more food than the normal rats in cholesterol supplemented group. However, the results showed a significant ($P < 0.05$) decrease in whole body weight and normalization of food intake from the administration of NjRBO to HFD-treated rats (Figures 1a and 1b).

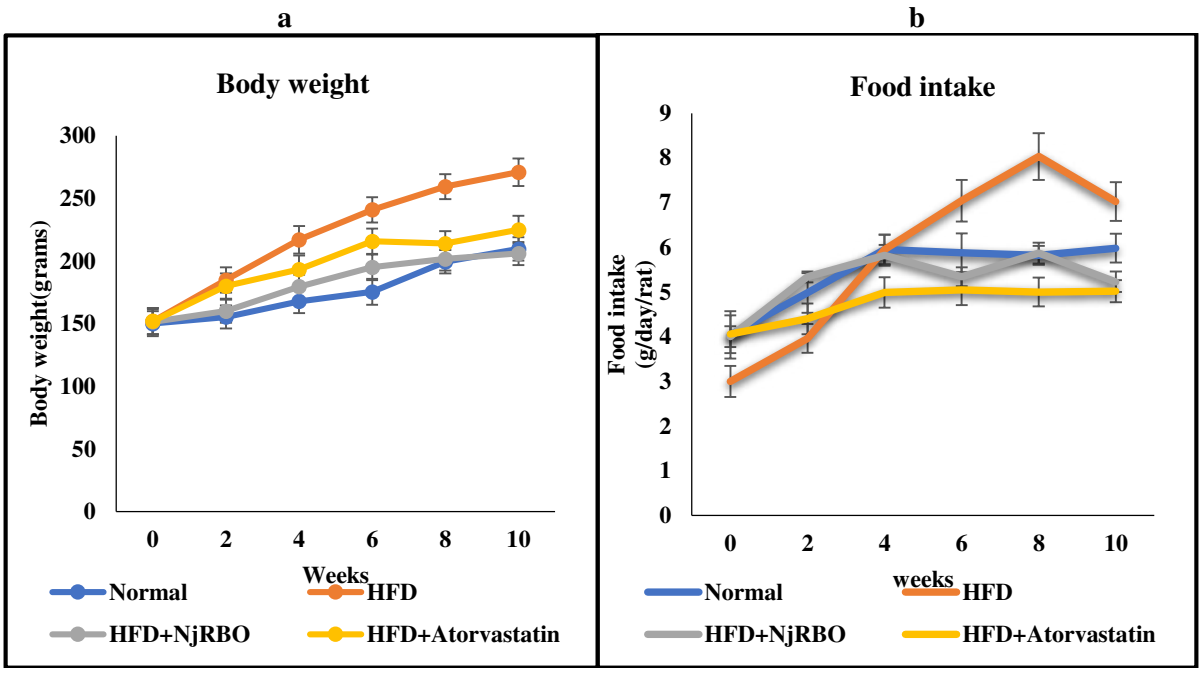

Fig 1: Effect of NjRBO on (a) body weight (grams) and (b) Food intake of Sprague-Dawley (SD) rats fed with high-fat diet (HFD) along the ten weeks of the study. Data are presented as mean ± SEM (n = 6 rats per group).

The dietary effects on serum lipid profile are shown in Table 3.

Table 3. Serum lipid profile of rats fed with the high-fat diet for two months

Parameters	Group I	Group II	Group III	Group IV
TC (mg/dl)	106.32 ± 3.02	243.04 ± 2.94[a]	112.11 ± 3.004[abc]	133.13 ± 2.99[ab]
TG (mg/dl)	78.57 ± 1.678	171.42 ± 1.765[a]	82.85 ± 1.643[abc]	107.14 ± 1.587[ab]
HDL-C (mg/dl)	48.00 ± 0.772	19.00± 0.770 [a]	43.670 ± 0.769[abc]	36.23± 0.765[ab]
LDL-C (mg/dl)	42.606 ± 0.993	123.756 ± 0.998[a]	49.54 ± 0.989[abc]	65.478± 0.991[ab]
Atherogenic index	0.887	6.513	1.134	1.807

TC: Total Cholesterol, TG: Triglycerides, HDL-C: High-Density Lipoprotein Cholesterol, LDL-C: Low-Density Lipoprotein Cholesterol. Group I- Normal, Group II- HFD, Group III- HFD + NjRBO (100mg/kg body weight), Group IV- HFD + Atorvastatin (10mg/kg body weight). A -a significant difference when compared with normal at p<0.05, b- a significant difference when compared with HFD at p<0.05, c- a considerable difference when compared with Atorvastatin at p<0.05. Values expressed as an average of ± SEM (n=6).

The total cholesterol (TC) levels in HFD groups were 243.04 ± 2.94mg/dl, which were significantly raised ($p<0.05$) as compared to the cholesterol levels of the control group, i.e. 106.32 ± 3.02mg/dl, which confirmed the development of hypercholesterolemia. The Njavara rice bran oil supplemented group (NjRBO) had significantly reduced ($p<0.05$) cholesterol levels to 112.11± 3.004mg/dl. The TG levels in the HFD group were significantly increased ($p<0.05$) compared to the TG levels of the control group. The NjRBO group had significantly low ($p<0.05$) TG levels (82.85 ± 1.643mg/dl) as compared with TG levels of the hypercholesterolemic group (171.42 ± 1.765mg/dl). Serum LDL levels (mg/dl) were analyzed in normal, HFD and NjRBO groups, as shown in Table 3. NjRBO administered group had significantly lowered the LDL levels to 49.54 ± 0.989mg/dl ($p<0.05$) compared to LDL levels of 123.756 ± 0.998 mg/dl in hypercholesterolemic control. However, NjRBO given group had significantly raised HDL levels to 43.670 ± 0.769 mg/dl as compared to HDL levels of 19.00± 0.770 mg/dl hypercholesterolemic control which showed more protection than standard drug received rats (36.23± 0.765mg/dl). In association with the hyperlipidaemia observed in the HFD group animals, the atherogenic index (AI) was significantly ($p < 0.05$) higher (6.513) in comparison to the normal group (0.887). NjRBO significantly ($p > 0.05$) decreased (1.134) the AI in the HFD fed rats reaching the control value.

