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RESEARCH ARTICLE

Evaluation of Protective effects of Fermented *Ficus racemosa* Fruit Extract against Oxidative Stress and Hyperglycemia

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ABSTRACT

Objective: Uncontrolled oxidative stress and diabetes are linked to multiple serious health issues. This study aimed to demonstrate the potency of *Aspergillus niger* solid-state fermentation (SSF) to enhance the preventive and therapeutic capacity of *Ficus racemosa* fruit extract (FRFE) against oxidative stress and hyperglycaemia.

Materials and Methods: SSF and post-SSF ultrasound-assisted extraction was utilised to prepare fermented FRFE (F-FRFE). F-FRFE was compared with unfermented FRFE (U-FRFE) in terms of the antioxidant activity and the protective effect against oxidative stress and hyperglycaemia. The antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl/2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging, ferric reducing antioxidant potential, and thiobarbituric acid reactive substance assays. The protective effect against oxidative stress was evaluated in human hepatocytes treated with the oxidative stress inducer, H_2O_2. The antioxidative stress and antihyperglycemic impact was further investigated in a streptozotocin-induced diabetic mouse model.

Results: Compared with U-FRFE, F-FRFE possessed higher *in vitro* and *ex vivo* antioxidant potential. In human hepatocytes, H_2O_2 treatment induced apoptotic cell death, which was alleviated by F-FRFE at a higher extent compared with U-FRFE. In the diabetic mouse, F-FRFE but not U-FRFE treatment completely subsidised hyperglycaemia and elevated oxidative stress.

Conclusion: Our findings demonstrated the protective effectiveness of F-FRFE against oxidative stress and hyperglycaemia with prospects in pharmaceutical applications.

Keywords: *Ficus racemosa*, Antioxidant, Hyperglycaemia, Diabetes, Oxidative stress, Solid-state fermentation

INTRODUCTION

Oxidative stress occurs when intrinsic antioxidant defences are overburdened by the production of reactive oxygen species (ROS) or due to the imbalance between ROS and the antioxidant level. ROS are produced by biological organisms to regulate various cellular processes such as stress response, cell death or survival, and inflammation.[1] Excessive accumulation of ROS or increased oxidative stress has been implicated in the development of multiple health issues such as ageing, obesity, diabetes, cardiovascular disease, osteoporosis, chronic kidney disease, chronic obstructive pulmonary disease, neurodegenerative diseases, and cancer.[1] Several reports have suggested that natural compounds, especially polyphenols extracted from plant sources, can remarkably reduce oxidative stress and attenuate oxidative stress-related health disorders such as diabetes and cancer.[2] Diabetes mellitus is a metabolic disorder with persistent hyperglycaemia, caused by the failure of insulin production (type 1) or insulin resistance (type 2). Diabetes is recorded as one of the most prevalent diseases worldwide with a high rate of premature morbidity and mortality.[3] Diabetic onset is linked to increased production of ROS, resulting in oxidative stress, which further escalates the progression of diabetes mellitus and leads to the development of other severe disorders.[4] Therefore, uncontrolled or untreated diabetic patients would develop serious complications, many of which would be caused by elevated oxidative stress. The current main treatment strategy for diabetes is the use of antihyperglycemic or hypoglycaemic drugs, such as metformin, thiazolidinediones, sulfonylureas, dopamine-2 agonists, glucagon-like peptide-1 agonists, dipeptidyl peptidase-4 inhibitors, and sodium-glucose co-transporter-2 inhibitors, either alone or in combination.[2] Nevertheless, chronic treatment with these chemical agents could lead to various undesirable side effects such as abdominal pain,

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nausea, and body weight gain.[5] Therefore, tremendous efforts have been made to identify alternative natural compounds in the management of hyperglycaemia and diabetes. Among these compounds, polyphenols extracted from plant sources, especially medicinal plants, are excellent candidates due to their low toxicity and high antioxidant and hypoglycaemic potential.[2]

