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Protection of Saururus Chinensis Extract against Liver Oxidative Stress in Rats of Triton WR-1339-induced Hyperlipidemia

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Saururus chinensis has been reported to contain compounds such as lignans, alkaloids, diterpenes, flavonoids, tannins, steroids, and lipids. Fermentation is commonly used to break down certain undesirable compounds, to induce effective microbial conversion, and to improve the potential nutraceutical values. Previous studies have reported that the fermentation process could modify naturally occurring constituents, including isoflavons, saponins, phytosterols, and phenols, and could enhance biological activities, specifically antioxidant and antimicrobial properties. The probiotic strains used for fermentation exert beneficial effects and are safe. In this study, the antioxidative effects of the Bacillus subtilis fermentation of Saururus chinensis were investigated in a rat model with Triton WR-1339-induced hyperlipidemia by comparing the measured antioxidative biological parameters of fermented Saururus chinensis extract to those of nonfermented Saururus chinensis extract. Fermentation played a more excellent role than nonfermentation in ultimately protecting the body from oxidative stress in the liver of the experimental rats with Triton WR-1339-induced hyperlipidemia.

Key words: Saururus chinensis, Oxidative stress, Hyperlipidemia, Fermentation

INTRODUCTION

Saururus chinensis has been reported to contain compounds such as lignans, alkaloids, diterpenes, flavonoids, tannins, steroids, and lipids. Fermentation is commonly used to break down certain undesirable compounds, to induce effective microbial conversion, and to improve the potential nutraceutical values (1,2).

Previous studies have reported that the fermentation process could modify naturally occurring constituents, including isoflavons, saponins, phytosterols, and phenols, and could enhance biological activities, specifically antioxidant and antimicrobial properties (3,4).

The probiotic strains used for fermentation exert beneficial effects and are safe (5).

Many oxygenated compounds, particularly aldehydes such as malondialdehyde (MDA) and conjugated dienes, are produced during the attack of free radicals to membrane lipoproteins and polyunsaturated fatty acids. Enzymic superoxide dismutase (SOD), glutathione peroxidase (GPx), and nonenzymic antioxidants play an important role in alleviating tissue damage due to the formation of free radicals.

Many studies have found that the level of serum MDA is higher in subjects with hyperlipidemia (6) and decreases following dietary supplementation with antioxidants. Similar observations have been reported in animal models of hyperlipidemia (7). That the risk of hyperlipidemia could be reduced by the consumption of flavonoids and their glycosides is supported by several studies (8-10).

Hyperlipidemia is one of the greatest risk factors contributing to the prevalence and severity of cardiovascular diseases. Therefore, it is very important to pay attention to the early-stage prevention and control of hyperlipidemia in a comprehensive way (11).

Inflammation is affected by hyperlipidemia, a robust risk factor for atherosclerosis. The endothelium preserves vascular integrity and prevents atherosclerosis by modulating the vasomotor tone, platelet activity, thrombosis, and inflammation. Increased vascular oxidative stress in hypercholesterolemia contributes to impaired endothelial function and atherogenesis (12).

In this study, the antioxidative effects of the Bacillus subtilis 168 fermentation of Saururus chinensis were investigated

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in a rat model with Triton WR-1339-induced hyperlipidemia by comparing the measured oxidative stress-related biological parameters of fermented Saururus chinensis extract to those of nonfermented Saururus chinensis extract.

MATERIALS AND METHODS

Measurement of Bacillus subtilis growth. The sterilized Saururus chinensis (SC) was inoculated with 1% Bacillus subtilis 168 at an absorbance of 1.0 at 600 nm, and the inoculated SC was fermented at 30°C and 40°C for 72 h. The cell growth of Bacillus subtilis 168 was identified.

Measurement of free-radical scavenging activity. 1 ml of the sample and 0.5 ml of 1,1-diphenyl-2-picrylhydrazyl (DPPH) alcoholic solution (0.2 mM) were mixed and incubated in a test plate at 37°C for 10 min. The absorbance at 517 nm was then measured.