4.2. *Liver weight and hepatic lipid profile*

Table 4 shows liver weight and hepatic lipid profile after eight weeks of feeding on a high-fat diet. In line with the changes in serum lipids, the effects of the NjRBO supplementation on triglyceride and total cholesterol contents of the livers in the rats given a high-fat diet were analyzed and compared with that of the standard drug atorvastatin. After eight weeks of

dietary treatment with NjRBO, the total cholesterol (TC) concentration of the liver was significantly ($p < 0.05$) lower (85.24 ± 3.14) compared to the high fat-fed group (99.32 ± 4.29). In addition, the liver triglyceride content was lowered (187.09 ±5.949) in the standard group. Still, it was significantly ($p < 0.05$) higher (226.12 ±8.92) in the HFD rats and was further reduced in the oil-treated group (193.34 ± 6.32).

Table 4: Liver weight and hepatic lipid profile

Parameters	Normal	HFD	HFD+NjRBO	HFD+Atorvastatin
Liver weight (g)	19.23± 0.82	31.098 ±1.09	22.10 ±0.987	27.00 ±0.890
Triglycerides(TGs) (mg/g)	187.09 ±5.949	226.12 ±8.92	193.34 ±6.32	201.53 ±7.627
Total cholesterol(TC) (mg/g)	83.47 ± 3.049	99.32 ± 4.29	85.24 ± 3.14	90.53 ± 3.65

4.3. *Lymphocyte proliferation assay and CD25 marker expression*

Lymphocyte proliferation assay was assessed to determine the efficiency of T cell proliferation. Splenocytes were isolated and stimulated with Concanavalin (ConA) at a medium dose of 1.5 or 3µg/mL. Levels of lymphocyte proliferation were detected using the MTT method, and the results were shown as absorbance at A490nm. As shown in Figure 2a, a significant ascending proliferative lymphocyte immune response was observed in the rats given HFD ($p<0.05$). However, downregulation in lymphocyte cell proliferative response was observed upon administration of dietary Njavara rice bran oil (NjRBO). To check proliferation efficiency, a CD25 surface marker was detected to analyze the activation of $CD4^+$ T cells (Figure 2b). It was found that HFD increased the expression of CD25 protein in isolated $CD4^+$ T cells, whereas dietary administration of NjRBO could mitigate the effect by lowering its protein levels.

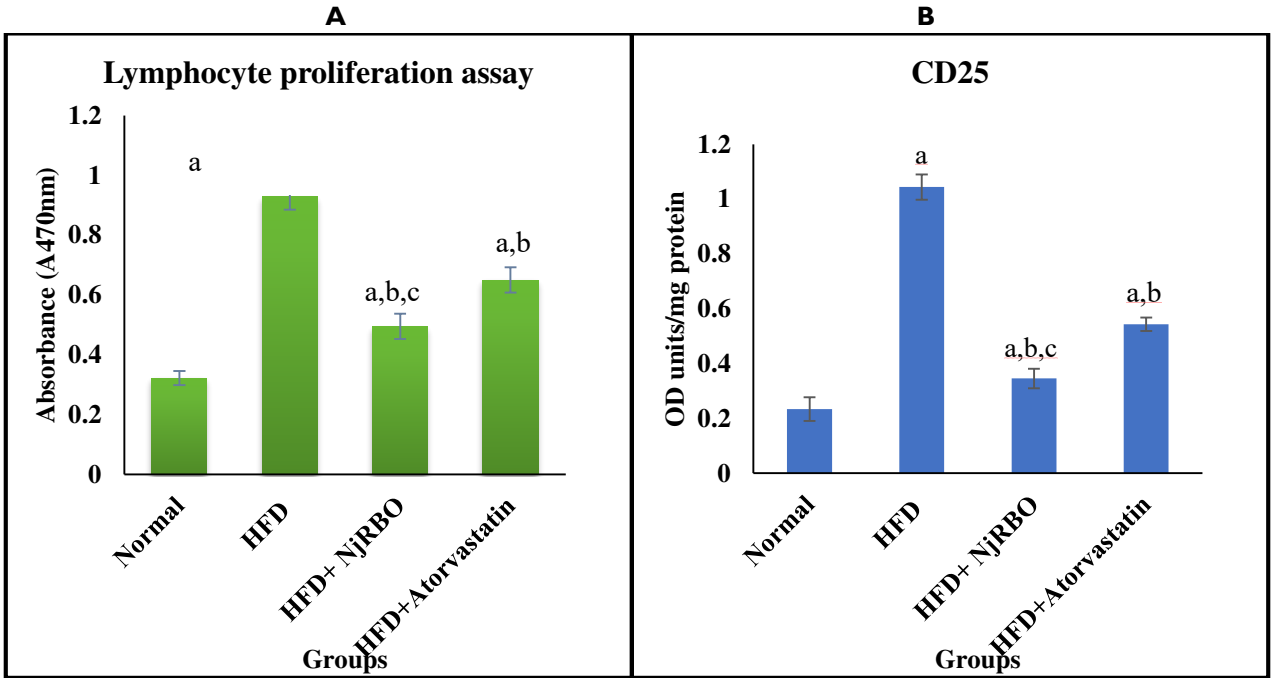

Fig 2. Effect of NjRBO on (a) Lymphocyte proliferation assay. Splenocytes were cultured in the presence of T cell mitogen