Ficus racemosa is cultivated in various countries, including Southeast Asia, China, Malaysia, India, Sri Lanka, Pakistan, New South Wales and Australia.[6] Different parts of *F. racemosa* tree have been used as a traditional medicine in many countries and reported to possess multiple biological activities such as antioxidant, antimicrobial, anti-diabetic, anti-cancer, hepatoprotective and anti-inflammatory capability.[6] The bioactive content and biological activities of various plant extracts have been shown to be augmented via solid-state fermentation (SSF).[7-11] Therefore, this study investigated the ability of SSF to enhance the bioactive values of *F. racemosa* fruit extract (FRFE) in terms of total phenolic content (TPC), total flavonoid content (TFC), the *in vitro* and *ex vivo* antioxidant activity, the protective capacity against oxidative stress injury in hepatocytes and the antioxidative stress and anti-hyperglycaemic potential in a diabetic mouse model.

MATERIALS AND METHODS

Sample Preparation for SSF

Ficus racemosa fruit was collected from the Tien Giang province in Vietnam. The sample was available at the herbarium of the International University–VNU HCM, Vietnam. *F. racemosa* fruit was cleaned, air dried and ground into a fine powder, which was the dried material (DM) for SSF.

SSF and Post-SSF Extraction

The *Aspergillus niger* strain was obtained from the microbial library of Nguyen Tat Thanh University, Vietnam, and was identified as *A. niger* by 18s rRNA gene sequencing. SSF and post-SSF ultrasound-assisted extraction was performed as described with modifications.[7] Briefly, DM was mixed with water in an Erlenmeyer flask covered with cotton plugs to obtain a moisture content of 70%. The samples were then sterilised at 121°C for 15 min, cooled, and inoculated with a fungal spore suspension (10^7 spores/g DM). The inoculated flasks were incubated at 30°C for 5 days. Next, 95% ethanol was added at 10:1 mL/g DM, followed by ultrasonication (40 kHz and 400 W) for 20 min at 30°C. The mixture was shaken for 5 min at room temperature and then centrifuged at 8,000 rpm for 20 min. The supernatant was filtered through a 0.2 µm membrane filter and concentrated by a vacuum evaporator at 40°C for 8 h. Unfermented *F. racemosa* fruit extract (U-FRFE) was prepared in the same manner as the fermented *F. rasemosa* fruit extract (F-FRFE), except for the absence of fungi. U-FRFE or F-FRFE was dissolved in deionised water to obtain the desired concentrations for the *in vitro, ex vivo, in vivo* and cell culture experiments.

Determination of the Total Phenolic Content

The TPC of the samples was measured by the Folin–Ciocalteu method as described in a previous study.[7] Briefly, 12 µL of the samples were mixed with 100 µL of diluted Folin&Ciocalteu's phenol reagent and incubated for 5 min at room temperature, followed by the addition of 100 µL of Na_2CO_3 (60 g/L). Next, the mixture was incubated for 1 h at room temperature and the absorbance was measured at 725 nm. TPC was expressed as mg of gallic acid equivalent per 1 g of DM (mg GAE/g DM), determined from the gallic acid calibration curve. All chemicals were purchased from Sigma (USA).

Determination of the Total Flavonoid Content

The TFC was determined as described in a previous study.[7] Briefly, 50 µL of the samples were mixed with 75 µL sodium nitrite (5%) and 75 µL aluminium trichloride (10%), and the mixture was incubated for 5 min before the addition of 100 µL sodium hydroxide (1%). After incubation for 1 h, the absorbance was measured at 510 nm. The total flavonoid content was expressed as milligrams of quercetin equivalent per 1 g of DM (mg QE/g DM) based on the calibration curve for quercetin. All chemicals were purchased from Sigma (USA).

Phytochemical Screening by Colour Tests

Colour tests for the presence of different phytochemical groups were performed according to previously described procedures.[7] The groups tested were as follows: alkaloid (Dragendorff test), flavonoid (Bate-Smith and Metcalf test), glycoside (Kedde reaction), phenol, saponin (foam test), terpenoid (Salowski test), triterpenoid and steroid (Liebermann-Burchard test).