Extraction methods. SC was purchased from Semyoung Oriental Co., Ltd. in Geochang, Gyeongnam (South Korea). 20 g SC and 200 ml myeolgyunsu were sterilized at 121°C for 20 min. Bacillus subtilis 168 was raised for 3 hr in Luria-Bertani (LB) broth. The sterilized SC was inoculated with 1% Bacillus subtilis 168 at an absorbance of 1.0 at 600 nm. The SC extract fermented with Bacillus subtilis 168 under the optimal conditions of temperature and time was used. For the nonfermented SC extract, dry SC was purchased from Semyoung Oriental Co., Ltd. and was reflux-condensed with 1 L 80% ethanol for 4 hr, after which it was concentrated with an evaporator and was freeze-dried.

Experimental animals. After the acclimation period, the rats were classified into groups. Triton WR-1339 (Sigma Co., USA), a specific cytotoxic agent for rats, was injected through tail vein at a dose of 200 mg/kg 40 hr before their anesthetization. The experiment group was divided into normal group (NOR), control group (TWR), fermented SC extract group (FSCT), and nonfermented SC extract group (NFSCT) (Table 1).

Blood and tissue collection. The animals were anesthetized with ether, and were dissected. The blood and liver were used for further analysis. The blood samples were centrifuged at 800 ×g for 10 min, at 4°C, to obtain serum samples, and were then stored at −80°C. The livers were rinsed with saline solution and were stored at −80°C. The liver tissues were homogenized in 1 : 5-volume PBS (pH 7.4). The homogenates were centrifuged at 800 ×g for 10 min, with the supernatant used as the liver total homogenate sample.

Assay of antioxidative activity.

Assay of SOD levels: The protein concentrations were determined according to a method described by Lowry *et al*. (13), using bovine serum albumin as the standard. 672 µl potassium phosphate buffer containing 200 mM KCl, 10 mM EDTA (0.2 M; pH 7.4), 100 µl xanthine (1 mM), 30 µl 1% DOC, 30 µl KCN (1.5 mM), and 150 µl cytochrome C (0.2 mM) were mixed with an 8-µl sample. After complete mixing, 10 µl xanthine oxidase (XOD) was added to each tube. The decrease in absorbance at 550 nm was measured for 2 min, and the SOD activity was expressed as U/mg protein.

Assay of catalase (CAT) levels: To 390 µl PBS (0.05 M; pH 7.0) was added 10 µl supernatant, and this mixture was used as the sample. PBS (1.9 ml; 0.05 M; pH 7.0) was mixed with 0.1 ml sample, and 1 ml H_2O_2 solution (30 mM) was added. The decrease in absorbance at 240 nm was measured for 90 s. The CAT activity was expressed as U/mg protein.

Assay of GPx levels: The reaction mixture consisted of 400 µl potassium phosphate buffer (0.1 M; pH 7.0), 70 µl NaN3 (0.01 M), 70 µl GSH (0.01 M), 70 µl NADPH (1.5 mM), 20 µl GSSG-redutase (1.8 U/ml), 360 µl double-deionized water, and 10 µl sample. It was kept at room temperature for 1 min, after which the reaction was initiated by adding 100 µl hydrogen peroxide (5 mM), and the absorbance was measured at 10 s intervals for 2 min at 340 nm. The GPx activity was expressed as U/mg protein.