Concanavalin A (Con A) or anti-T cell receptor (anti-CD3; 1 µg/ml) with soluble anti-CD28 (1 µg/ml) (CD3/CD28) for 72hr at 37°C with 5% CO_2. Levels of lymphocyte proliferation in each group were detected using the MTT method, and the absorbance was read at 490 nm (A 490nm). (b) CD25 surface marker expression by ELISA. Values expressed as the average of 6 values ± SEM in each group. A significant difference when compared with normal at ($p < 0.05$), b- considerable difference when compared with HFD at ($p < 0.05$), c- substantial difference when compared with Atorvastatin at ($p < 0.05$).

4.4. *CD28 expression on activated $CD4^+$ T cells*

CD28 is a co-stimulatory receptor expressed by activated $CD4^+$ T cells. Its coordination with TCR is essential for T-cell activation, differentiation and survival. $CD4^+$ T-cell co-receptor signalling molecule CD28 protein expression was studied by ELISA and Western blot on isolated $CD4^+$ T cells. Mean CD28 levels in T lymphocyte fraction compared to the control are higher in the HFD-supplemented group. Njavara rice bran oil (NjRBO) downregulated the expression (Figure 3a), resulting in a marked decrease in its co-receptor activity. This change was further confirmed using western blot analysis, as shown in Figure 3 b.

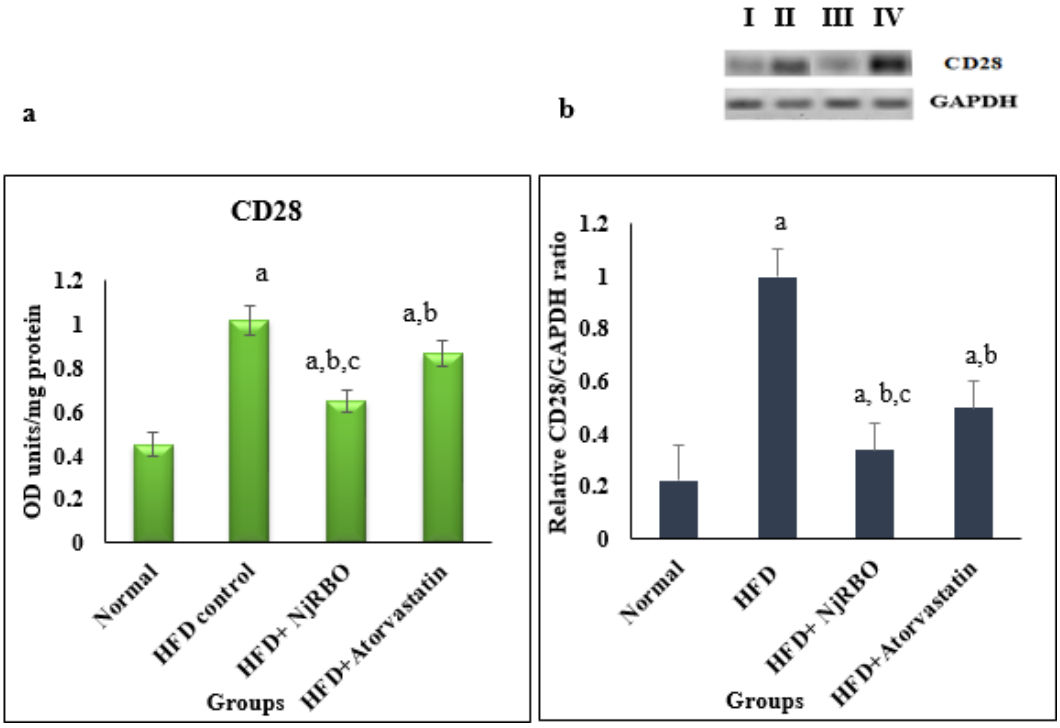

Fig 3: Expression of co-stimulatory molecule CD28 on activated CD4^{+}T cells through (a)ELISA and (b) Western blot.

Group I- Normal, Group II - HFD, Group III - HFD + NjRBO, Group IV - HFD +Atorvastatin. Values expressed as the average of 6 values ±SEM in each group. A significant difference when compared with normal at (p < 0.05), b- a considerable difference when compared with HFD at (p < 0.05), c- a significant difference when compared with Atorvastatin at (p < 0.05).

4.5. *CTLA-4 expression on activated T cells*

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a co-inhibitory receptor molecule, and its protein expression showed a significant upregulation in control rats compared to HFD-treated rats. Whereas Njavara rice bran oil (NjRBO) treatment resulted in increased expression of CTLA-4, it could potentially inhibit further activation of T cells by blocking signals initiated by T-cell receptors and CD28 (Figure 4a and Figure 4b).