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Radical Scavenging Assay

The ABTS radical scavenging capacity was determined as previously described.[7] Briefly, the ABTS radical solution was prepared by mixing the ABTS solution (7 mM) with the potassium persulfate solution (2.45 mM). The mixture was kept in the dark at room temperature for 16 h before being diluted to obtain a working ABTS radical solution with an absorbance at 734 nm of 0.7. Then, 200 µL of the samples were added to 600 µL of ABTS radical working solution and the mixture was incubated for 30 min at room temperature in the dark. The absorbance at 734 nm was then measured. The percentage of scavenging activity (% SA) was calculated from the absorbance of the control (A_c) and ascorbic acid/extract sample (A_s) as follows: % SA = $(A_c - A_s) \times 100 / A_c$.

The half-maximal inhibitory concentration (IC_{50}) value was

then determined based on the linear curve showing the % SA at different sample concentrations, which was the concentration at which % SA = 50. A higher IC_{50} value indicated a lower radical scavenging ability. All chemicals were purchased from Sigma (USA).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging capacity of FRFE was determined as previously described.[7] Briefly, 20 µL of the samples were mixed with 180 µL of DPPH (6×10^{-5} M, Sigma, USA). The mixture was incubated for an hour at room temperature in the dark, and the absorbance was measured at 517 nm. DPPH solution was used as a control. The % SA and IC_{50} were calculated as in the ABTS radical scavenging assay.

Ferric Reducing Antioxidant Potential (FRAP) Assay

The FRAP assay was performed as previously described.[7] Briefly, the FRAP working reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6) with 1 volume of 2,4,6-tri(2-pyridyl)-s-triazine (10 mM) prepared in 40 mM hydrochloric acid and 1 volume of 20 mM ferric chloride. Then, 200 µL of the samples were added to 10 µL of the FRAP working solution and the mixture was incubated at 37°C for 30 min. The absorbance was then measured at 593 nm. The reducing power was calculated from the ferrous sulphate standard curve. FRAP values were expressed as millimoles of ferrous sulphate equivalent per 1 g of sample (mmol FSE/g). All chemicals were purchased from Sigma (USA).

Ex Vivo Lipid Peroxidation or Thiobarbituric Acid Reactive Substance (TBARS) Assay using Mouse Brain Homogenates

The assay was performed as previously described.[12] Trolox (Sigma, USA) was used as an antioxidant control. The *ex vivo* assay was based on the determination of the relative amount of malondialdehyde (MDA). The MDA level was determined based on the reaction with TBA, resulting in products with maximum absorbance at 532 nm. Briefly, mouse brains were homogenised in phosphate buffer (pH 7.4) at a 1:10 ratio at 4°C. Brain homogenates were incubated with the extract samples and $FeSO_4/H_2O_2$ mixture ($FeSO_4$ 0.1 mM:H_2O_2 15 mM at 1:1 ratio) at 37°C for 15 min. Trichloracetic acid 10% was added to the mixture, which was then centrifuged at 12,000 rpm for 5 min. The supernatant was collected and incubated with 0.8% TBA in a 2:1 ratio at 100°C for 15 min. The absorbance was measured at 532 nm and the IC_{50} was determined. All chemicals were purchased from Sigma (USA).

Cell Culture

HepG2 cells (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma, USA), 100 U/mL penicillin (Sigma, USA), and 100 µM streptomycin (Sigma, USA). The cells were maintained in a 5% CO_2/95% air incubator at 37°C. All experiments were performed at less than 10 passages. Cells were tested for mycoplasma every 2 months (Universal Mycoplasma Detection Kit, ATCC, USA).

WST1 Cell Survival Assay

Cell viability was determined using the cell proliferation reagent WST-1 (Sigma, USA) in a 96-well plate format according to the manufacturer's instructions.