Assay of MDA levels: Lipid peroxidation was assayed by measuring the amount of MDA that reacted with thiobarbituric acid (TBA) at 535 nm. Briefly, 1 ml sodium dodecyl sulfate (7% SDS) was added to 0.5 ml total liver homogenate or mitochondrial fraction. The tubes were mixed and incubated for 30 min at 37°C, after which 2 ml 0.67% TBA (mixed 1 : 1 with acetic acid) was added to the tubes. The tubes were mixed and placed in boiling water (100°C) for 50 min, after which 5 ml butanol was added. The tubes were mixed again and were centrifuged at 800 ×g for 10 min. The absorbance of the resulting supernatant was

Table 1. Experimental design of the hyperlipidemic rats

Experimental group (n)	Day 1~20 treatment (dose)	Day 21 treatment (dose)
NOR (7)	1.5 ml/kg 0.9% saline, i.p.	0.9% saline (1.5 ml/kg, i.p.)
TWR (7)	1.5 ml/kg 0.9% saline, i.p.	Triton WR-1339 (200 mg/kg, through tail vein)
FSCT (7)	1.5 ml/kg FSC extract (5 mg/kg), i.p.	
NFSCT (7)	1.5 ml/kg NFSC extract (5 mg/kg), i.p.	

NOR: normal group; TWR: Triton WR-1339-treated group; FSCT: fermented Saururus chinensis extract and Triton WR-1339-treated group; NFSCT: nonfermented Saururus chinensis extract and Triton WR-1339-treated group; i.p.: intraperitoneally; n: number of animals.

then measured at 535 nm. For this experiment, 1,1,3,3-tetraethoxypropane was used as the standard.

Histological analysis. The liver tissue from the experiment groups were immediately fixed in 10% formalin then treated with conventional grades of alcohol and xylol, embedded in paraffin, and sectioned with 4~6 μm thickness. The sections were then stained with hematoxylin and eosin (H&E) stain for the examination of the histopathological changes, which was done under a light microscope (NIKON CP 4500, Tokyo, Japan). An experienced histologist who was unaware of the treatment conditions made histological assessments (14).

Statistical analysis. All the grouped data were statistically evaluated with the SPSS version 12kor software. One-way analysis of variance (ANOVA) followed by a least-significant difference (LSD) test was used to determine the significance of the differences between the groups. Statistical analysis was performed with student's t test. The results were considered statistically significant when $p < 0.05$, 0.01, and 0.001. All the results were expressed as mean ± SD.

RESULTS AND DISCUSSION

Establishment of the optimal temperature of fermentation. As favorable conditions during fermentation result in an accelerated growth phase, the maximum growth rate is the response that defines the fermentation process. The optimal temperature of Bacillus subtilis 168-fermented Saururus chinensis extract was 40°C showing maximum growth rates higher than that of 30°C (Fig. 1).

Establishment of the optimal time of fermentation in optimal temperature of 40°C. Free radicals are known to be a major factor in biological damages, and DPPH has been used to evaluate the free radical scavenging activity of natural antioxidants. DPPH is a radical itself with a purple color, which changes into a stable yellow-colored compound by reacting with an antioxidant, and the extent of the reaction depends on the hydrogen-donating ability of the

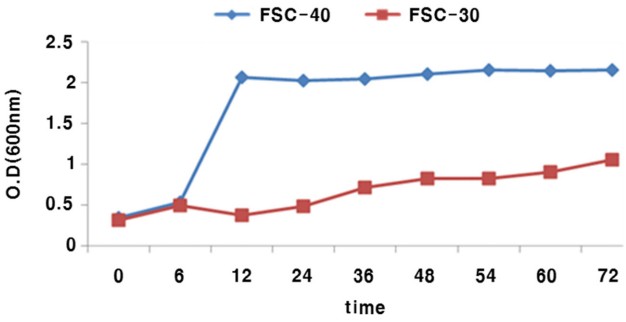

Fig. 1. Cell Growth of Bacillus subtilis 168 in temperature condition FSC-40: fermented Saururus chinensis extract-40°C. FSC-30: fermented Saururus chinensis extract-30°C. This was for the confirmation of optimal fermentation temperature, which was 40°C.