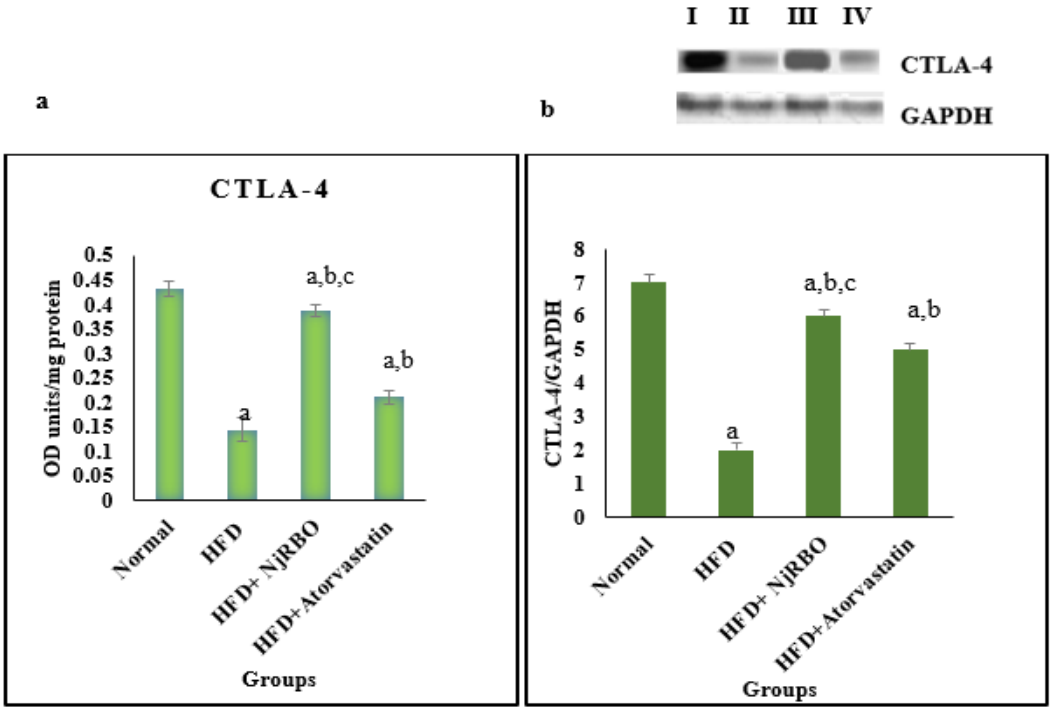

Fig 4. (a) Protein expression of CTLA-4 as determined by(a) ELISA (b) Western blot.

Group I- Normal, Group II - HFD, Group III - HFD + NjRBO, Group IV - HFD +Atorvastatin. Values expressed as the average of 6 values ±SEM in each group. A significant difference when compared with normal at (p < 0.05), b- considerable difference when compared with HFD at (p < 0.05), c- a significant difference when compared with Atorvastatin at (p < 0.05).

4.6. *Th1/Th2 cytokine analysis through ELISA*

It is well known that IL-2, IFN-γ, TNF-α and IL-4, IL-10, and IL-5 are the key cytokines representing Th1 and Th2 cells, respectively. Thus, ELISA detected its protein expression in activated $CD4^+$ T cells. The Th1-specific cytokines showed a much higher expression in the HFD group than the normal, suggesting a Th1 dominant response in the diseased (Figure 5a). Upon Njavara rice bran oil (NjRBO) supplementation resulted in a considerable decrease in pro-inflammatory cytokine milieu in activated T cells. Conversely, a significant increase in the production of anti-inflammatory cytokines IL-4, IL-5 and IL-10 was observed in the oil-treated group compared to the diseased (Figure 5b).

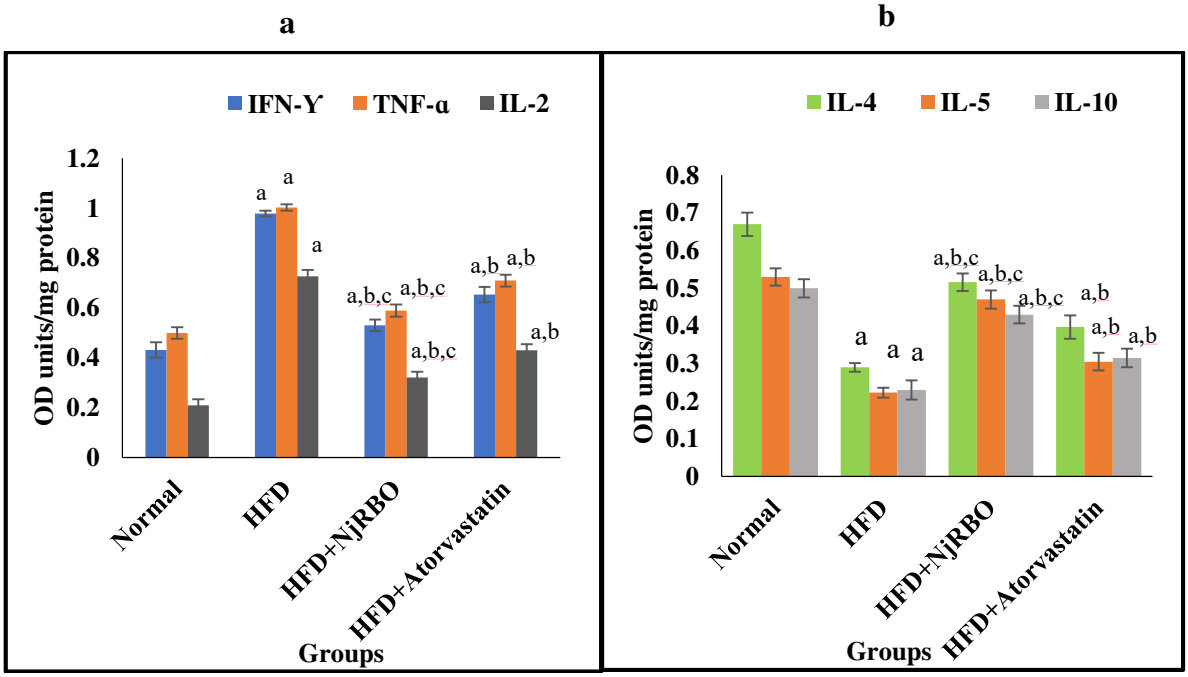

Fig 5. Protein expression of Th1 and Th2 polarization cytokines quantified through ELISA.