Western Immunoblotting

Protein extract preparation and subsequent sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE)/Western immunoblotting were performed as previously described.[13-16] Briefly, the cells were lysed in cell lytic M buffer and the protein concentration was determined by Bradford. The total protein lysate was then subjected to sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting for cleaved caspase-9 and β-actin as a loading control. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies were used as secondary antibodies. All antibodies were purchased from Cell Signalling Technology (USA).

Animal Studies and Sample Collection

Male Swiss albino mice were obtained from the Nha Trang Institute of Vaccines and Biological Products in Vietnam after six weeks of age. The mice were acclimated to standard environmental conditions for a week and fed standard rodent chow and water ad libitum. All mouse studies were conducted under the supervision and approval of the Animal Research Committee of the International University, Vietnam National University of Ho Chi Minh City (No. BT03/2023, http://crl.bio.hcmiu.edu.vn/about-us/facilities/) and according to ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Examination of the Acute Toxicity

To determine acute toxicity, mice (five male mice per group) were given a single oral dose of the vehicle control, U-FRFE or F-FRFE at various doses (0.2, 1 and 5 g/kg). Mortality and general behaviour were reported daily for 14 days.

Examination of the Antioxidative Stress and Antihyperglycemic Effect

Mice were IP injected with a single dose of the vehicle control or streptozotocin (STZ, 70 mg/kg BW, Sigma, USA). After 7

days of vehicle or STZ treatment, mice with blood glucose levels above 200 mg/dL were treated daily with vehicle control, U-FRFE or F-FRFE at 0.2 g/kg body weight (BW), or glibenclamide (5 mg/kg BW). The experiments were performed in two independent times and there were four mice per group in each replicate for a total of eight mice per group.

Quantification of Serum Glucose

Serum glucose levels were determined using the GOD-PAP Kit (Human Diagnostic Ltd. Co., Uganda) following the manufacturer's instructions.

Quantification of MDA in Livers in Livers and Kidneys

MDA levels in the livers and kidneys were determined as previously described.[2] Briefly, the livers and kidneys were homogenised in KCl 1.15% for 1 min at 13,000 rpm. The homogenates were then incubated with Tris-HCl (pH 7.4) at 37°C for 1 h, and TCA 10% was added to stop the reaction. The supernatant obtained by centrifugation reacted with TBA 0.8% at 100°C for 15 min and the absorbance was measured at 532 nm. The concentration of MDA was determined from the MDA standard, normalised to total liver or kidney protein (determined by Bradford) and expressed as nmol per 1 g of protein. All chemicals were purchased from Sigma (USA).

Quantification of Reduced Glutathione in Livers and Kidneys

The reduced glutathione (GSH) level in the livers and kidneys was determined as previously described.[2] Briefly, the livers and kidneys were homogenised in KCl 1.15% for 1 min at 13,000 rpm. The homogenates were then incubated with Tris-HCl (pH 7.4) at 37°C for 1 h, and TCA 10% was added to stop the reaction. The supernatant obtained from the centrifugation reacted with the Elman reagent in phosphate-EDTA buffer for 3 min at room temperature, and the absorbance was measured at 412 nm. The GSH concentration was determined from the GSH standard, normalised to total liver or kidney protein (determined by Bradford) and expressed as nmol per 1 g of protein. All chemicals were purchased from Sigma (USA).

Statistical Analysis

Student's t-test was used for significant comparisons between the two groups. ANOVA followed by the post hoc Tukey's honestly significant difference (HSD) test was used for comparisons between three or more groups. All statistical analyses were performed using the R program. A p-value of less than 0.05 was considered a significant difference.

RESULTS

Improvement of the Bioactive Phytochemical Content and Antioxidant Activity after SSF

As shown in Table 1, TPC and TFC were significantly increased after the fermentation duration of 5 days. Besides polyphenols, the levels of other bioactive phytochemicals such as terpenoids and sterols could be increased post- SSF, as indicated by the enhanced signals in the phytochemical screening (Table 2). The increased bioactive content in FRFE after SSF was congruent with the increase in *in vitro* and *ex vivo* antioxidant activity (Table 1).