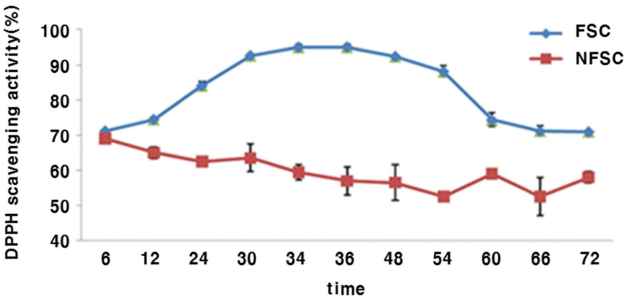

Fig. 2. Free radical scavenging effect in FSC and NFSC at 40°C. FSC: fermented Saururus chinensis extract; NFSC: nonfermented Saururus chinensis extract. This was for the confirmation of optimal fermentation time, which was 34 hr.

Table 2. SOD, CAT, GPx levels in liver total homogenate and mitochondrial fraction of Triton WR-1339-induced hyperlipidemic rats

Experimental group		NOR (unit/mg protein)	TWR (unit/mg protein)	FSCT (unit/mg protein)	NFSCT (unit/mg protein)
MF	SOD	57.93 ± 3.09^{c}	31.02 ± 0.59^{a}	53.95 ± 1.09^{c} (85.21)	46.36 ± 2.95^{b} (57.02)
LH	SOD	50.18 ± 4.78^{d}	15.57 ± 1.35^{a}	42.02 ± 0.61^{c} (76.42)	35.53 ± 0.61^{b} (57.65)
	CAT	276.72 ± 13.54^{d}	52.28 ± 5.20^{a}	128.93 ± 10.97^{c} (34.15)	101.62 ± 11.93^{b} (21.98)
	GPx	91.00 ± 4.09^{d}	27.13 ± 9.79^{a}	64.27 ± 4.95^{c} (58.15)	53.04 ± 7.71^{b} (40.56)

NOR: normal group, TWR: Triton WR-1339-induced group, FSCT: fermented Saururus chinensis extract and Triton WR-1339-treated group, *NFSCT: nonfermented Saururus chinensis* extract and Triton WR-1339-treated group, MF: mitochondrial fraction (unit/mg protein), LH: liver total homogenates (unit/mg protein), SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase.
The number of pqrenthesis is the rate of inhibition(%).
Results are presented as the mean ± S.D. (n = 7).
a, b, c, d are different group by one-way ANOVA with post-hoc test.

antioxidant (15). DPPH scavenging activity was used to establish the optimal time of fermentation in Saururus chinensis extract fermented at 40°C by Bacillus subtilis 168. The optimal time of fermentation was 34 hr showing more than 95% of DPPH scavenging activity (Fig. 2).

Antioxidative biological parameters: Antioxidative biological parameters associated with oxidative stress were showed in Table 2.

SOD converts $\bullet O_2^-$ into H_2O_2, preventing the attack of PUFA by OH, which is produced by the reaction of $\bullet O_2^-$ with H_2O_2. The SOD levels in liver total homogenates were lower in the TWR group than in the NOR group and the FSCT and NFSCT group. The mitochondrial SOD and liver SOD levels were also lower in the TWR group than in the NOR group and the FSCT and NFSCT group. SOD levels in mitochondrial fraction and liver total homogenates are shown in Table 2. SOD level (31.02 unit/mg protein and 15.57 unit/mg protein) of TWR group was found to be lower than that (57.93 unit/mg protein and 50.18 unit/mg protein) of NOR group. SOD level (53.95 unit/mg protein and 42.02 unit/mg protein) of FSCT group was higher than that (46.36 unit/mg protein and 35.53 unit/mg protein) of NFSCT group. Although FSC did not prevent the deactivation of SOD to the levels of the NOR group, FSC prevented the deactivation of SOD resulting from oxidative stress more than NFSC.