(a) Th1 cytokines IFN-γ, TNF-α and IL-2 in CD4 $^+$T cells. (b) Th2 cytokines IL-4, IL-5, IL-10 in $CD4^+$T cells. Values expressed as an average of 6 values ±SEM in each group. A significant difference when compared with normal at ($p < 0.05$), b- a significant difference when compared with HFD at ($p < 0.05$), c- a significant difference when compared with Atorvastatin at ($p < 0.05$).

4.7. *Th1/Th2 transcription factor T-bet and GATA3 expression*

Since T-bet and GATA3 are reported to represent Th1 or Th2 immune response, the role of Njavara rice bran oil (NjRBO) on the mRNA expression of T-bet and GATA3 in activated $CD4^+$ T cells was elucidated. In this regard, the expression level of T-bet mRNA in HFD rat was significantly higher than in the control rats, whereas GATA3 expression reflecting anti-inflammatory Th2 Cell's predominancy were lowered. NjRBO treatment of HFD rats increased GATA3 mRNA expression levels and decreased pro-inflammatory T-bet mRNA expression compared to the diseased groups (Figures 6a and 6b). The presence and localization of Th1 and Th2 specific transcription factors, T-bet and GATA3 in activated $CD4^+$ T cells was confirmed using immunofluorescence (Figure 6c), which allows visualization of antibody staining for both markers separately. Herein, compared to the control, a predominance of T-bet expression was observed in the cells of HFD-received rats. At the same time, GATA3-expressing cells were found to be decreased upon disease induction. Furthermore, treatment with NjRBO resulted in a marked increase in GATA3 expressive cells than T-bet expression, confirming the increased occurrence of Th2 cells and decreased inflammatory Th1 cells.