FRFE with Enhanced Protective Capacity against Oxidative Stress Injury in Hepatocytes

H_2O_2 was utilised to induce oxidative stress in hepatocytes. H_2O_2 treatment resulted in decreased cell survival and increased cellular levels of the apoptotic marker, cleaved caspase-9, which was completely blocked by pretreatment with F-FRFE. In contrast, pretreatment with U-FRFE (Figure 1) partially alleviated oxidative stress-mediated apoptotic cell death.

Acute Toxicity Examination

In the acute toxicity study, no mortality, signs of toxicity, or significant behavioural changes, including grooming, hyperactivity, loss of righting reflex, sedation, and convulsions, were observed after oral administration of either U-FRFE or F-FRFE for up to 14 days, even at the highest dose of 5 g/kg BW (Table 3).

FRFE with Enhanced Protective Capability against Oxidative Stress and Hyperglycaemia in Streptozotocin-Induced Mouse Model

In mice treated with a single dose of streptozotocin, an increase in blood glucose levels (Figure 2) and oxidative stress, as indicated by increased MDA levels and decreased GSH levels in the kidneys and livers (Figure 3), were observed. GSH is considered to be one of the most important antioxidants protecting cells from ROS and oxidative stress damage. A decrease in the GSH level is therefore an indicator of increased oxidative stress. Another well-studied marker of oxidative stress is MDA, which is one of the end products of lipid peroxidation. All of these disturbances were reversed by treatment with either F-FRFE or glibenclamide, a hypoglycaemic agent commonly used for treating type 2 diabetes. However, the mitigating effect of U-FRFE on oxidative stress and hyperglycaemia was to a lesser extent compared with that of F-FRFE, suggesting SSF-mediated enhancement of the protective efficacy of FRFE against oxidative stress and hyperglycaemia.

Table 1. Total phenolic content, total flavonoid content and the antioxidant activity of unfermented and fermented *Ficus racemosa* fruit extract.

	TPC (mg GAE/g DM)	TFC (mg QE/g DM)	ABTS IC_{50} (µg/mL)	DPPH IC_{50} (µg/mL)	FRAP (mmol FSE/g)	*Ex vivo* IC_{50} (µg/mL)
U	16.30 ± 0.56	1.06 ± 0.08	126.80 ± 5.87	159.17 ± 8.94	0.28 ± 0.03	54.14 ± 4.39
F	34.13 ± 1.33*	2.33 ± 0.13*	55.18 ± 2.55*	76.53 ± 5.08*	0.75 ± 0.04*	21.45 ± 2.14*
AA	-	-	1.76 ± 0.17	2.27 ± 0.15	19.70 ± 0.79	-
Trolox	-	-	-	-	-	23.92± 1.38

TPC, total phenolic content; GAE, gallic acid equivalent; DM, dry material; TFC, total flavonoid content; QE, quercetin equivalent; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant potential; FSE, ferrous sulphate equivalent; IC_{50}, half-maximal inhibitory concentration; U, unfermented *Ficus racemosa* fruit extract; F, fermented *Ficus racemosa* fruit extract (F); AA, ascorbic acid; data were means ± standard deviation from 3 independent replicates; * $p<0.05$, compared to U.

Table 2. The presence of phytochemical groups in unfermented and fermented *Ficus racemosa* fruit extract.

Group	U	F
Alkaloid	+	-
Flavonoid	+	++
Glycoside	+	++
Phenol	+	++
Saponin	-	-
Tannin	+	++
Terpenoid	+	++
Triterpenoid and steroid	+	++

U, unfermented *F. racemosa* fruit extract; F, fermented *F. racemosa* fruit extract; (-), undetected; (+) and (++), detected; group marked with (++) had higher signal intensity in colour tests than group marked with (+).