CAT converts H_2O_2 into H_2O, preventing the attack of polyunsaturated fatty acids (PUFA) by OH, which is generated by the reaction of $\bullet O_2^-$ with H_2O_2. The CAT levels in the liver total homogenates were lower in the TWR group than in the NOR group and FSCT and NFSCT group. CAT level (52.28 unit/mg protein) of TWR group was found to be lower than that (276.72 unit/mg protein) of NOR group. CAT level (128.93 unit/mg protein and 101.62 unit/mg protein) of FSCT group and NFSCT group were found to be higher than that of TWR group. CAT level of FSCT group was higher than that of NFSCT group. Triton WR-1339 decreased the activation of CAT, but FSC and NFSC treatment restored the activation of CAT.

GPx converts hydroperoxide (ROOH) into GSSG. GPx prevents the attack of PUFA by RO• and •OH, which are derived from unstable ROOH. GPx levels in liver total homogenate were shown in Table 2. GPx level (27.13 unit/mg protein) of TWR group was found to be lower than that (91.00 unit/mg protein) of NOR group. GPx level (64.27 unit/mg protein) of FSCT group was higher than that of NFSCT group (53.04 unit/mg protein). Triton WR-1339 decreased the activation of GPx. FSC treatment increased the reactivation of GPx more than NFSC treatment.

Antioxidative enzyme (SOD, CAT and GPx levels in mitochondrial fraction and total liver homogenate) in the FSCT group was higher than that in the NFSCT group (Fig. 4).

MDA is one of the end-products of the lipid peroxidation process and of oxidative stress. MDA and lipid peroxide produced during oxidative stress cause or exacerbate various diseases related to aging and hepatotoxicity. The levels of MDA, a marker of lipid peroxidation increased, but FSC and NFSC prevented an increase in the MDA level in the liver total homogenate fraction of the FSC- and NFSC-treated rats (Table 3). The MDA level (4.24 nmol/mg protein) of the TWR group increased compared with that of the NOR group (1.14 nmol/mg protein). The MDA levels of the FSCT and NFSCT groups (1.90 and 2.72 nmol/mg protein, respectively) decreased 2.22- and 1.55-fold compared with that of the TWR group. The Triton WR-1339 increased the levels of MDA, a marker compound in the process of lipid

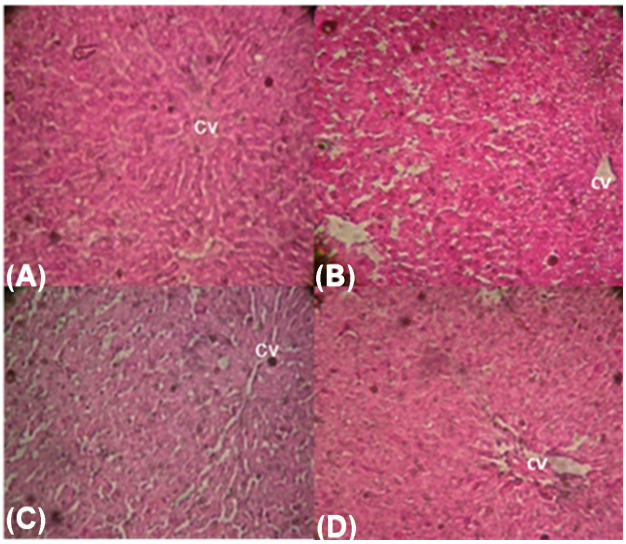

Fig. 3. Histological examination of liver tissue (H&E staining, 200x). (A) NOR: normal group (B) TWR: Triton WR-1339-treated group (C) FSCT: fermented Saururus chinensis extract and Triton WR-1339-treated group (D) NFSCT: nonfermented Saururus chinensis extract and Triton WR-1339-treated group; original magnification: 200x; H&E: hematoxylin and eosin.