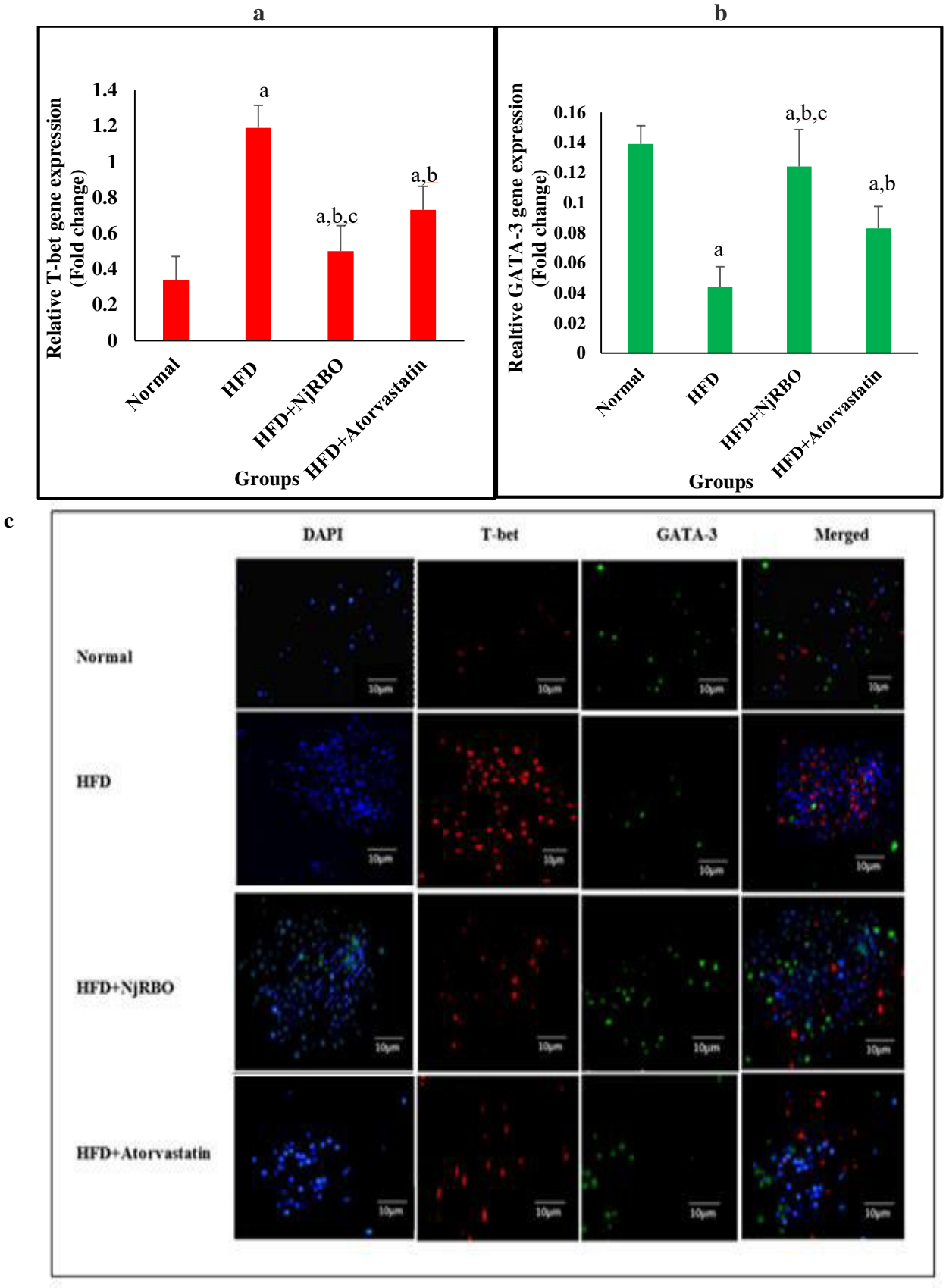

Fig 6. Effect of NjRBO on Th1/Th2 cell lineage markers.

*Real-time PCR analysis of (a) T-bet and (b) GATA3 expression in T cells. RNA was extracted from cells activated for 4hr with plate-bound CD3/CD28 antibodies (2µg/ml), followed by cDNA preparation and quantitative PCR analysis. The results are expressed in fold increase ($2^{\Delta Ct}$) comparing basal with activated conditions. Values are the average of 6 values ± SEM in each group. A significant difference when compared with normal at ($p < 0.05$), b- a considerable difference when compared with HFD at ($p < 0.05$), c- a significant difference when compared with Atorvastatin at ($p < 0.05$). (c) Co-expression of T-bet and GATA3 in $CD4^+$ T cells. $CD4^+$ T cells were cultured for 12 to 24hr on immobilized anti-CD3 with soluble anti-CD28 antibodies. Immunofluorescent images of $CD4^+$ T cells stained with antibodies to the Th1 specific transcription factor T-bet (red) and Th2 specific transcription factor GATA-3 (green) indicate T-bet cells and GATA3 cells were distinct cell populations. Cell nuclei were labelled with DAPI(blue). (Scale bar -10µm; *400X).*

4.8. Th1/Th2 ratio

T-bet and GATA3 expression is the reflection of the Th1/Th2 ratio. Compared with the standard group, the Th1/Th2 in HFD diseased group was higher, whereas dietary Njavara rice bran oil (NjRBO) administration caused a reduction in the Th1/Th2 ratio (Figure 7).

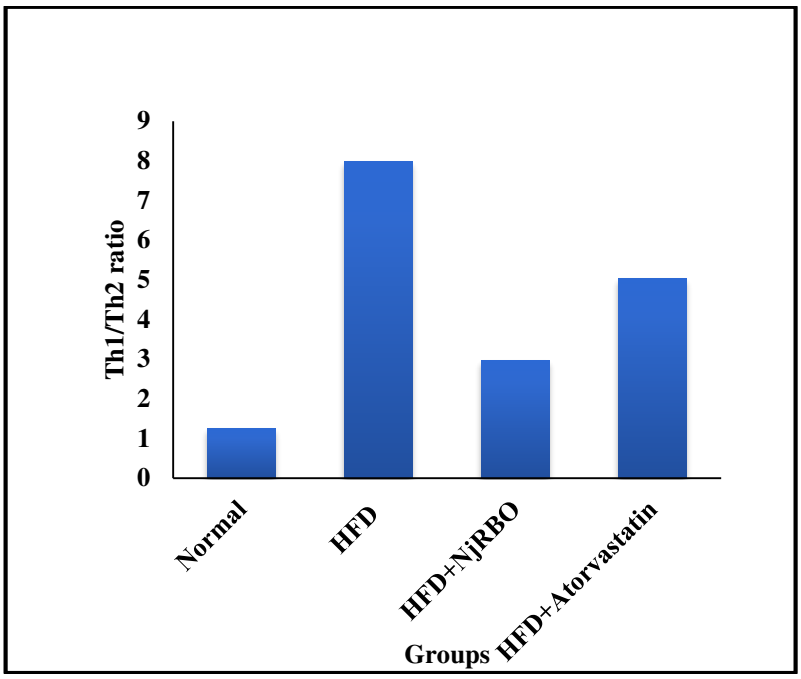

Fig 7: Dietary effect of NjRBO on Th1/Th2 ratio.

Results were expressed as an average of 6 values ± SEM in each group. A significant difference when compared with normal at (p < 0.05), b- Significant difference when compared with HFD at (p < 0.05), c- a significant difference when compared with Atorvastatin at (p<0.05).