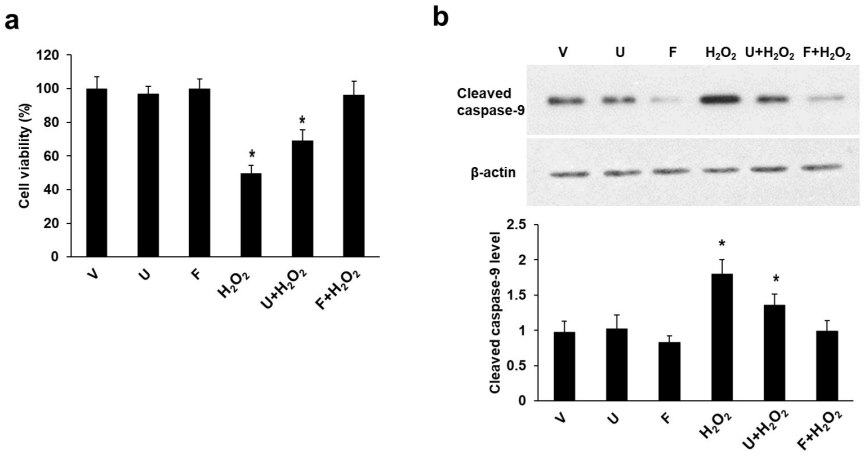

Figure 1. Protective efficacy against oxidative stress injury in hepatocytes. HepG2 cells were treated with vehicle (V), unfermented *Ficus racemosa* fruit extract (U), or fermented *Ficus racemosa* fruit extract (F) (0.2 mg/mL) for 30 min before being treated with vehicle or H_2O_2 (200 µM) for 6 h. Then, the cells were subjected to the WST1 cell viability assay (a) or Western immunoblotting for cleaved caspase-9 or β-actin (b). Data in graphs were means ± standard deviation. In (a), the cell viability for the vehicle group (V) was normalised to 100%; n = 5 from two independent occasions. In (b), the cleaved caspase-9 density was normalised to β-actin density and caspase-9/β-actin density ratio was then normalised to 1; n = 3 from two independent occasions. * $p<0.05$, compared to all other groups.

Table 3. Observation of the mortality and signs of toxicity after 14 days of administration of unfermented and fermented *Ficus racemosa* fruit extract.

Dose (mg/kg BW)		Mortality	Grooming	Hyperactivity	Loss of the righting reflex	Sedation	Convulsion
U	0.2	N	N	N	N	N	N
	1	N	N	N	N	N	N
	5	N	N	N	N	N	N
F	0.2	N	N	N	N	N	N
	1	N	N	N	N	N	N
	5	N	N	N	N	N	N

BW, body weight; U, unfermented *Ficus racemose* fruit extract; F, fermented *Ficus racemose* fruit extract; N, no mortality or no abnormal sign for all five mice in a group.

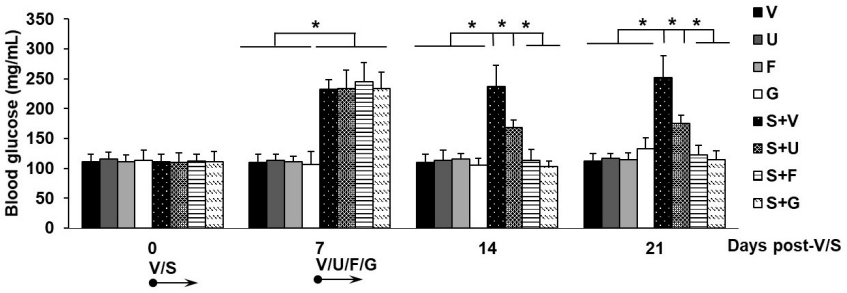

Figure 2. Protective effect against hyperglycaemia in streptozotocin-induced diabetic mouse model. After 7 days of vehicle (V) or streptozotocin (S) treatment, the mice were treated with vehicle (V), unfermented *Ficus racemosa* fruit extract (U), fermented *Ficus racemosa* fruit extract (F), or glibenclamide (G). Serum samples were collected on days 0, 7, 14, and/or 21 after V/S treatment for the determination of glucose levels. Data were means ± standard deviation; n = 8; * $p<0.05$, compared to all other groups.