Table 3. MDA levels in liver total homogenate of Triton WR-1339-induced hyperlipidemic rats

	Liver total homogenate	
Experimental group	MDA contents (nmol/mg protein)	Inhibition (%)
NOR	1.14 ± 0.24^a	-
TWR	4.24 ± 0.17^d	-
FSCT	1.90 ± 0.19^b	75.42
NFSCT	2.72 ± 0.13^c	48.98

NOR: normal group, TWR: Triton WR-1339-treated group, FSCT: fermented Saururus chinensis extract and Triton WR-1339-treated group, NFSCT: nonfermented Saururus chinensis extract and Triton WR-1339-treated group, MDA: malondialdehyde.
Results are presented as the mean ± S.D. (n = 7).
a, b, c, d are different group by one-way ANOVA with post-hoc test.

peroxidation, but the FSC and NFSC treatments decreased them.

Histological examination of the liver tissue. The treatment of rats with Triton WR-1339 to induce liver fibrosis is an accepted model for mimicking human disease. As shown in Fig. 3, treatment with Triton WR-1339 caused diffused and massive intracytoplasmic vacuolation of the hepatocytes, indicating fat globule deposition in the hepatocytes (as evidence of fatty-liver degeneration) and venous congestion of the hepatic central vein compared with the normal liver architecture.

The FSCT and NFSCT groups significantly attenuated the histological features of fatty-liver degeneration, as evidenced by the mild intracytoplasmic hepatocyte vacuolation.

Oxidation is an essential biological process to many organisms for the production of energy. The uncontrolled production of oxygen-derived free radicals, however, is involved in the onset of many diseases, such as cancer, rheumatoid arthritis, and atherosclerosis, as well as in degeneration processes associated with aging (16).

Cells must maintain a proper balance between the levels of free radicals and antioxidants to ensure the structural integrity of the critical components. When the levels of free radicals exceed those of the antioxidants during oxidative stress, sensitive biomolecules such as lipids, proteins, and DNA, in particular, can be damaged (17).

The free radicals derived from oxidative stress lead to the accumulation of MDA as an end-product of lipid peroxidation, resulting in the deactivation of the antioxidant enzymes SOD, CAT, and GPx (18).

The nonionic detergent Triton WR-1339 has been widely used to block the uptake of triacylglycerol-rich lipoproteins from plasma by the peripheral tissues, to produce acute hyperlipidemia in animal models, which are often used for a number of objectives, particularly for screening natural or chemical hypolipidemic drugs (19-21).