5. DISCUSSION

NjRBO is known to induce anti-inflammatory effects, but much of the research to date has focused on the effect of NjRBO on the activity of endothelial cells and peritoneal macrophages through regression studies in a rabbit model.[17] As aberrant immune cell activation promotes the progression of atherosclerotic complications, immunomodulatory strategies have become emerging treatments targeting its root cause. The protective effect of Njavara in treating various inflammatory conditions is known. Still, no scientific data on the molecular mechanisms by which the plant offers protection has been studied concerning $CD4^{+}$T cells. The effect of NjRBO on the balance of the two mutually inhibitory T helper cell subtypes, Th1 and Th2 cells, respectively, has hitherto received little attention. Hence, in this study, we elucidated the dietary effect of Njavara rice bran oil (NjRBO) on Th1 and Th2 cell subtypes in the high-fat diet-induced atherosclerotic rat model. Imbalanced lipid metabolism and a dysfunctional immune response entail chronic inflammation, characteristic of atherosclerosis. [29]Evidence of NjRBO to help eliminate the increased amounts of lipid through inhibiting cholesterol synthesis and enhancing the HDL-mediated removal of excess cholesterol from peripheral tissues and its subsequent transport to the liver for removal is well established. [18] To begin with, the hypolipidemic properties of NjRBO were evaluated by analyzing serum and hepatic lipid markers. Following the 60 days of experimental study, HFD-received rats showed a significant decrease in HDL-C levels and increased serum TC, TAG, LDL-C and atherogenic index, respectively, consistent with previous findings. [30] These pro-inflammatory lipid markers showed a significant decrease upon NjRBO supplementation. Hence the present findings underscore that NjRBO supplementation would alter lipid-related inflammatory and atheroprotective processes critical for limiting the progression of atherosclerosis. Further, the TGs and TC in liver tissue were significantly decreased in the NjRBO-treated group. Therefore, this, in line with previous findings, establishes the hypolipidemic effect of NjRBO, which could alter lipid-related atheroprotective processes critical in inhibiting the progression of atherosclerosis. The presence of activated T lymphocytes in all stages of atherosclerotic lesion development implies their involvement in vascular disorders. T cells proliferate, and the proliferation rate reflects the level of activation accompanied by the production of various cytokines.[31] Here, firstly, we looked into the immune activation of Th1 and Th2 lineages of T cell response in cultured spleen lymphocytes from experimental-induced atherosclerotic rats. Their presence has functional consequences since their complete absence reduces lesion formation during hypercholesterolemia.[32,33] Stimulation of cells under the influence of Concanavalin A(ConA) is a reliable method to study the immunomodulatory effects. ConA is a mitogenic lectin with the ability to stimulate T-cell proliferation was used. [34] The immune mechanism prevailing during as suggests a key role for aberrant alterations in T cell activation during the initiation and perpetuation of disease. [35] T cell activation requires two distinct signals. Concanavalin A (ConA) is an antigen-independent signal one inducer, leading to polyclonal proliferation and CD28, a major co-stimulatory receptor provides signal two in the ConA-induced T cell proliferation. [36] Here, we have studied the implication of petroleum ether extract of NjRBO on the ConA-mediated T cell proliferation. Exploration of lymphocyte proliferation showed that NjRBO almost completely suppressed Con A-stimulated splenocyte proliferation, resulting in decreased T-cell expansion compared to the HFD group, which showed

enhanced proliferative ability. The amelioration of Th1 immune response by NjRBO was further confirmed by quantification of inflammatory activation markers on the cellular surface. T-lymphocyte activation requires CD28 and CTLA-4, prototypal co-stimulatory and co-inhibitory cell surface molecules, which provides signals critical to T-cell proliferation. [37] CD28 is the major co-stimulatory molecule on T cells which interacts with CD80 and CD86 on antigen-presenting cells, causing enhanced T-cell responses by up-regulating cytokine secretion and promoting cell proliferation. [38] As per our findings, Njavara rice bran oil (NjRBO) had a potent inhibitory effect on CD28 expression in $CD4^+$T cell activation following polyclonal stimulation with ConA. CD28 and CTLA-4 mutually control the level of the immune response. Therefore CTLA-4 co-inhibitory molecule protein levels were studied, and an upregulation upon receiving NjRBO was evident, which could potentially inhibit further activation of T cells by blocking signals initiated by T-cell receptors and CD28, hence could maintain the T-cell balance. Under normal conditions, there exists a balance between Th1 and Th2 cells. However, the pathological consequences of imbalance in Th1/Th2 response are reflected during atherosclerotic disease progression. To accurately analyze the Th1/Th2 balance, Th1 and Th2 cytokines concentrations need to be analyzed. Upregulation of Th1 cytokines is associated with a compensatory downregulation of Th2 cytokines and vice versa. [39,40] In this study, $CD4^+$ T cells were sorted and analyzed for signature cytokines representing Th1/Th2 cells to verify whether this supplementation contributed to the polarization of the immune response, manifesting as changes in the levels of individual cytokine levels. A previous study showed conclusive data explaining that components from rice bran affected the immunomodulatory function by altering T lymphocyte activity. [41] Experimental data obtained from mouse models of atherosclerosis have shown that inhibition of pro-inflammatory cytokines suppresses atherosclerosis development and progression. [42] The effects of NjRBO supplementation on the production of selected Th1 (IL-2, TNF-α and INF-γ) and Th2 cytokines (IL-10, IL-5 and IL-4) in rats fed on high cholesterol diet were studied. Results recapitulated the previous studies as IL-2, IFN-ɣ and TNF-α production were lower in the NjRBO group. Meanwhile, NjRBO supplementation also showed an anti-inflammatory effect via an elevation in Th2 cytokine levels (IL-10, IL-4 and IL-5. Hence, the therapeutic efficacy of Njavara rice bran oil (NjRBO) in inhibiting splenocyte proliferation correlated with a decreased level of Th1 pro-inflammatory cytokines and an up-regulated Th2-specific cytokine milieu, thus revealing its immunomodulatory role of keeping the Th1/Th2 balance upon antigenic stimulation. The ratio of the Th1/ Th2 Cell in HFD rats increased significantly, and a Th1 cell type predominance was proven, which was restored upon NjRBO incorporation into the diet. T cell lineage decisions are orchestrated by the interplay of T-bet and GATA3, which are the master regulators determining Th1 and Th2 differentiation. [43] The influence of T-bet deficiency on the functions and phenotype of diet-induced atherosclerosis was assessed in mice models through earlier investigation. [44] Here in our findings, an upregulation of T-bet and downregulation of GATA3 were seen in high cholesterol-fed groups. At the same time, the Njavara rice bran oil (NjRBO) administration could regulate the expression of T-bet and GATA3, shifting the Th1/Th2 balance towards the anti-inflammatory Th2 response through mRNA expression and immunofluorescence studies. These results were also reflected in the decreased Th1/Th2 ratio in the Njavara rice bran oil (NjRBO) supplemented group compared with HFD diseased. Putting this together, we conclude that a shift toward the Th1 pathway of Th cell activation occurs in HFD feeding and that NjRBO can counter this shift in the Th1/Th2 balance and hence produce its therapeutic effects.In summary, the current study clearly illustrates the immunomodulatory action of dietary Njavara rice bran oil (NjRBO) in enhancing a Th2 response which is proven effective as an anti-atherogenic treatment therapy. Keeping a balance of Th2 cells can greatly reduce the severity of atherosclerosis (Figure 8). Dietary introduction of NjRBO hence could correct Th1/Th2 imbalance which eventually alters the cytokine environment during atherosclerotic progression. These findings provide new insight into the immunomodulatory role of NjRBO in terms of its effects in HFD-induced rat models. They also broaden current perspectives in understanding the importance of Njavara rice bran oil.