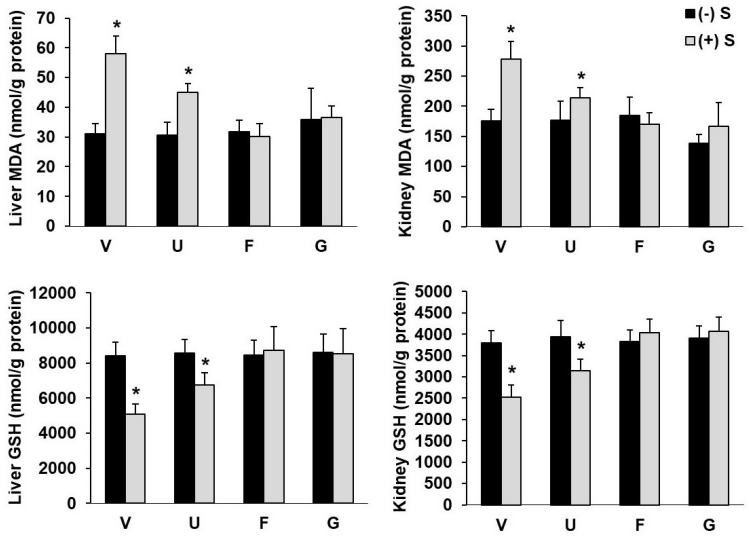

Figure 3. The protective effect against oxidative stress in a streptozotocin-induced diabetic mouse model. After 7 days of vehicle (V) or streptozotocin (S) treatment, the mice were treated with vehicle (V), unfermented *Ficus racemosa* fruit extract (U), fermented *Ficus racemosa* fruit extract (F), or glibenclamide (G). Livers and kidneys were collected on day 21 post-V/S treatment for the determination of malondialdehyde (MDA) or reduced glutathione (GSH) levels. Data were means ± standard deviation; n = 8; * $p<0.05$, compared to all other groups.

DISCUSSION

This study implicated *A. niger* SSF as an effective and environmentally friendly approach to prospectively augment the release of various bioactive compounds in FRFE. The mechanistic action of SSF for increasing the bioactive content of the plant extract has not been thoroughly investigated. It is likely that enzymatic conversion and/or cleavage of the material matrix contributes to the enhanced release of bioactive compounds in the plant extract,[7] which requires further investigation to substantiate the suggested mechanisms and determine the specific change in the phytochemical profile after SSF.

The results of the phytochemical composition and *in vitro* antioxidant activity of U-FRFE were congruent with those of a previous study[17] using FRFE prepared in the same manner. Specifically, the TPC, TFC, and DPPH IC_{50} values determined in the present study, as well as the presence of alkaloids, flavonoids, tannins, and steroids, were consistent with the previous study. However, the DPPH radical scavenging activity of U-FRFE was lower than that of FRFE in another study,[18] probably due to differences in the extraction method and in the material source.

Different types of polyphenols may be associated with the antioxidant and antidiabetic potentials of the plant extract.[18] Regarding the polyphenolic profile, a previous study reported that FRFE contains significant amounts of caffeic acid, catechin, (-)-epicatechin, gallic acid, p-coumaric acid, rutin, syringic acid, trans-ferulic acid, vanillin and vanillic acid,[19] some of which may be increased after SSF. In addition to the polyphenolic compounds, other phytochemicals in FRFE may contribute to the protective capacity against oxidative stress and hyperglycaemia. Further research is essential to clarify the relationship between the change in phytochemical composition after SSF and the enhanced effect of FRFE on antioxidative stress and anti- hyperglycaemia. Since F-FRFE was shown to have higher free radical scavenging activity and increased intrinsic GSH level, it is plausible that it prevented oxidative stress or ROS accumulation through multiple actions: limiting ROS formation, removing existing ROS by direct scavenging activity and/or enhancing intrinsic antioxidant defence to limit ROS accumulation. Further studies are needed to determine the mechanistic action of F-FRFE in suppressing oxidative stress.