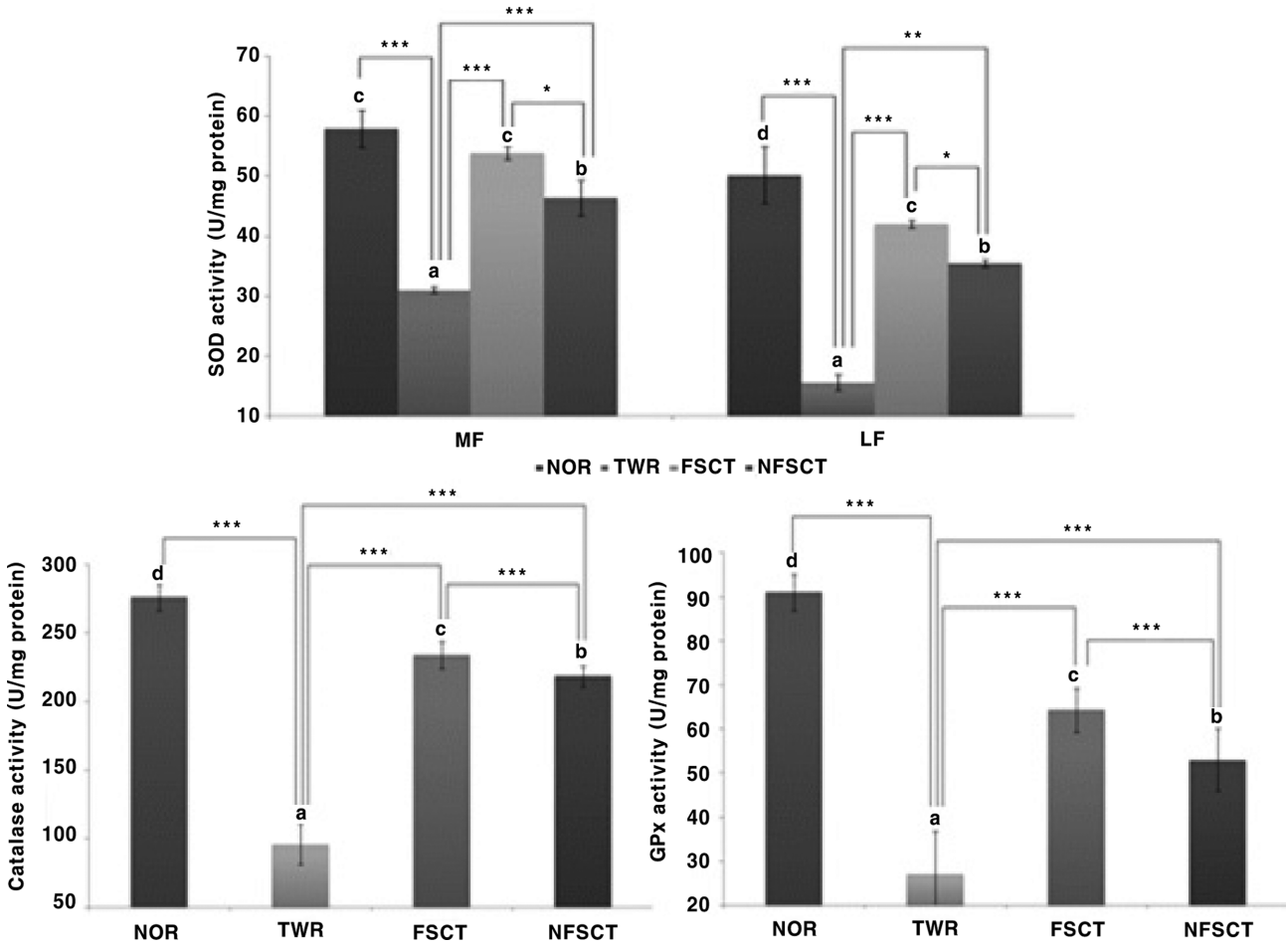

Fig. 4. Presentation of significant differences for SOD, CAT, GPx levels in liver total homogenate NOR: normal group; TWR: Triton WR-1339-treated group; FSCT: fermented Saururus chinensis extract and Triton WR-1339-treated groups; NFSCT: nonfermented Saururus chinensis extract and Triton WR-1339-treated group. MF: mitochondrial fraction; LF: liver total homogenate fraction. The results are presented as mean ± SD (n = 7). Significantly different from the value of the TWR group at $^{***}p < 0.001$ and $^{*}p < 0.01$.

Triton WR-1339 intake increased the MDA levels in the TWR group. The deactivation of antioxidative enzymes like SOD, CAT, and GPx enabled the abundant •OH^- radicals and the excessive RO• radicals with the originally produced •O_2^- to attack the PUFA, which initiated the lipid peroxidation, ultimately leading to the increase of MDA, the end-product of oxidative stress in the liver.

Of course, the SC treatment decreased the MDA levels in both FSCT and NFSCT groups, regardless of whether they were fermented or not, as shown in Table 3. The treatment of SC ultimately led to the decrease of MDA, the end-product of oxidative stress, by scavenging ROS, such as the •OH and RO• radicals, and by preventing the attack of the PUFA, which suppressed the oxidative stress.

As shown in Fig. 4, there were significant differences between fermented Saururus chinensis group (FSCT) and nonfermented Saururus chinensis group (NFSCT) for major biological parameters of oxidative stress such as SOD, CAT, and GPx. The fermented group had the more useful effect than the nonfermented group.

Considering all the aforementioned results, it can be said that fermentation played a more excellent role than nonfermentation in ultimately protecting the body from oxidative stress in the liver of the experimental rats with Triton WR-1339-induced hyperlipidemia.

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