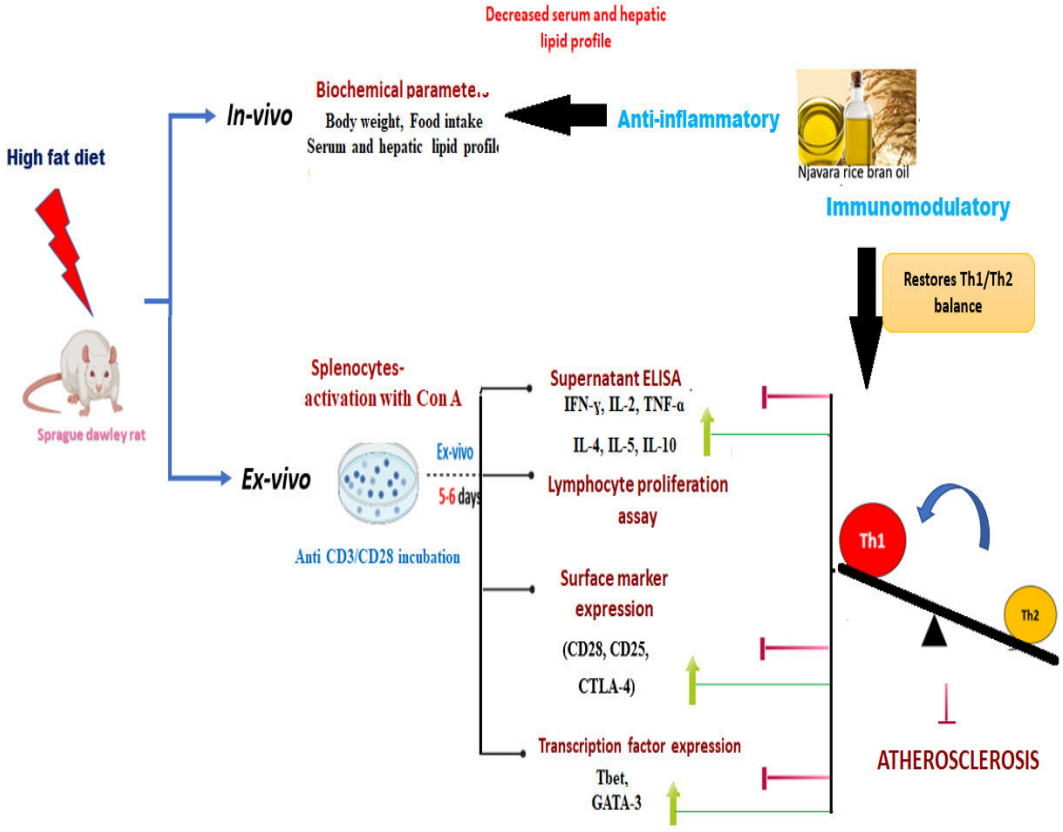

Fig 8: Immunomodulatory role of Njavara rice bran oil in Th1/Th2 lineage imbalance in atherosclerotic rats.

6. CONCLUSION

Aberrant alterations in T cell activation during the initiation and perpetuation of disease and the therapeutic effectualness of NjRBO in controlling $CD4^+$ T cell lineage decisions were looked into in this study. It identified that NjRBO could hinder dysregulated activation of $CD4^+$ T cells by maintaining the Th1/Th2 cell balance, restoring T-cell homeostasis and significantly recovering HFD-induced inflammatory response. Hence, Njavara rice bran oil (NjRBO) could be an effective anti-atherogenic dietary supplement for the treatment of atherosclerosis. Furthermore, its immunomodulatory effect, regulating T Cell immune balance during atherosclerosis, was established for the first time.

7. AUTHORS CONTRIBUTIONS STATEMENT

Lal Preethi SS and A Helen conceived the original idea. The experiments were conducted and analyzed by Lal Preethi SS and Vidya Sabu. Helen conceptualized the main theories regarding the investigations. Lal Preethi SS wrote the manuscript. The manuscript was revised and edited by A Helen. All authors contributed to the writing and editing of the final draft.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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