The human hepatoma cell line HepG2 was used in this study as a cellular model of oxidative stress injury. HepG2 is originally derived from hepatocellular carcinoma but still retains several biological characteristics of hepatocytes. It has been used to study oxidative injury induced by H_2O_2, iron arachidonic acid, CYP2E1, alcohol, and hepatitis C virus.[20] Regarding H_2O_2, it has been widely used to induce oxidative stress injury in hepatocytes and other cell types.[21-23] In particular, H_2O_2 has been reported to induce apoptotic cell death, which is consistent with the results of this study. Importantly, the induced apoptotic cell death was completely "rescued" by F-FRFE, but not by U-FRFE pretreatment, likely due to the higher antioxidant activity of F-FRFE compared to U-FRFE. H_2O_2-induced oxidative injury in HepG2 cells was also reported to be completely rescued by cocoa phenolic extract in a previous study,[24] indicating the significant effect of the antioxidant-rich extract in ameliorating oxidative injury. Regarding the mechanism of cell death suppression, it is plausible that F-FRFE prevents cell death by inhibiting the apoptotic pathway activation. However, further investigation would be required to exclude the involvement of other cell death/survival pathways in the effect of F-FRFE on cell viability.

For hyperglycaemic mouse models, STZ and alloxan, the cytotoxic glucose analogues, are the most prominent chemical agents used to induce diabetes. Alloxan has been used in previous studies to create a hyperglycaemic mouse or rat model.[17,25] In these studies, FRFE at a dose of 0.2 g/kg BW was shown to partially alleviate the hyperglycaemia, which is consistent with the observation in this study for the effect of U-FRFE at the same dose. Compared with U-FRFE, F-FRFE completely reversed the elevated blood glucose levels induced by STZ treatment, indicating a higher protective effect against hyperglycaemia.

The onset of diabetes is associated with increased oxidative stress and increased oxidative stress has been reported to accelerate the progression of diabetes and lead to the development of other serious health disorders.[4] Findings in this study also supports speculation regarding the interactive effect of F-FRFE on oxidative stress and hyperglycaemia. It is likely that FRFE stabilises blood glucose levels by reducing oxidative stress. However, the possibility of an oxidative stress-independent hypoglycaemic effect of F-FRFE could not be ruled out without further investigation. If the effect of F-FRFE on blood glucose levels depends on its antioxidant activity, another unclear mechanism is how F-FRFE modulates blood glucose levels by controlling oxidative stress. Regardless of these unrevealed mechanisms, the preventive efficacy of F-FRFE against oxidative stress injury and hyperglycaemia is conclusive, highlighting its potential applications in nutraceutical and pharmaceutical products.

CONCLUSION

Diabetes and other oxidative stress-related health disorders can potentially be alleviated by natural compounds from plant sources. F-FRFE with enhanced TPC, TFC and antioxidant capacity was demonstrated in this study to effectively protect hepatocytes from oxidative stress and subsidise the oxidative stress and hyperglycaemia in STZ-induced mouse model. The mechanistic dual action of F-FRF was also revealed: enhancement of intrinsic antioxidant defence and direct scavenging of free radicals. Most importantly, the F-FRF extract was shown to be safe, highlighting its potential to replace or reduce the use

of current chemically synthesised drugs with undesirable side effects.

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Ethical Approval: All mouse studies were conducted under the supervision and approval of the Animal Research Committee of the International University, Vietnam National University of Ho Chi Minh City (No. BT03/2023, http://crl.bio.hcmiu.edu.vn/about-us/facilities/) and in accordance with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and the National Research Council's Guide for the Care and Use of Laboratory Animals.

Conflict of Interest: No conflict of interest was associated with this work.

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